Assessment of Reproductive Morphology, Cytology, and Metabolic Profiling of Indigenous and Under-exploited Species, *Zingiber barbatum* (Wall.) from Myanmar

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Assessment of Reproductive Morphology, Cytology, and Metabolic Profiling of Indigenous and Under-exploited Species, *Zingiber barbatum* (Wall.) from Myanmar

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ABBREVIATIONS

ANOVA – Analysis of Variance

CCMN – Cross-Contribution Compensating Multiple Standard Normalization

EGS – Estimated Genome Size

FAO – Food and Agriculture Organization

FDR – False Discovery Rate

FFNSC – Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds

GC-MS – Gas Chromatography–Mass Spectrometry

GC-TOF-MS – Gas Chromatography–Time-of-Flight–Mass Spectrometry

HS – Headspace

HCA – Hierarchical Cluster Analysis

H-MCR – Hierarchical Multi-Curve Resolution

HS-SPME – Headspace Solid-Phase Microextraction

JST – Japan Standard Time

ID – Identification Number

IR – Illegitimate Recombination

ITPGRFA – The International Treaty on Plant Genetic Resources for Food and Agriculture

LIMMA – Linear Models for Microarray Data

MS – Mass Spectra

MVDA – Multivariate Data Analysis

NIST – National Institute of Standards and Technology

OPLS-DA – Orthogonal Partial Least Square Projection to Latent Structure Differential Analysis

PCC – Pearson Correlation Coefficient

RI – Retention Index

SD – Standard Deviation

SIMCA – Soft Independent Modelling by Class Analogy

SPME – Solid-Phase Microextraction

SPSS – Statistical Package for the Social Sciences

TEs – Transposable Elements

UN – The United Nations

UR – Unequal Intra-Strand Homologous Recombination

VOCs – Volatile Organic Compounds

WGD – Whole-Genome Duplications

SUMMARY

Myanmar possesses great biodiversity due to its broad geographical location, climatic condition and ethnic diversity. The varied climatic conditions and topography of Myanmar enable the growth of plenty of temperate, tropical and subtropical species. Diversity of the ethnic tribes with their own cultures, traditions and preferences on consumption of plant species also influence the ways of plant cultivation and production systems. Myanmar plant genetic resources are still under-explored due to the low investment for exploration and conservation initiatives. This study was carried out to provide information on the assessment of Zingiber barbatum species from Myanmar, which is one of the less-studied species to date.

The Zingiberaceae is a monocotyledonous family of perennial, mostly rhizomatous herbs. The family comprises species valued as aromatic and spice crops and as having medicinal value. The members of Zingiberaceae are used in the ethnic medicine of Myanmar due to their healing properties, both dry and fresh. The genus *Zingiber* comprises 144 species of which 37 species have been reported from Myanmar. Taxonomically, *Zingiber* is classified into four sections based on their inflorescence habits. Most *Zingiber* species have medicinal value due to their biological activity. Despite wide use as a food, a spice, a dietary supplement, and a traditional remedy only several species are well studied.

The current study focused on the assessment and characterization of the underexploited medicinal species *Zingiber barbatum* Wall. endemic to Myanmar and broadly used as a traditional herbal remedy in Myanmar. *Z. barbatum* belongs to the section *Cryptanthium* Horan. of the genus *Zingiber*. It is an indigenous medicinal species and endemic only to Myanmar. Myanmar is considered the center of its diversification. The rhizome is a valuable part of the plant and has applications as a healing anti-inflammatory and analgesic in the ethnomedicine of Myanmar. A few studies on morphological and genetic diversity characterization have revealed a high degree of variability among *Z. barbatum* genotypes originating from Myanmar. It is a species reported as one of the most troublesome taxa due to very variable morphological features.

There is still ambiguity that occurs in the characterization of *Z. barbatum* species due to the lack of comprehensive investigations. Studies on the cytology and phytochemistry of *Z. barbatum* are absent. Characterization of flower biology is limited to a few old reports and is not sufficiently comprehensive. *Zingiber* herbaria specimens are not enough for the study of flower biology due to the complexity of characterizing their flowers as they are delicate and ephemeral, and liable to rot while exsiccating in the bracts. Given this fact, a substantial part of this study encompassed the

assessment and characterization of reproductive morphology, cytology, and characterization of the volatile organic compounds in the *Z. barbatum* species. The study was carried out using the *Z. barbatum* plant collection available in the Gene Research Center of the University of Tsukuba (GRC UT) (Tsukuba, Japan). The plant materials were obtained during a field exploration of plant genetic resources in Myanmar. The *Z. barbatum* genotypes introduced to the collection were recorded as an accession with an appropriate identification code number and maintained as a living collection in the greenhouse of the GRC UT.

Two *Z. barbatum* genotypes, designated as accessions ZO113 and ZO223, were assessed to characterize the reproductive morphology. Accession ZO113 was obtained from the Nay Pyi Taw region and accession ZO223 was obtained from the Mandalay region of Myanmar. The study was conducted based on the conventional method of observation, evaluation, and morphological description of the inflorescence habits and flowers. The minimum quantitative and qualitative parameters were applied for assessment and characterization. The dissection of flowers carried out to characterize and to describe the morphology of the flowers. The study does not include statistical analysis due to the absence of replications for each flowering plant. Two qualitative parameters were different between the two *Z. barbatum* genotypes. These differences included the phenotypic variations with regards to the shape of inflorescence during growth and development. Accession ZO113 formed a conical shape inflorescence with the acuminate apex at the stage of emergence, which was gradually changed to an ovate-oblong with an obtuse-acute apex at the blossom stage, and to a wide-fusiform with an acute apex at the final stage of flowering. Accession ZO223 formed an elliptic shape inflorescence on a short peduncle, which also changed gradually into an ovate-oblong with an obtuse-acute apex at the blossom stage, and to a fusiform with an acuminate apex at the final stage of flowering. The variation of the central labellum lobe which is characterized as its bifurcation on the middle and the presence of pinkish dots at the base was observed in accession ZO113. It can be assumed that the observed phenotypic variation between the two *Z. barbatum* genotypes might be due to genetic divergence driven by reproductive isolation, or as a result of collected mutations due to continuous re-creation of the asexual form.

The non-targeted method was applied to evaluate and characterize the volatile organic compounds in the rhizome of six *Z. barbatum* accessions from Myanmar. The volatile organic compounds (VOCs) were identified by the application of gas chromatography combined with time-of-flight-mass spectrometry (GC-TOF-MS). The adjusted mass spectra obtained by the hierarchical multi-curve resolution (H-MCR) method were matched against the reference mass spectra in

different libraries for the peak annotation and identification. In total, 81 VOCs were identified in the profile of *Z. barbatum* consisting mainly of monoterpene (21%) and sesquiterpene (30%) hydrocarbons. Twenty-four identified VOCs were significantly different between six *Z. barbatum* accessions based on Tukey's HSD test. The hierarchical cluster analysis resulted in clear between-groups separation based on 81 identified VOCs forming two clusters. Cluster I comprised accessions ZO63 and ZO160 from the Bago region, while accessions ZO105 and ZO223 from the Mandalay region and ZO191 and ZO217 from Shan state were clustered together in Cluster II. Four accessions collected from Shan state (ZO191 and ZO217) and the Mandalay region (ZO105 and ZO223) of Myanmar had similar VOC profiles in comparison. VOC composition of two accessions (ZO63 and ZO160) from the Bago region were different in 14 compounds compared to the other four accessions. Although all evaluated accessions were grown in uniform conditions (in the field of the GRC UT) at the same altitude and under the same ecological conditions, they possessed variation in their VOC composition. This chemical diversity can possibly be attributed to "plant memory", which is possibly facilitated by biotic and abiotic stresses that a plant faced in its natural environment in the past.

The DNA content, genome size, and ploidy level in *Z. barbatum* species have not been reported to date. Cytological assessment was carried out using twenty accessions of *Z. barbatum* and one accession of *Z. officinale* as a reference standard. Estimation of the nuclear DNA content (2C-value) was analyzed by the application of the flow cytometry method. Two accessions (ZO208 and ZO189) showed significant differences in nuclear DNA content based on Tukey's HSD test. The 2C-values among the assessed *Z barbatum* accessions ranged from 2.90 ± 0.26 pg (ZO208) up to 5.98 ± 0.05 pg (ZO189), with an average value of 5.04 ± 0.22 pg. The estimation of the genome size showed that *Z. barbatum* accessions have a relatively small genome size (1C<3.5 pg) which is a generic characteristic of the family Zingiberaceae. Assessment of the inferred ploidy levels revealed that all evaluated accessions were triploid except two accessions, which were characterized as a diploid (ZO208) and tetraploid (ZO189). In perspective, the sequenced genome analysis can be suitable to confirm and confront the cytological findings for a better understanding of the mechanisms involved in genome size increment and evolution in *Z. barbatum*.

Z. barbatum genotypes from Myanmar displayed variation despite the limited number of accessions that were compared for the specific objectives. The current study provides comprehensive information regarding flower biology, cytological status, and volatile metabolite composition of the *Z. barbatum* species. The considerable observed variation regarding each

objective for the studied *Z. barbatum* species might be the result of human impact, natural selection, a long re-creation of the asexual reproduction and traditional cultivation approaches as has been reported for other economically important crops from Myanmar. In perspective, future studies on *Z. barbatum* genetic diversity in Myanmar need to increase with the purpose of exploration and exploitation. Moreover, conservation measures and programs need to be developed both at the regional and the country levels for better sustainable utilization of *Z. barbatum* genetic resources in Myanmar. Information about this study could be considered in conservation and sustainable utilization programs for indigenous *Z. barbatum* species due to its ethnobotanical and medicinal value in Myanmar. The study could be useful in taxonomic, systematic, phytochemical, and genetic diversity studies of this underexploited species.

CHAPTER 1
General Introduction

1.1 Introduction

Interest in traditional medicine and its importance to public health has increased in essence in many countries during the last few decades (Awale *et al.*, 2006; Gude, 2013; Ekor, 2014; DeFilipps and Krupnick, 2018). Plants and plant-based products are widely used in ethnomedicine, in the food industry, in aromatherapy, cosmetics (oils and essences), and as coloring agents. Medicinal plants have benefits compared to the chemical alternatives, because they are often cheaper, locally available and easy to consume raw or as simple prepared medicine. Great attention is paid to those under-exploited species, which can be a source of new biologically active compounds for modern pharmacognosy and phytochemistry, conservation or breeding programs.

The members of the Zingiberaceae family occupy a special place and have a long history of use as medicinal remedies in ethnomedicine of different countries due to their anti-cancer, anti-inflammatory, anti-bacterial and analgesic properties (Aggarwal and Shishodia, 2006; Sharifi-Rad *et al.*, 2017). They are consumed both dry and fresh, as vegetables, spices, pickled or used for culinary purposes and remain integral components of many types of cuisine even today. Some cultivated gingers are exploited for the cosmeceutical, nutraceutical and pharmaceutical industry (Ibrahim *et al.*, 2007). Myanmar is also considered one of the centers for Zingiberaceae genetic diversity (Myers *et al.*, 2000; Krupnick and Kress, 2003).

Biodiversity of the Myanmar region faces severe threats of loss at an alarming rate due to ecological factors (climate change, deforestation, loss of habitats) and low investment in conservation initiatives (Myers *et al.*, 2000; Aung *et al.*, 2020).

1.2 Eco-geography of Myanmar

Myanmar is situated in the Indochinese Peninsula of South East Asia between latitudes 9° 32′ N and 28° 31′ N and longitudes 92° 10′ E and 101° 11′ E. The country itself is divided into Lower Myanmar and Upper Myanmar. Lower Myanmar comprises coastal areas, represented by a long coastline bordering on the Andaman Sea and the Bay of Bengal, while Upper Myanmar consists of the interior parts of the country. Topographically Myanmar is divided into four regions: the mountainous and coastal areas in the north and west; the Shan highlands in the east, and plains in

central Myanmar, bounded by the Salween River in the east and the Irrawaddy and Sittang rivers in the west and south.

The climate of Myanmar is tropical monsoon with three seasons, which is influenced by its geographical position and monsoon winds: the cool season runs from November to February with warm to hot daytime temperatures and relatively dry air; the intensely hot season runs from March to May throughout most of the country, and the monsoon season runs from June to October with high rainfall and humidity.

Myanmar possesses various types of ecosystems due to climatic and topographical factors, and wide latitudinal range (tropical to subtropical). The Myanmar ecosystems span southern tropical evergreen rainforest, the northern subtropical montane forest, mixed deciduous, savanna and alpine types, supporting a wealth of plant diversity that constitutes a significant component of the Indo-Burma biodiversity hotspot (Tanaka, 2005; Ding *et al.*, 2019; Aung *et al.*, 2020).

1.3 Ethnobotanical investigation of *Zingiber* diversity in Myanmar

Given the wide variety of climates, geographical features, and being situated in an Indo-Burma biodiversity hotspot, the Myanmar region is exceedingly rich in its diversity of flora and fauna that comprises an abundant number of unique species. Kress *et al*. (2003) reported about 11,800 species representing 2,371 genera and 273 families, out of which 1,071 species are endemic to Myanmar. On the grounds of a comprehensive compilation done by DeFillipps and Krupnik (2018), 123 families, 367 genera, and 472 species are utilized as medicinal plants in Myanmar.

The genus *Zingiber* Mill., comprising *Zingiber barbatum*, is the second-largest genus in the family Zingiberaceae with an accepted 144 species (The Plant List, 2019; World Checklist of Zingiberaceae, 2019). The genus is distributed throughout the tropical and subtropical warm-temperate South and South East Asia with the center of the diversity in monsoon continental Asia (Theilade and Mood, 1997; Theilade, 1999; Myers *et al.*, 2000; Wu and Larsen, 2000; Kress *et al.*, 2002). The result of the extensive inventory studies of the genus *Zingiber* led to the discovery and confirmation of 37 species in Myanmar (Kress *et al.*, 2003; Tanaka, 2012; Aung *et al.*, 2015, 2016, 2017; Aung, 2016; Aung and Tanaka, 2019). Despite this, Myanmar is still known as a “floristic blank” in the Indo-Burma Hotspot due to the lack of research on its vegetation compared to other Southeast Asian countries where rich flora is well investigated and known (Tanaka *et al.*, 2018). The plant diversity and botanical exploration of the flora of Myanmar is still going on since the identification of the natural status of the many species remains incomplete.

1.4 Ethnic diversity and traditional medicine in Myanmar

Myanmar possesses a total of 135 ethnic groups, each with its own culture, traditions, and dialect (Awale *et al.*, 2006). The plant resources are an essential part of the culture and well-being of indigenous communities; however, less attention and interest have been paid to their conservation (Shin *et al.*, 2018). The ethnic diversity influences the distribution and diversification of plant species (Ahmad, 2008).

The belief in traditional medicine is strong in Myanmar and traditional treatments have been followed for generations and are still popular even today. Traditional medicine is dominated by Buddhist philosophy, Chinese medicine and Ayurvedic concepts, which in turn have an impact on the daily lifestyle and the art of traditional healing (Myanmar Insider, 2015). According to local beliefs, the medicinal plants can cure 96 different diseases affecting the human body. Medicinal plants are found in abundance and all their parts serve as highly affordable remedies for diseases. The systematic evaluation of species used by indigenous groups varies and is specific to each ethnicity, but indicates the existence of some criteria leading to selecting plants as medicine (Awale *et al.*, 2006).

1.5 The entity of *Zingiber barbatum* Wall.

1.5.1 Taxonomy and phylogeny

Zingiber barbatum Wall. is a geophyte, aromatic, perennial that grows as an annual flowering plant, varies in height and size, has an erect stem and horizontal tuberous rhizomes (Figures 1.1 and 1.2) and has a forced dormancy period. The species is well known by its vernacular names “Pwe-au” or “Meik-thalin” in Myanmar (Aung, 2016); there are no synonyms.

Taxonomic and phylogenetic studies reveal that *Z. barbatum* belongs to a section of *Cryptanthium* Horan. of the monophyletic genus *Zingiber*, family Zingiberaceae (Kress *et al.*, 2002). Traditional classification of *Zingiber* genus to the sections is made based on some morphological features and inflorescence habits: terminal or radical (Kress *et al.*, 2002; Theerakulpisut *et al.*, 2012). The section *Cryptanthium* Horan. to which *Z. barbatum* belongs comprises species with radical inflorescence (Newman, 2015).

1.5.2 Origin, distribution, habitat, and ecology

Z. *barbatum* is a species endemic to Myanmar and distributed throughout the country; however, it was also reported in Chiang Mai Province of northern Thailand (Theilade, 1999; Govaerts, 2004). Myanmar is considered to be the center of *Z. barbatum* diversification (Aung, 2016). *Z. barbatum* was for the first time described by Danish botanist Nathaniel Wallich in Plantae Asiaticae Rariores (Wallich, 1830). The species was found abundantl in Rangoon (current Yangon of Myanmar) in the hills along the Irrawaddy River near Prome (current Pyay City).

Nowadays, *Z. barbatum* is kept as a backyard plantation in households in different provinces of Myanmar. Theilade (1999) described *Z. barbatum* as endemic to northern Thailand, growing in hilly areas of 100-150 m, easily recognized by the villous character of the plant. Aung (2016) reported the distribution area as lowland and central Myanmar, from south to southeast.

Z. barbatum is found wild in hilly areas, in the Dipterocarp and Shorea forests, at altitudes from 75 m up to 1050 m. It prefers light with moderately shady terrain and high humidity; major pollinators are bees and moths. The local population keeps *Z. barbatum* as a backyard plantation in households in different provinces of Myanmar. Local farmers grow plants in small places like near a fence or levee, more for personal consumption as a medicine than for commercial production or for food (Daisy Myint, University of Tsukuba, pers. comm.).

1.5.3 Morphological and genetic diversity studies: variation and lectotypes

Morphological and genetic diversity studies of *Z. barbatum* remain controversial and limited information is available regarding this issue. Morphological features are used as a key character to visually identify and distinguish the Zingiberaceae species in taxonomic and phylogenetic studies (Kress *et al.*, 2002).

The vegetative mode of propagation is peculiar for the members of the genus *Zingiber*, as well as for most Zingiberaceae. This type of reproduction limits the gene flow, due to which the low genetic diversity among the species exists (Ravindran *et al.*, 2005). Different factors, such as environmental and genetic factors, and artificial selection affect the morphological characteristics, which can be the reason for variability and diversity.

Wallich (1830) described the *Z. barbatum* species from Myanmar as having a very faint aroma and taste. The stem is rather erect with lanceolate leaves, acuminate at the apex. Spikes of 5.4-8.1 cm, barely upborne from the ground, growing on the creeping roots, near the base of the stem,

radical, ovate, cuneate at the bottom part. Bracts are broad-ovate, with convex-ventricose, villous, apex subulate-curved and open. Lip is ovate, blunt and convex on the top. Unripe fruits were observed in September.

The most comprehensive study on morphological characterization and genetic diversity was done by Wicaksana (2012). The genetic diversity studies on the original *Z. barbatum* collection from Myanmar, based on morphological and molecular markers, revealed high genetic variability among *Z. barbatum* genotypes at intra- and interspecific levels (Jatoi *et al.*, 2008; Wicaksana *et al.*, 2011). Reproductive morphology was not included due to the absence of flowers during the experimental periods.

Aung (2016) described *Z. barbatum* as one of the most problematic taxa due to its very variable morphological characteristics, and reported on three main types of *Z. barbatum*:

- **Type A:** *Z. barbatum* Wall. - coincides with those described in the original protologue by Wallich (1830) (Figure 1.3);
- **Type B:** Plant has linear leaves and fusiform inflorescence with cuspidate bracts covered with long white hairs, described as a distinct species, *Z. popaense*, related to *Z. barbatum* (Figure 1.4);
- **Type C:** This type has not been described before. The plant is characterized as very small, up to 30 cm tall and densely arranged leaves with short internode and a larger broad labellum. This type is also found in central Myanmar and described as *Z. pygmaea* (Figure 1.5).

Thereby, it is supposed that although *Z. barbatum* includes these three taxa, each type can be clearly distinguished and assigned to three independent species (Aung, 2016).

The original *Z. barbatum* protologue in Plantae Asiaticae Rariores provided an illustration (t. 55) drawn by Wallich (1830); however, the type of material was not designated. The illustration (Figure 1.6) made by Wallich (1830) is only a single material for the designation of the nomenclatural for *Z. barbatum*. In this connection, these illustrations were designated as lectotypes (Aung, 2016) in accordance with the International Code of the Botanical Nomenclature (Art. 9.1), and Type A was referred to as the original *Z. barbatum* species described by Wallich (1830).

1.5.4 Cytological studies

Polyploidy played a significant role in the evolution and diversification of Zingiberaceae resulting in a blurring of morphological boundaries between taxa (Leong-Skornickova *et al.*, 2007).

The existence of polyploidy depends on the basic chromosome number (Jatoi *et al.*, 2007). Frequently, closely related species belonging to the same genus, constantly save the basic number of chromosomes. The basic number reported for members of *Zingiber* is x=11 or x=12 (Ravindran *et al.*, 2005; Jatoi *et al.*, 2007). The complex chromosome structural changes during the evolution ensued to increase or decrease the chromosome numbers, and the ploidy level respectively, in the order Zingiberales (Jatoi *et al.*, 2007).

The variation of genome size has significant consequences at the cellular, tissue and organismal levels leading to influences on the phenological and ecological behavior of the plants (Leong-Skornickova *et al.*, 2007). A literature review and search in the Plant DNA C-values database (Leitch *et al.*, 2019) determined the absence of records and cytological studies for the *Z. barbatum* species.

1.5.5 Phytochemical studies

The *Zingiber* species is valuable as a medicinal plant due to its possession of various biological properties such as anti-inflammatory (Ozaki, *et al.*, 1991; Singh *et al.*, 2005; Zhang *et al.*, 2016), antioxidant (Nile and Park, 2015; An *et al.*, 2016), antimicrobial (Pithayanukul *et al.*, 2007; Sasidharan and Menon, 2010; Mesomo *et al.*, 2013; Kumar *et al.*, 2014) and anticancer (Murakami *et al.*, 2002; Kirana *et al.*, 2003; Takada *et al.*, 2005; Citronberg *et al.*, 2013).

The unique aroma, flavour and bioactivities are related to the combination of the phenolic compounds (gingerols, shogaols, and paradols) and essential oils, mainly consisting of volatile organic compounds (VOCs) (Vernin and Parkanyi, 2005; Mao *et al.*, 2019). The essential oil of the *Zingiber* species is a complex mixture of VOCs, mainly consisting of monoterpenoids (C10) and sesquiterpenoids (C15) such as α-zingiberene, ar-curcumene, β-bisabolene, sabinene, zerumbone, β-phellandrene, pinene, and terpenes, whereas the bioactivities relate mostly to the phenolic compounds (Vernin and Parkanyi, 2005; Wohlmuth *et al.*, 2006; Mao *et al.*, 2019).

Although, *Z. barbatum* has a long history of use as a herbal remedy very little known about this species. The composition of both volatile and non-volatile constituents of *Z. barbatum* has never been screened or reported before.

1.5.6 Application in ethnomedicine

Z. barbatum is used by the different ethnic communities as traditional medicine due to its anti-inflammatory and analgesic properties (Wicaksana, 2012). The rhizome is a valuable part of the

plant. The steam-heated medicinal product “Sayar Ye’s Meik Tha Lin” (Figure 1.7) from milled rhizome is used for treating gout, to cure skin problems like eczema, rashes and itchiness, as a carminative agent, against fever and to relieve joint, bone and muscle pains. Rhizome is ground with water and applied to scalds and taken orally to cure dysentery.

1.5.7 Mode of reproduction

Since many species of Zingiberaceae are rhizomatous, vegetative reproduction is peculiar for the members of this family. The vegetative mode of propagation is also typical for *Z. barbatum*. The vegetatively reproductive species have low genetic variation because of their asexuality, which limits the gene flow. However, asexual species often harbour an abundance of variation coming from new mutations caused by an allelic variation at loci at which neutral mutations occur or are due to genotypic variation, which is caused by somatic mutations, balancing selection, or continuous re-creation of the asexual form (Bengtsson, 2003). These variations often lead to an increase in the homogeneity of individuals in the population or tend to increase the divergence between gene copies resulting in differentiation of individuals (Olden, 2006).

1.6 Problem addressed

Nowadays, most of the plant species are vulnerable and at risk of extinction due to lack of attention, comprehensive investigations, anthropogenic activities and/or ecological factors. The eco-geographical factors, artificial selection and/or traditional agricultural approaches used by ethnic communities perhaps influence the reproductive isolation of *Z. barbatum* leading to its diversification and observed variability. Limited information has led to a neglect of this species that can threaten its subsequent existence.

To solve the taxonomic ambiguities and as an addition to already existing research on genetic diversity, a substantial part of this study focused on the evaluation of the reproductive morphology, cytology and volatile metabolite profiling of *Z. barbatum* from Myanmar.

1.7 Hypotheses

The hypotheses proposed for the current study are:

1. The vernacular name "Meik-thalin" used by local ethic communities of Myanmar regarding *Z. barbatum* is also used to refer to *Z. montanum*, which is probably a reason for persisting

confusion about the description of these taxa. Information regarding reproductive morphology of *Z. barbatum* are very limited and old. There might be a phenotypic variation regarding reproductive morphology among *Z. barbatum* individuals representing different geographical regions of Myanmar.

2. The various environmental conditions have impacted the production and emission of the volatile organic constituents in plants. The volatile metabolites of *Z. barbatum* have never been screened before and information is absent. Variation might be found regarding volatile metabolites among *Z. barbatum* species collected from different eco-geographical regions of Myanmar.
3. The ethnic agricultural approaches and diverse environmental condition may impact *Z. barbatum* genetic resources, resulting in phenotypic plasticity or/and genetic variability. Therefore, cytological analysis is fundamental for classification of the plant species. Cytological evaluation of *Z. barbatum* has never been done and has not been reported before. It is assumed, that intraspecific variation of nuclear DNA content might occur among *Z. barbatum* species.

1.8 Objectives

To support the proposed hypotheses the following objectives were determined:

1. Assessment and description of the morphological features regarding the inflorescence habits and flower features to confirm the taxonomic affiliation of *Z. barbatum* at the interspecific level.
2. Identification and characterization of the VOC composition in *Z. barbatum* species to detect chemical variation which may exist among individuals collected from different eco-geographical regions of Myanmar
3. Estimation and designation of the relative nuclear content, genome size and inferred ploidy level of *Z. barbatum* to describe its cytological status.

The summary of the general research methodology and specific approaches are shown in Figure 1.8.

Figure 1.1 General view of the *Zingiber barbatum* plant from the collection of the Gene Research Center at the University of Tsukuba (Tsukuba, Japan).

Figure 1.2 General view of the *Zingiber barbatum* rhizome. The photograph was taken after harvesting the *Zingiber barbatum* from the plant collection in the field of the Gene Research Center at the University of Tsukuba (Tsukuba, Japan).

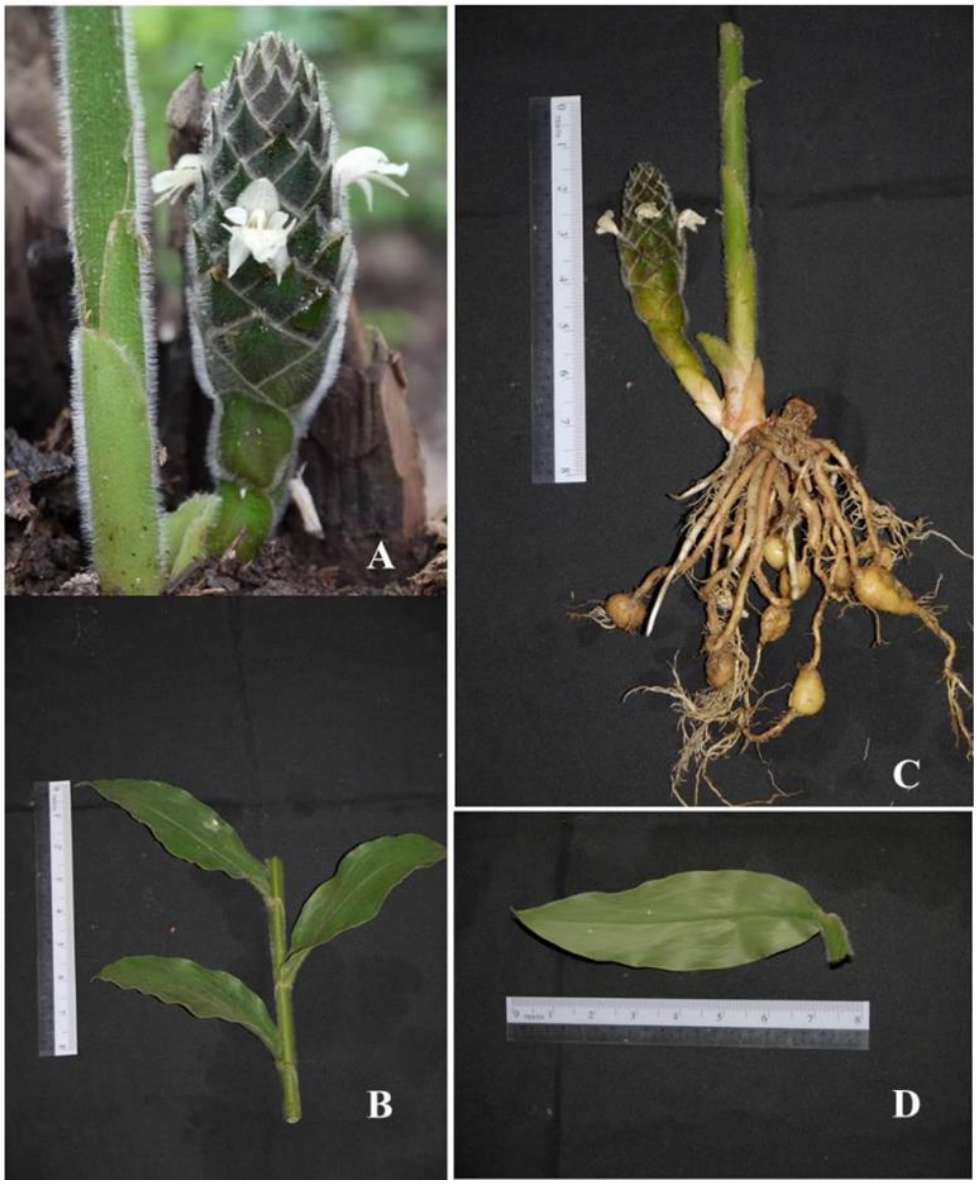

Figure 1.3 *Zingiber barbatum* Wall. Type A, growing wild in the central and south-eastern parts of Myanmar. Type A coincides with the original protologue described by Wallich (1830). A, inflorescence with flowers; B, leaves and ligules; C, inflorescence and rhizome with tubers; D, leaf blade. Reprinted from "Taxonomic study of the genus *Zingiber* Mill. (Zingiberaceae) in Myanmar", by Mu Mu Aung, 2016, the PhD Thesis (p. 141), Kochi University, Japan.

Figure 1.4 *Zingiber popaense* Nob. Tanaka, Type B, a distinct species related to the *Z. barbatum* group of species found wild in central Myanmar. Type B is characterized by linear leaves and fusiform inflorescence with cuspidate bracts covered with long white hairs. A, inflorescence with flowers; B, leaves and ligules; C, close view of flower and inflorescence; D, roots with tuber. Reprinted from "Taxonomic study of the genus *Zingiber* Mill. (Zingiberaceae) in Myanmar", by Mu Mu Aung, 2016, the PhD Thesis (p. 142), Kochi University, Japan.

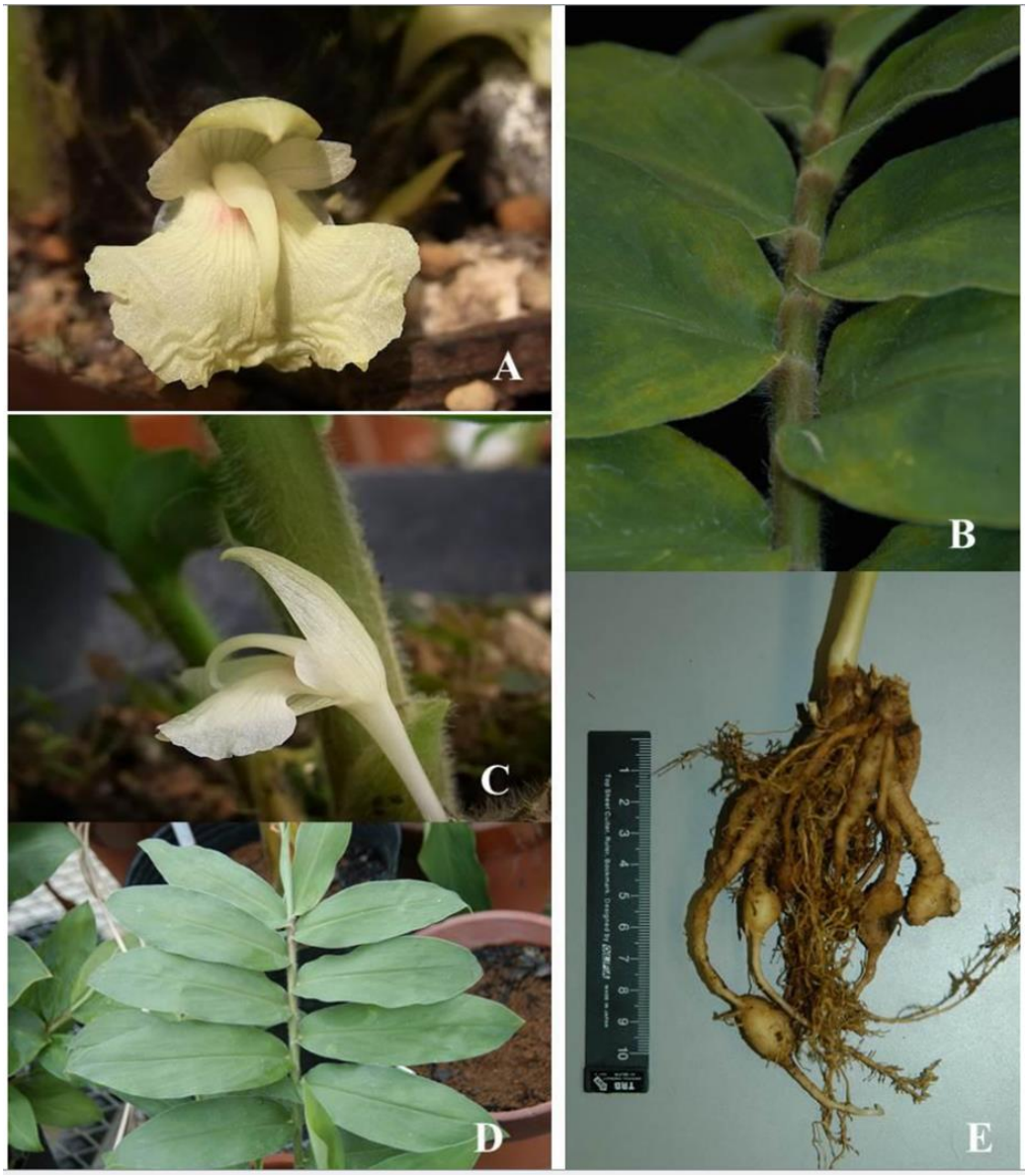

Figure 1.5 *Zingiber pygmaea* M. M. Aung & Nob. Tanaka, sp. nov., Type C, found in wild in central Myanmar. Type C is described as a variation of the *Z. barbatum* species and is characterized by densely arranged leaves with short internode, larger broad labellum and up to 30 cm in height. A, front view of flower; B, leafy stem and ligules; C, side view of flower and inflorescence; D, stem with leaves; E, roots with tubers. Reprinted from "Taxonomic study of the genus *Zingiber* Mill. (Zingiberaceae) in Myanmar", by Mu Mu Aung, 2016, the PhD Thesis (p. 143), Kochi University, Japan.

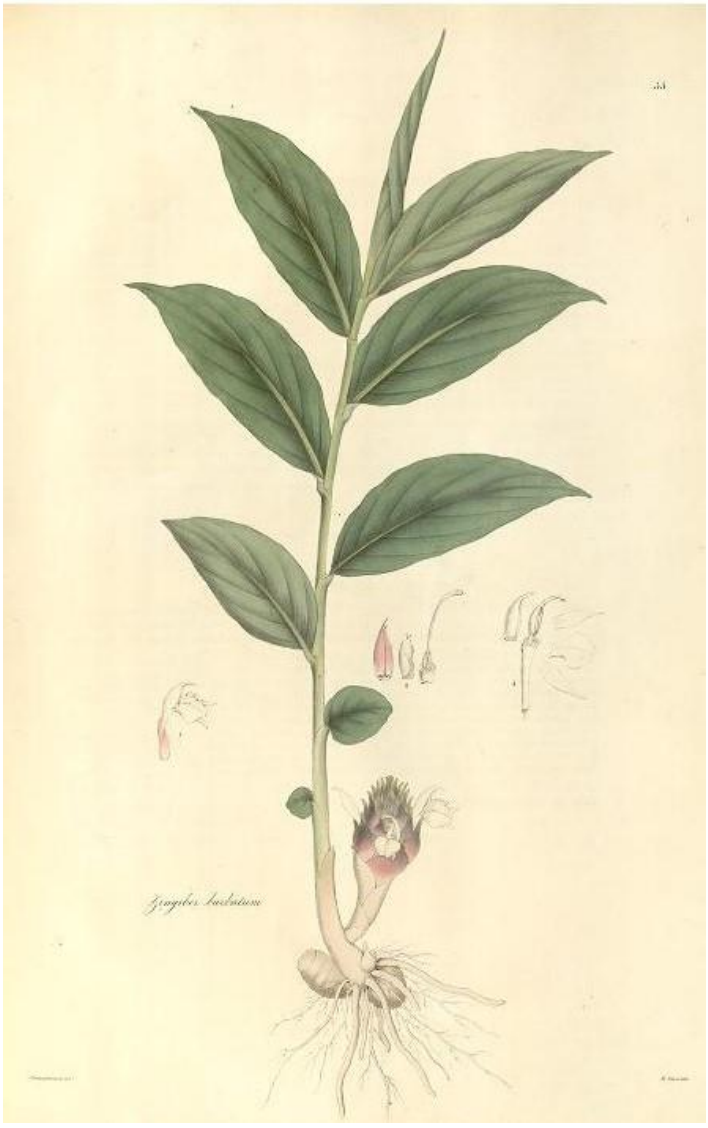

Figure 1.6 Original illustration of *Zingiber barbatum* drawn by Nathaniel Wallich. Reprinted from *Plantae Asiaticae Rariores, or, Descriptions and Figures of a Select Number of Unpublished East Indian Plants* (vol. I, t. 55) by N. Wallich, 1830. London: Treuttel and Würtz.

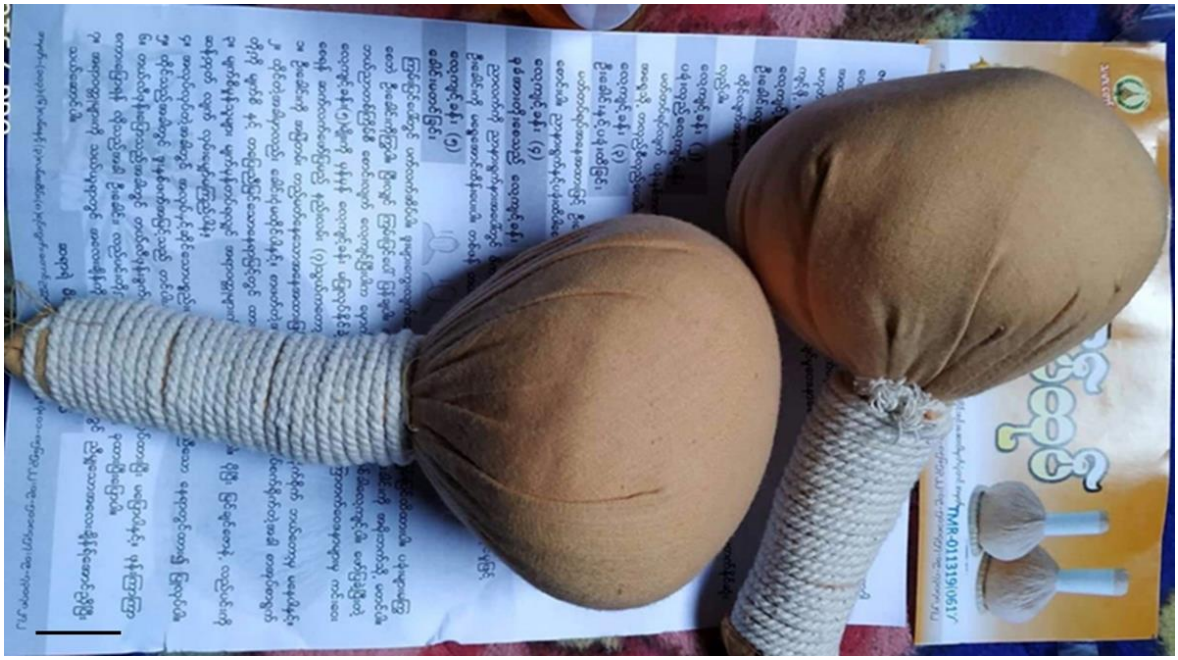

Figure 1.7 The medicinal product “Sayar Ye’s Meik Tha Lin” made from the milled rhizome of *Zingiber barbatum*. Scale bar: 2.0 cm. Photograph provided by Daisy Myint, Graduate Student of the University of Tsukuba. Tsukuba, Japan (2019).

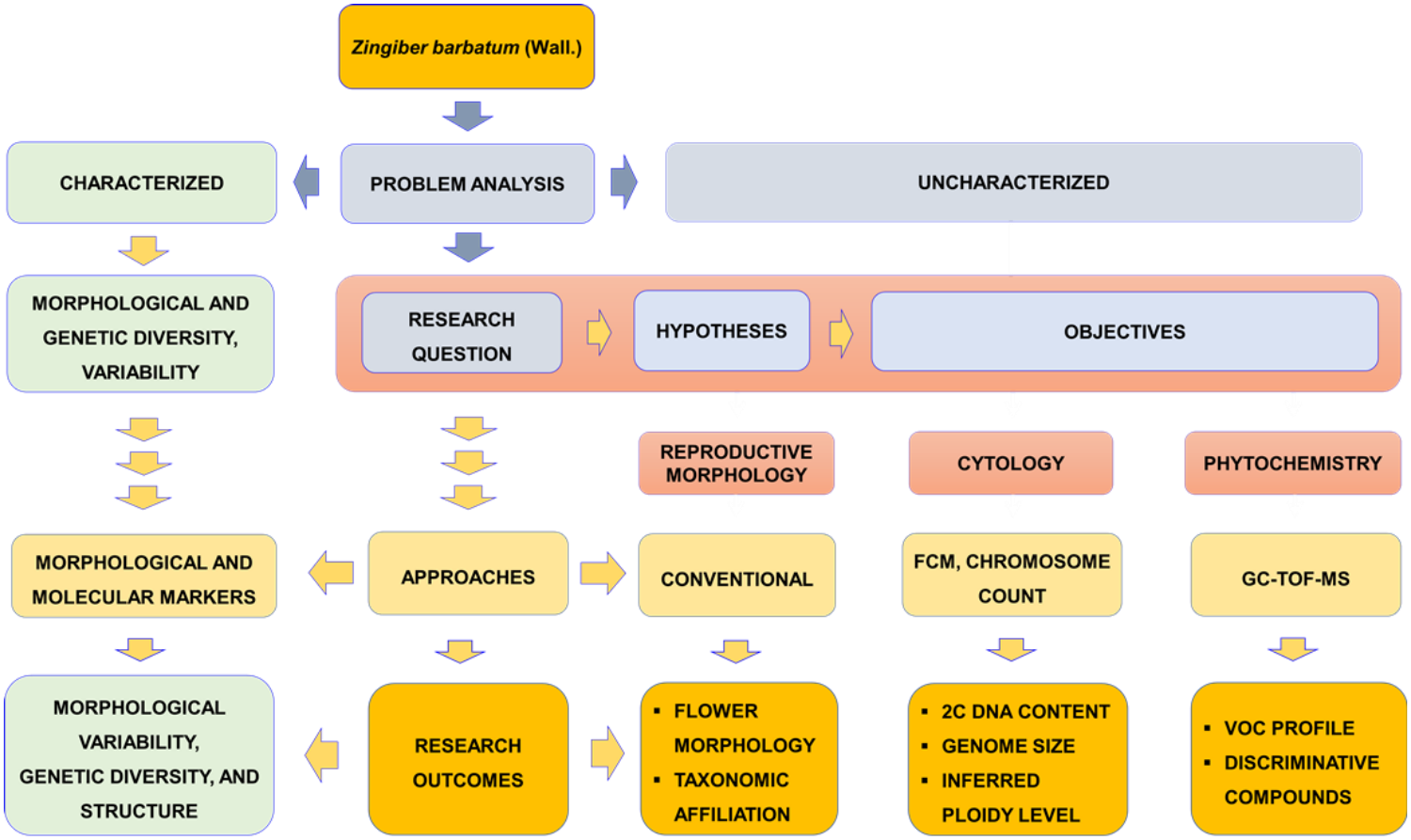

Figure 1.8 Flow-chart describing the research methodology.

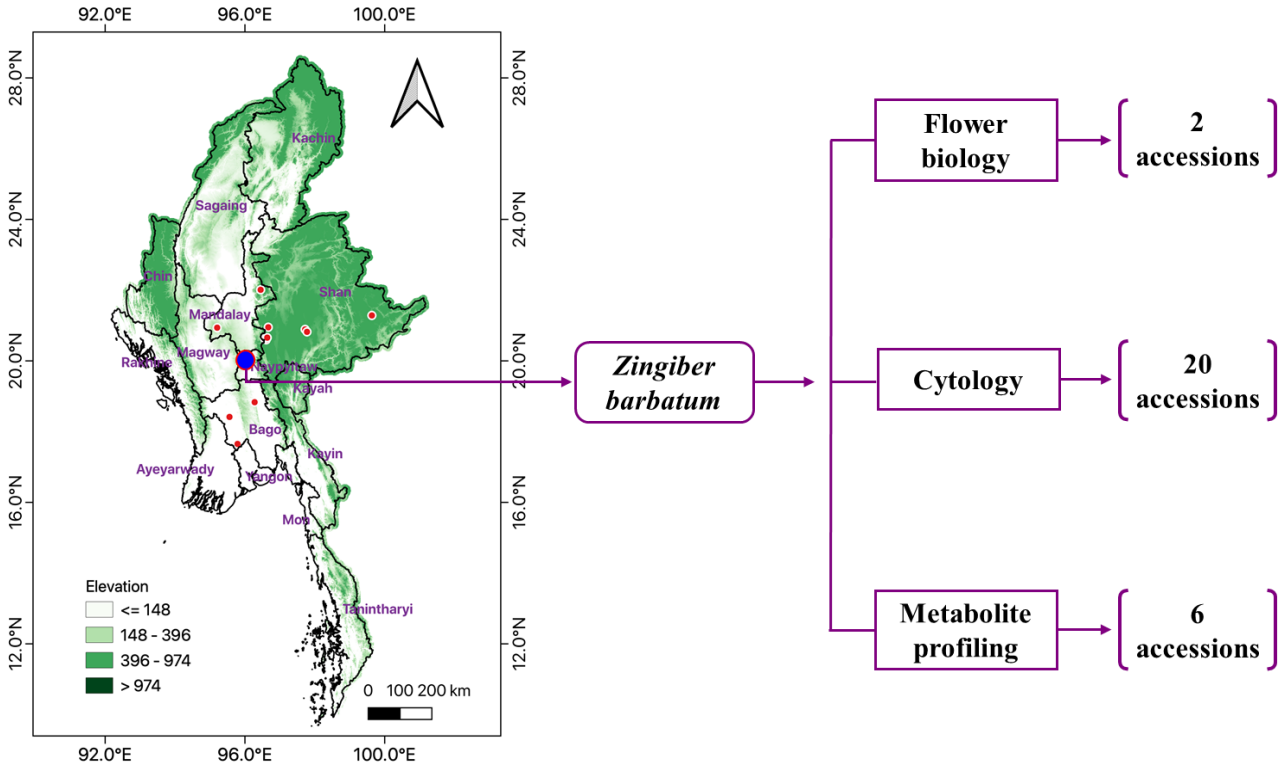

Figure 1.9 Map of Myanmar indicating the collection site of *Zingiber barbatum* accessions applied for the current study.

CHAPTER 2

Descriptive Characterization of the Reproductive Morphology of *Zingiber barbatum* Wall.

2.1 Introduction

Characterization of the reproductive morphology remains relevant to classical taxonomic studies. Most species in the genus *Zingiber* Mill. are phenotypically similar and difficult to distinguish at the non-flowering stage. Therefore, observation and characterization of the flowers' biology and the habit of the inflorescence remain relevant to classical taxonomic studies in *Zingiber* species at the first stage of the evaluation and the description.

Zingiber barbatum Wall is an aromatic, perennial, endemic species in Myanmar and the north part of Thailand (Theilade, 1999; Govaerts, 2004). *Z. barbatum* has been neglected for a long time and its biology is poorly known as evidenced by the rather scarce studies. Reports, regarding the reproductive morphology of *Z. barbatum*, which are mainly derived based on the herbarium collections, are insufficient for a detailed morphological characterization.

Z. barbatum is characterized as a geophyte, flowering plant, varying in height and size, with horizontal tuberous rhizomes. It is a perennial plant, but grows as an annual, with a forced dormancy period. The plant goes dormant and loses all the vegetative aboveground parts with the onset of winter when the habitat temperature decreases to below 15^{o}C at night. The plant grows wild in hilly areas at altitudes from 75 m up to 1,050 m of the Dipterocarp and Shorea forests, prefers light with moderate shade and a high humidity environment.

2.1.1 Taxonomic affiliation based on inflorescence habits

Z. barbatum belongs to the genus *Zingiber*, section *Cryptanthium* and is characterized by inflorescence consisting of a spike on a short procumbent peduncle (Theerakulpisut *et al.*, 2012). Taxonomically, the genus *Zingiber* is classified into four sections based on the inflorescence habit: i) section *Dymczewiczia* (Horan.) Benth., a species with a terminal inflorescence; ii) section *Pleuranthesis* Benth., a species with a terminal inflorescence; iii) section *Zingiber* Mill. and iv) section *Cryptanthium* Horan., which comprise species with radical inflorescence (Theerakulpisut *et al.*, 2012; Triboun *et al.*, 2014; Newman, 2015). However, some species of section *Zingiber* (e.g. *Z. gramineum*, *Z. junceum*) and section *Cryptanthium* Horan. (e.g. *Z. barbatum*) have been reported

to produce inflorescence both on a terminal and/or on a radical procumbent peduncle (Theerakulpisut *et al.*, 2012; Newman, 2015).

The fusion of two sterile stamens into a labellum and the presence of a single anther with a horn-like appendage (anther crest) embracing the upper part of the style (Sabu *et al.*, 2013) are the general distinguishing features of the flowers in the genus *Zingiber*. The complexity of characterizing *Zingiber* flowers lies in the fact that flowers are delicate and ephemeral, and liable to rot if exsiccated in the bracts, thus the herbaria specimens have little value for study of flower morphology (Theilade and Mood, 1997).

2.1.2 Phenotypic variation of inflorescence

Aung (2016) described *Z. barbatum* as a troublesome taxa due to very variable morphological characteristics and reported about three main types of variation in *Z. barbatum*. The species belonging to the different types were identified as *Z. barbatum* (Type A) and this corresponds to the original description of Wallich (1830), while *Z. popaense* (Type B) and *Z. pygmaea* (Type C) are described as distinct species, related with *Z. barbatum* (Tanaka, 2012; Aung, 2016).

The genetic diversity studies on the original *Z. barbatum* collection from Myanmar, based on morphological and molecular markers, revealed high genetic variability among *Z. barbatum* genotypes in intra- and interspecific levels (Jatoi *et al.*, 2008; Wicaksana, 2012). At the same time, the phylogenetic analysis revealed that almost half of the *Z. barbatum* accession collected from Myanmar are closely related to *Z. montanum*, but actually it is two different species (Wicaksana, 2012). Controversy about the inflorescence habits, species ambiguity and reported intraspecific genetic variability also complicates descriptions of the species. The traditional agricultural approaches might be influencing the distribution and diversification of the *Z. barbatum* species in Myanmar. The general morphology of *Z. barbatum* was described by Wicaksana (2012); however, the description of the flowers was not included in the study due to the absence of flowers.

The objective of this chapter is to characterize the reproductive morphology of *Z. barbatum* species more precisely; the study focused on the characterization of the inflorescence architecture and the phenotypic features of the flower, with records of some phenological parameters. The current study is the first attempt to characterize the reproductive morphology of *Z. barbatum* from Myanmar based on a living collection from the Gene Research Center of the University of Tsukuba (GRC UT), Japan.

2.2 Materials and Methods

2.2.1 Plant materials

Two accessions of *Z. barbatum* from the collection of the GRC UT (Tsukuba, Japan) were used as plant material to conduct this study. Accession ZO113 was obtained from the Nay Pyi Taw region and accession ZO223 was obtained from the Mandalay region (Figure 2.1) of Myanmar. The rhizomes were obtained during field exploration of plant genetic resources in Myanmar under a Grand-in-Aid for Overseas Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Watanabe *et al.*, 2007; Kawase *et al.*, 2011). The accessions were grown in plastic pots of 30 x 50 cm and maintained as living plants in the greenhouse of the GRC UT (Tsukuba, Japan). The study was conducted from July to November in 2019.

2.2.2 Descriptive parameters for characterization of the inflorescence and flowers

The characterization of the reproductive morphology of *Z. barbatum* was done based on the screening of inflorescence architecture and flower features using quantitative and qualitative descriptions.

The quantitative description was done based on seven parameters: plant height, inflorescence length, peduncle length, spike length, peduncle width, spike width, and the number of inflorescences per accession. Measurements were done every three days, starting from the day that inflorescence first emerged up to the day when the last three measurements showed the same index.

The qualitative characters included a description of the form and habit of the inflorescence and peduncle, the shape, color and pubescence of the spike's bracts and sheathing bracts of the peduncle, the color of flowers and the phenotypic features of the flowers. The flower features were characterized based on the dissection of flower parts and their description.

The life span of flowers was described based on phenological observation. The phenology of the flower was screened and recorded every day: from the first bloomed flower until the last one. The parameters recorded during phenological observation were the number of flowers per spike, the duration of flower span per plant, the time of the flower appearance from the bract, and the time period necessary for the flower to fully open.

2.2.3 Data analysis

Statistical analysis was not possible because of the absence of replication for each flowering plant. Due to this, the results comprise only general measurements of quantitative parameters and recordings of the observed changes in qualitative characteristics and phenology that were done every three days.

2.3 Results

The current study included two accessions of *Z. barbatum*, ZO113 and ZO223, representing two geographical regions of Myanmar: Mandalay and Nay Pyi Taw. These accessions formed flowers from the beginning of July up to the beginning of September of 2019.

Accession ZO223 (Figure 2.2) produced one and accession ZO113 produced two inflorescences (Figure 2.3). The plant height and inflorescence length and width measurements showed the growth of both the plant itself and the inflorescence continued throughout the entire flowering period. The summary characteristics of the similarities and/or differences in *Z. barbatum* species in comparison with those reported in other studies are shown in Table 2.1.

2.3.1 Qualitative description of the inflorescence features

Although the two accessions both belong to *Z. barbatum*, some phenotypic differences were observed regarding the shape of inflorescences at the stage of emergence and during growth, which were visually easy to detect. The emerged inflorescence of ZO223 had an elliptic shape on a short peduncle (scape) with an obtuse apex (Figure 2.2B), while emerged inflorescences of ZO113 had a conical shape with an acuminate apex without a peduncle (Figure 2.3B).

Along with growth and development, the size and shape of the spikes changed gradually; their width and length increased along with the peduncles' growth and lengthening. This tendency continued despite flowering resulting in significant change in the shape of the spikes from the moment of appearance. The spike shape of accession ZO223 changed gradually from wide-elliptic to ovate-oblong with the obtuse-acute apex at the stage when blossoming began, and to the fusiform with an acuminate apex at the final stage of blossom (Figure 2.2C-E). The same tendency was observed in accession ZO113, the shape of the spike changed gradually from conical to ovate-oblong with an obtuse-acute apex at the stage when blossoming began, and to the wide-fusiform with an acute apex at the final stage of blossom (Figure 2.3C-E).

Z. barbatum's spikes are dense and carry helically arranged bracts. Mature bracts broadly ovate with a papery margin, apices obtuse or obtuse-acute, protuberant, dark red color, enclosing a single flower per bract. The lowermost 2-4 bracts are sterile and do not produce flowers.

The rigid peduncle is erect, consists of internodes and is covered by sheathing bracts. The sheathing bracts ovate, with acute apices, protuberant, light green at apices and reddish close to the base. The bracts and sheathing bracts have pubescence of approximately 1.0-2.0 mm, are soft and velvety in appearance, but reduce during growth.

The senescence of inflorescences started with the changing color of the bracts from dark red to dim brownish-red, followed by a change in color in the middle of the bracts to light dull brownish and to light red at the edges, then to orange-red and ending by drying (Figure 2.4). The drying of the inflorescence began from the top to the bottom and was accompanied in parallel by the drying of the sheathing bracts from the bottom to the top. The observed senescence was identical in the both accessions.

2.3.2 Quantitative description of the inflorescence features

The total inflorescence length was 30.0 cm in accession ZO223 and ranged between 35.0-37.0 cm among the two inflorescences of accession ZO113. The length of peduncles ranged from 13.0 to 15.0 cm and the width of peduncles ranged from 9.0 to 9.4 mm between the two accessions. The spike length ranged from 15.0 to 22.0 cm and the width of the spikes were 34.4 mm in accession ZO223 and 38.1-38.6 mm in accession ZO133. Figure 2.5 displays the data of the quantitative measurements of the morphological features of plant and inflorescence growth.

The span time of the inflorescence senescence was different between the examined accessions. The inflorescence senescence in accession ZO223 began 15 days after completion of flowering and ended in 25 days. Accession ZO113 had a prolonged inflorescence senescence. The senescence began 21 days after completion of flowering and extended up to 49 days.

2.3.3 Description of morphological features and phenology of *Zingiber barbatum* flowers

The flowering began at the end of July, after 28-34 days of inflorescences formed and lasted up to 19-22 days for both examined accessions. In total, accession ZO223 formed 40 flowers, and accession ZO133 formed 47-48 flowers during the entire flowering period. The life span of each flower was very short; it began to appear from the bracts early in the morning around 4:00 AM and began fading by evening. Flowers fully opened after 4-6 hours from the moment of appearance. In

inflorescence, they bloomed sequentially from the bottom to the top and one to three flowers opened simultaneously, rarely four.

In the current study, *Z. barbatum* produced flowers of a pale to light-yellow color (Figure 2.6) with a very light bitter citrus scent. Flower length ranged from 4.7 to 5.0 cm between the two examined accessions (Figure 2.6C). The floral tube was approximately of 0.3 cm in diameter, slender and externally and internally white and glabrous. The calyx was light-yellow, glabrous, membranous, apex shortly serrated, tubular and unilaterally split. The corolla lobes were subequal in length, lanceolate, pale-yellow and longitudinally striped. The dorsal lobe facing the inflorescence axis was slightly concave in the hood. The dorsal lobe was 2.5 × 1.5 cm and the lateral lobes were 2.5 × 1.0 cm.

The stamen, which belongs to the inner circle of the androecium, was placed at the base of the corolla. The other two sterile members of the androecium were fused into petal-shaped staminodes, called the labellum, and were placed opposite the stamen. The base of the labellum is tubularly folded and attached to the corolla tube. The labellum was soft-velvety in appearance and light-yellow in color. The central labellum lobe was 2.8 × 2.2 cm, obcordate, apex emarginate, margins were slightly undulate-reflexing; the lateral staminodes were 1.5 × 0.6 cm, ovate-lanceolate, apex acute in accession ZO223 and acuminate in accession ZO113, connate to the labellum by basal 1/2 - 1/3 (Figure 2.6D). It should be noted that the central labellum lobe of accession ZO113 was bifurcated in the middle (Figure 2.6D) with pinkish dots at the base of the labellum (Figure 2.7).

The stamen was approximately 1.5-1.7 cm in length (without the anther crest straightened), light yellow; carried 2 single-nested anthers. The anthers were light-yellow in color, 1.6 cm long (excluding anther crest), thecae cylindrical, parallel, dehiscence longitudinal, anther crest light-yellow, hooded, wrapped around the style, leaving the stigma free (Figure 2.6C, F). The style was white, glabrous and filiform. The stigma was approximately 0.15 × 0.1 cm, white, scarcely wider than the style, tubular, downwards facing and ostiole ciliate (Figures 2.6C, F and 2.7). Seeds were not observed during this study.

2.4 Discussion

The assessment of the reproductive morphology of *Z. barbatum* was done in order to characterize inflorescence architecture (habit) and to describe the phenotypic features of the flower. The phenological observation was conducted to describe the growth cycle of inflorescences and the life span of the flower.

The conducted study revealed that *Z. barbatum* produces inflorescences on radical erect peduncles (Figures 2.2 and 2.3). The conducted study confirmed the taxonomic affiliation of *Z. barbatum* to the *Zingiber* section *Cryptanthium* Horan. as it was reported in former studies (Theerakulpisut *et al.*, 2012; Triboun *et al.*, 2014). According to Wallich (1830) inflorescences are formed from the creeping roots near the stem or from the base of the stem and barely elevated above the ground surface. The current study revealed that inflorescences arise directly from a rhizome near the stem; however, they were formed on a long enough erect peduncle, corresponding to the description of the inflorescence habit of *Z. barbatum* reported by Theilade (1999). According to Theilade (1999), *Z. barbatum* accessions produced inflorescences on the erect peduncle of 2.0-6.0 cm in length, while according to Aung (2016), *Z. barbatum* (belonging to Type A) produces inflorescence on the erect peduncle of approximately 3.0 cm in length (Table 2.2). However, the current study revealed that two *Z. barbatum* genotypes produced inflorescences on the erect peduncle of 13.0 to 15.0 cm in length, which is the opposite of the description reported in former studies.

The reported distinguishing feature of *Z. barbatum* inflorescence is the presence of the high pubescence (Wallich, 1830; Aung, 2016) and this corresponds to its etymology "barbatum" - having long, weak hair. However, I did not observe the high pubescence on inflorescences among the two examined accessions during this study. Moreover, to the contrary, the observed pubescence decreased during the process of inflorescence growth.

Assessed quantitative parameters did not display any differences among the two accessions, except the time span that inflorescences underwent senescence. However, the qualitative parameters showed some variation regarding the shape of inflorescences at the stage of appearance and the shape of the labellum (bifurcation of the central labellum lobe in ZO113) between the two assessed accessions.

The comparative assessment of the observed phenotypic variability regarding the shape of the spike during growth, at the stage of blossom beginning and at the last stage of blossom (Figures 2.2 and 2.3) revealed similarity corresponding with the description of the spike given for both *Z. barbatum* and *Z. montanum* (Aung, 2016). The observed variation regarding reproductive morphology in *Z. barbatum* species might be due to the influence of collected mutations through the continuous asexual mode of propagation which resulted in increasing genetic divergence of individuals in the population (Bengtsson, 2003; Olden, 2006). From another perspective, the observed differences regarding the shape of inflorescence and the labellum can be either a

genetically determined feature, when the activity and position of the shoot apical meristem (SAM) are determining a degree of the inflorescence architecture (Benlloch *et al.*, 2015), or as a result of phenotypic plasticity, when due to various environmental conditions the variation of the same traits among the species of the same taxa could be induced (Thompson, 1991). The visual reduction of the indumentum (pubescence) could also be the result of phenotypic plasticity and/or as a consequence of the terminated trichomes metabolic activity, which, after they die, may either persist or be shed (Johnson, 1975; Thompson, 1991).

The study revealed that *Z. barbatum* has zygomorphous, bisexual, ephemeral, epigynous flowers, with a light bitter citrus scent and with the presence of a horn-like anther crest embracing the upper part of the style. The observed life span of flowers was less than one day. Wallich (1830) reported, that the plants introduced into Calcutta Garden blossom freely during the cold and rainy season. The current study showed that *Z. barbatum* flowered mainly during August when the average daily temperature was above +30°C and the average humidity above 80%. The current result corresponds with those reported by Aung (2016) for *Z. barbatum* Type A when the flowers and fruits were observed from July to September.

The study, being conducted under relatively homogeneous conditions, has the advantage of eliminating factors that could influence the flow of the experiment. Although the examined plants were grown under greenhouse conditions, about a 5- to 6-day gap in flowering of accession ZO113 was observed. According to Ravindran *et al*. (2005) the rarity of flowering in *Zingiber* is influenced by photoperiodic and climatic factors. Thus, the observed trend was probably triggered by temperature differences on those days, when the average daily temperature ranged from +21 to +28°C, and the weather was mostly cloudy and cool.

During this study the assessed *Z. barbatum* accessions did not produced seeds. The absence of seeds could be due to the high pollen sterility of many *Zingiber* species hence resulting in no sexual reproduction (Ravindran *et al.*, 2005) influenced by a single or complex set of factors. The dominant xenogamy, entomophily and different types of breeding systems have been reported for Zingiberaceae (Sakai *et al.*, 1999; Li *et al.*, 2002; Jatoi *et al.*, 2007; Specht *et al.*, 2012). The heterostyly with a gametophytically controlled self-incompatibility system have been reported for *Z. officinale* (Dhamayanthi *et al.*, 2003) and a partial self-incompatibility has been reported for *Z. densissimum* (Fan *et al.*, 2015). Future studies are needed to reveal the mechanisms behind the formation of seeds that will lead to a better understanding of the reproductive biology of the *Z. barbatum* species.

2.5 Proposed practical application

The morphological descriptive method used in this study is easy, low budget and does not require high-tech equipment. The conducted study revealed notable phenotypic variations of inflorescence and central labellum of the flower among the two examined *Z. barbatum* accessions, but quantitative parameters were not, in essence different. Therefore, the descriptive morphology can be useful in first step screening and characterization of the underexploited and/or unknown species in the genus *Zingiber*.

The detailed description along with color photographs of inflorescences and flowers can be considered as useful visual and informative material for future systematic, taxonomic and genetic diversity studies on *Z. barbatum* species.

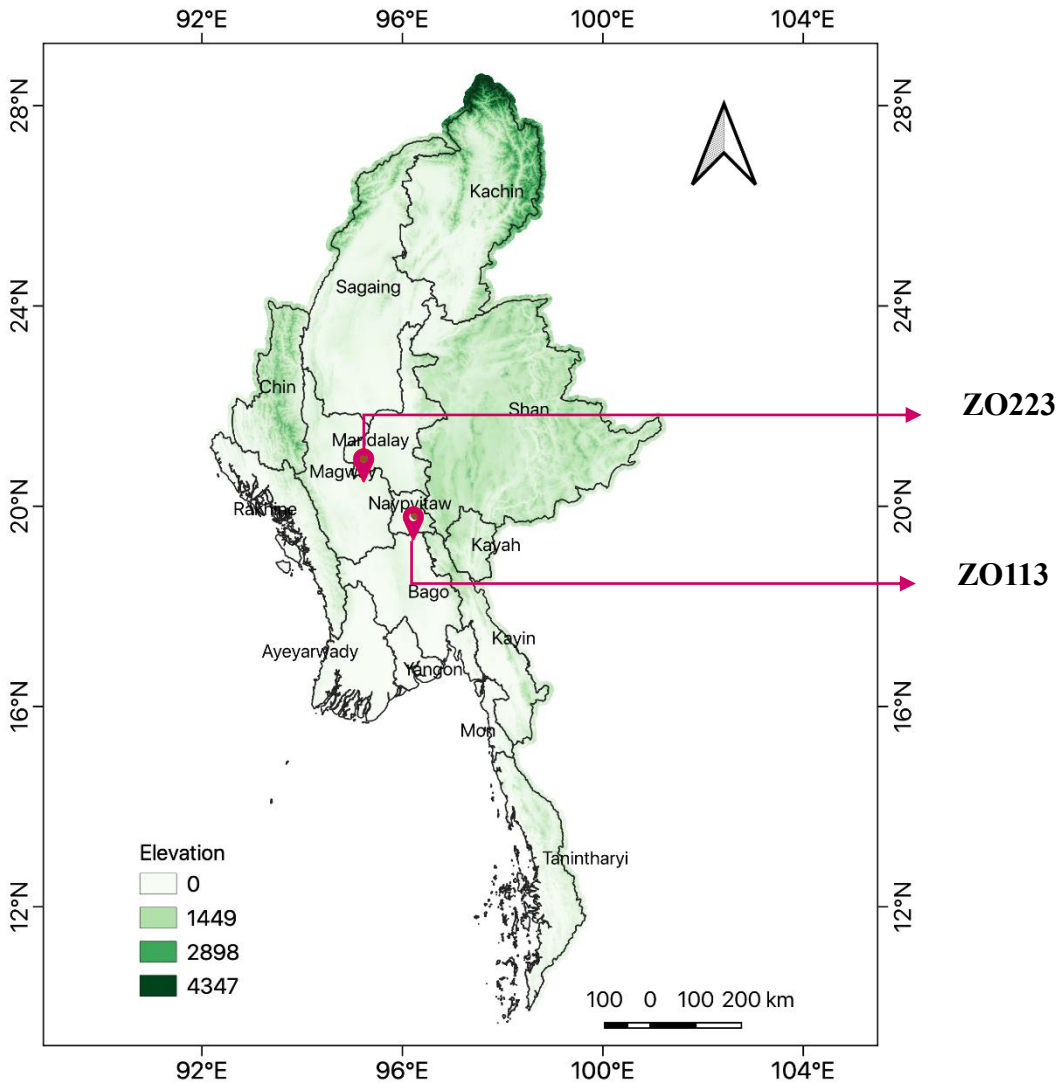

Figure 2.1 Map of Myanmar with the indicated collection site of *Zingiber barbatum* accessions used in the current study. Accession ZO113 was collected in Yezin of Nay Pyi Taw region (former Mandalay region) at elevation 213 m a.s.l; and accession ZO223 was collected in Kyauk Pa Daung township of Mandalay region at elevation 595 m a.s.l.

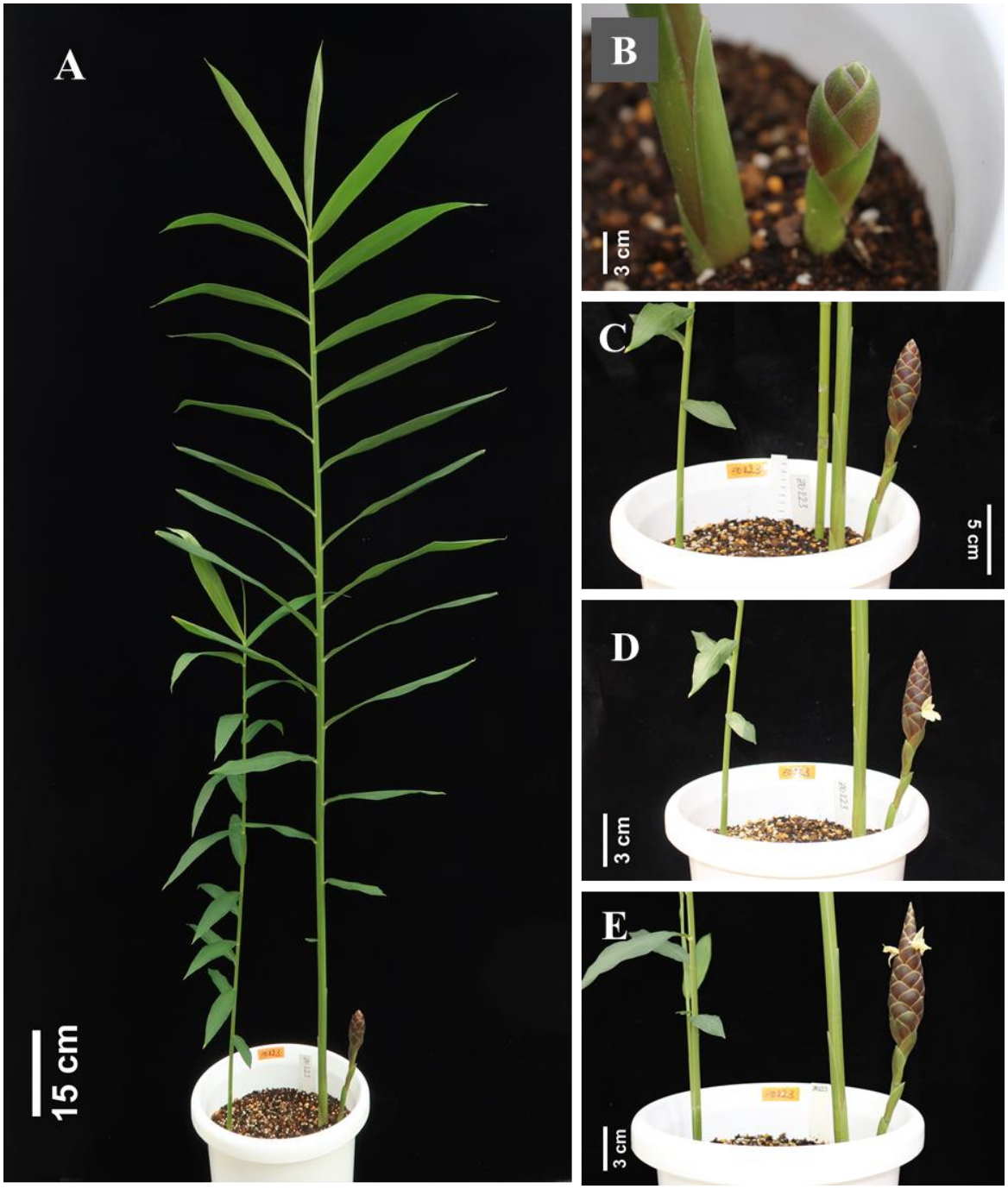

Figure 2.2 Inflorescence of *Zingiber barbatum* accession ZO223 at the different stages of growth and flowering. A, Plant with inflorescence; B, Emerged inflorescence of 5 days old; C, Inflorescence of 24 days old; D, Inflorescence with flowers at the stage of blossom beginning (35 days old); E, Inflorescence almost at the stage of blossom ending (48 days old).

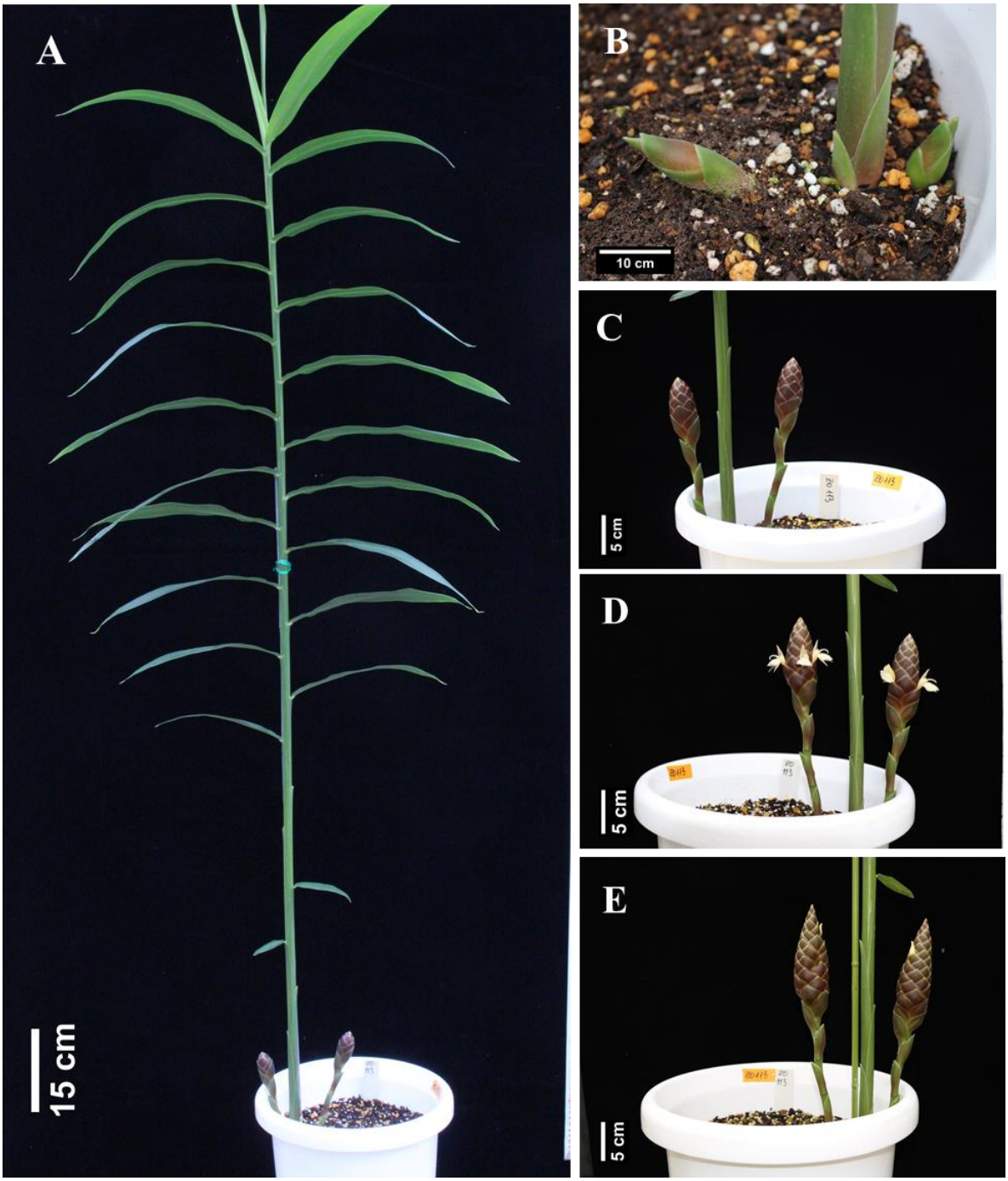

Figure 2.3 Inflorescence of *Zingiber barbatum* accession ZO113 at the different stages of growth and flowering. A, Plant with inflorescences; B, Emerged inflorescences of 3-5 days old; C, Inflorescences of 26-28 days old; D, Inflorescences with flowers at the stage of blossom beginning (35-37 days old); E, Inflorescence almost at the stage of blossom ending (55-57 days old).

Figure 2.4 Senescence of the inflorescence in *Zingiber barbatum* accession ZO113. A, Inflorescence is gradually changing color to orange-red; B, Dried inflorescence.

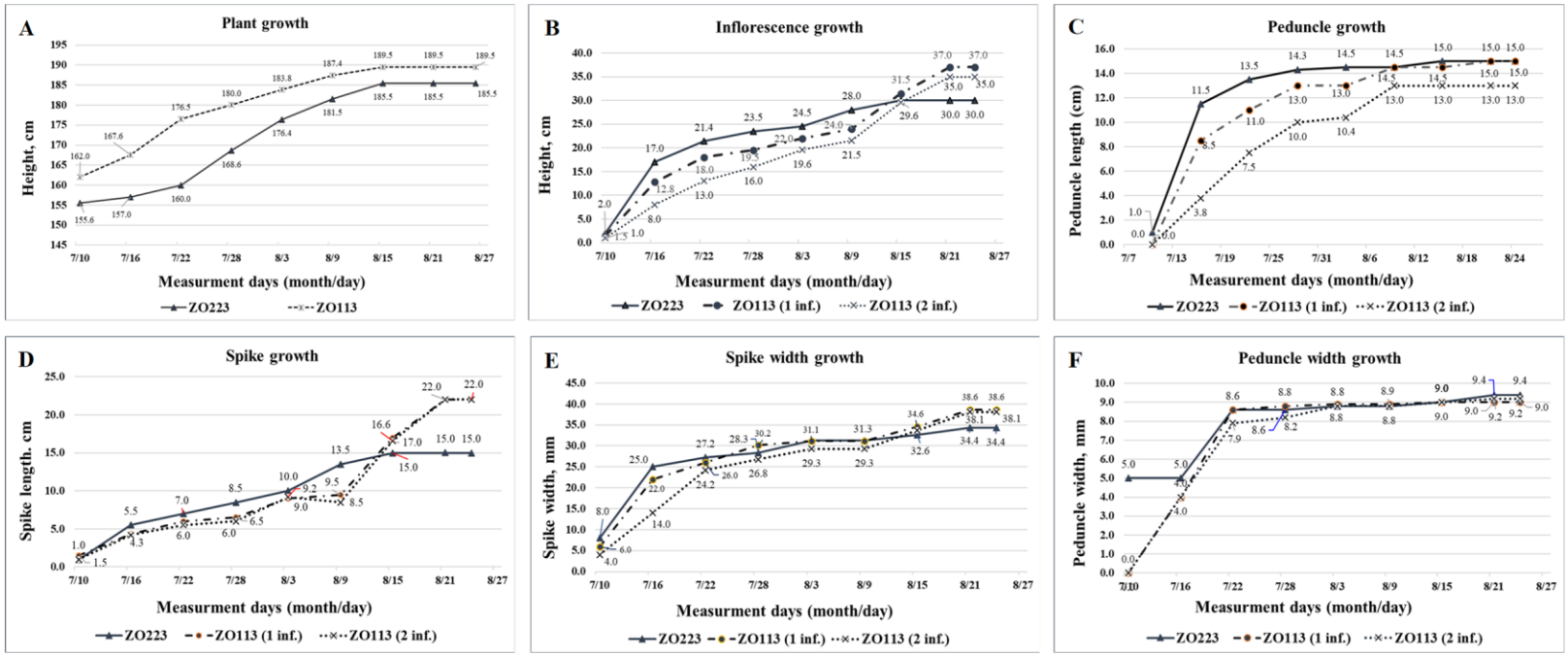

Figure 2.5 Comparative score data on the morphological quantitative measurements between accessions ZO113 and ZO223. Measurements were taken every three days, starting from the day of inflorescence that emerged up to the day when the last three measurements showed the same index. The charts are made based on a six-day measurement index. A, Plant height (cm); B, Total inflorescence height (cm); C, Peduncle length (cm); D, Spike length (cm); E, Spike width (mm); F, Peduncle with (mm). Abbreviation: inf., inflorescence.

Figure 2.6 *Zingiber barbatum* flowers and its dissected parts in comparison between accessions ZO113 and ZO223. A, Close up view of flower of accession ZO223; B, Close up view of flower of accession ZO113; C, General view of flowers in comparison, flower of accession ZO223 (left, slightly bigger) and flower of accession ZO113 (right); D, Dissected parts of flowers, upper row accession ZO223, lower row, accession ZO113: 1, labellum with two lateral staminodes; 2, dorsal corolla lobes; 3, two lateral corolla lobes; 4, stamens. E, Anthers of flowers in comparison (front side), upper row accession ZO223, lower row, accession ZO113. F, Stigma.

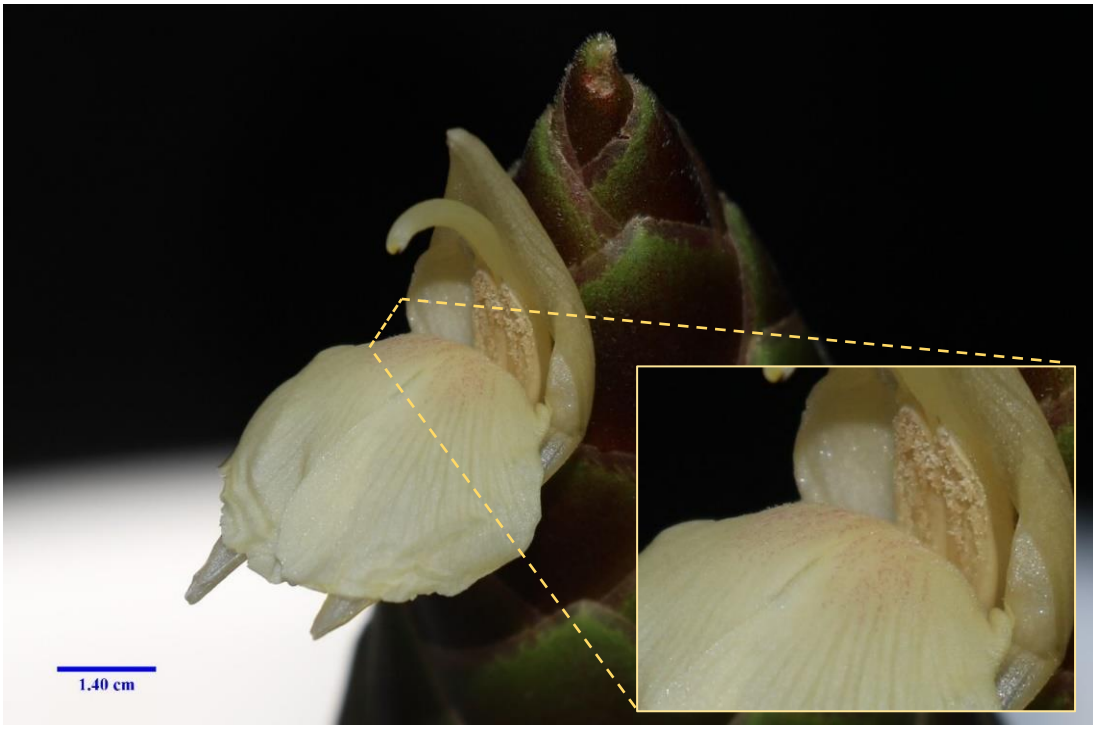

Figure 2.7 Flower of *Zingiber barbatum* accession ZO113 displaying the pinkish dots at the base of the central labellum lobe.

Table 2.1 Comparative morphological characteristics of similarities and/or differences of *Zingiber barbatum* based on quantitative and qualitative assessments.

Attributes	***Z. barbatum* (ZO113)**	***Z. barbatum* (ZO223)**	***Z. barbatum***	***Z. barbatum***
Literature source for description	This study	This study	Wallich N., 1830	Theilade I., 1999
Distribution (region/country)	Nay Pyi Taw	Mandalay	Prome, Burma	Chiang Mai, Thailand
Elevation	213 m a.s.l.	595 m a.s.l.	not reported	100-150 m a.s.l.
Number of inflorescences	2	1	not reported	not reported
Plant total height	185.5 cm	189.5	121.9-152.4	0.6 - 1.0
Inflorescence features	radical, on erect, rigid peduncle, dense, ovate-oblong, apex acute	radical, on erect, rigid peduncle, dense, elongated ovate-oblong, apex acuminate	radical, on thick and rigid, short peduncle, ovate, apex acute	radical, on erect peduncle, ovate to conical, apex acute
Inflorescence length	35.0-37.0 cm	30.0 cm	not reported	not reported

Table 2.1 Continued

Attributes	*Z. barbatum* (ZO113)	*Z. barbatum* (ZO223)	*Z. barbatum*	*Z. barbatum*
Peduncle length	13.0-15.0 cm	15.0 cm	5.0 cm	2.0-6.0 cm
Peduncle width	9.0-9.2 mm	9.4 mm	not reported	not reported
Spike length	22.0 cm	15.0 cm	5.0-7.5 cm	5.0-10.0 (12.0) cm
Spike width	38.1-38.6 mm	34.4 mm	n/a	3.0-4.0 mm
Bract features	imbricated, broadly ovate, with papery margin, apex obtuse-acute, protuberant, velvety-villous, dark red	imbricated, broadly ovate, with papery margin, apex obtuse, protuberant, velvety-villous, dark red	imbricated, broadly ovate, convex, ventricose, apex cuspidate, hairy at all part, dull dark reddish or greenish	ovate to cuspidate, upper ones narrower and more pointed, villous
Flowering month	August	August	August - February (Calcutta Garden)	not reported
Duration of flowering	21-22 days	19 days	not reported	not reported
Number of flowers	47-48	40	not reported	not reported

Table 2.1 Continued

Attributes	*Z. barbatum* (ZO113)	*Z. barbatum* (ZO223)	*Z. barbatum*	*Z. barbatum*
Flower features	pale-yellow, zygomorphous, monoclinous, ephemeral, epigynous, with light fragrance, longistylous		big in size, white to pale pink	white
Calyx features	glabrous, membranous, apex shortly serrated		two-dentate	white in colour
Corolla features	lobes subequal in length, lanceolate, pale-yellow, longitudinally striped, slightly concave in hood		lobes lanceolate, dorsal lobe ascended, rostrate-acuminate, apex concave	white, dorsal lobe larger than lateral lobes
Labellum features	pale-yellow, pinky tinge in the bottom of mid-lobe, soft-velvety, mid-lobe obcordate, margin slightly undulate-reflexing, mid-lobe bifurcated in the middle, lateral staminode ovate-lanceolate, apex acute	light-yellow, soft-velvety, mid-lobe obcordate, margin slightly undulate-reflexing, lateral staminode ovate-lanceolate, apex acute	labellum ovate, apex emarginate, notched, bellow convex	labellum white with a yellow tinge inside; mid-lobe obovate, emarginate; side lobes small

Table 2.1 Continued

Attributes	*Z. barbatum* (ZO113)	*Z. barbatum* (ZO223)	*Z. barbatum*	*Z. barbatum*
Stamen and carpel features	anther c. 1.6 cm long, light yellow, thecae cylindrical, parallel, dehiscence longitudinal, anther crest hooded, wrapped around style; style white, glabrous, filiform; stigma white, scarcely wider than style, tubular, downwards-facing, ostiole ciliate		anther large, subsessile, anther crest protruded over curved lip, elevated above anther; style clavate, apex curved, slightly protuberate; stigma convex, ciliate	not reported
Capsule (fruit/seed)	not observed		capsule obovate, tufted, glabrous, cherry-size; seeds, black, aril big, white	not reported

CHAPTER 3

Identification and Characterization of Volatile Organic Compounds in *Zingiber barbatum* Wall. from Myanmar

3.1 Introduction

Volatile organic compounds (VOCs) are involved in the various processes of the plant's vital activity. Plants produce, store and emit VOCs for communication and interaction, which manifests as a quick defense signaling between distant organs for enhancing resistance to an upcoming stress (Conrath *et al.*, 2002; Baldwin *et al.*, 2006; Bennaoum and Benhassaini, 2019). VOC emission strongly depends on the plant species (Llusia *et al.*, 2002) and separate plant lineages often resolve the same problem (for example, attracting pollinators, enhancing resistance to stress, plant-to-plant interaction) by adopting different chemical solutions (Vivaldo *et al.*, 2017). As secondary metabolites, VOCs have an important chemical diversity and are differently represented in specific taxa, thus they can also serve as chemotaxonomic markers (Franz and Novak, 2015; Bennaoum and Benhassaini, 2019).

From the taxonomic or systematic point of view, the distinctive feature is not the production of the essential oil itself, but the biosynthetically specific group of substances (e.g. flavonoids, sulfur compounds, terpenoids) characteristic to specific species; as more substances are derived in the biosynthetic pathway, the more specific it can be for certain taxa (Franz and Novak, 2015).

3.1.1 Volatile organic compounds characteristic to the members of the genus *Zingiber*

The wild medicinal species are valued as a source of useful bioactive constituents possessing medicinal properties. *Zingiber* species have been commonly consumed as a spice, a food, a dietary supplement and have been used as a traditional remedy in Asian and Southeast Asian countries since ancient times Among *Zingiber* species, the phytochemical composition of *Zingiber officinale* (edible ginger), *Z. montanum* (cassumunar ginger), *Z. zerumbet* (bitter ginger), and *Z. mioga* (Japanese ginger) is quite well reported. The rhizome has been accepted as a valuable part of the plant due to various biological activities such as anti-inflammatory (Ozaki, *et al.*, 1991; Singh *et al.*, 2005; Zhang *et al.*, 2016), antioxidant (Nile and Park, 2015; An *et al.*, 2016), antimicrobial (Pithayanukul *et al.*, 2007; Sasidharan and Menon, 2010; Mesomo *et al.*, 2013; Kumar *et al.*, 2014)

and anticancer (Murakami *et al.*, 2002; Kirana *et al.*, 2003; Takada *et al.*, 2005; Citronberg *et al.*, 2013).

The unique aroma, flavor and various bioactive properties of *Zingiber* species are related to the combination of their chemical constituents: volatile compounds (constituting essential oil) and phenolic compounds (gingerols, shogaols, paradols) (Vernin and Parkanyi, 2005; Mao *et al.*, 2019). The essential oil of *Zingiber* species is a complex mixture of VOCs, mainly consisting of mono- and sesquiterpenes with different functional groups varying in structures (Vernin and Parkanyi, 2005; Wohlmuth *et al.*, 2006; Mao *et al.*, 2019). The species-related "unique" volatile substances are reported for *Z. officinale*, α-zingiberene, geranial, ar-curcumene; *Z. montanum*, sabinene, (*Z*)-ocimene, terpinen-4-ol; *Z. zerumbet*, zerumbone, pinene; and *Z. mioga*, β-phellandrene and β-elemene (Kurobayashi *et al.*, 1991; Wohlmuth *et al.*, 2006; Sukatta *et al.*, 2009; Sasidharan and Menon, 2010; Sharma *et al.*, 2016; Tan *et al.*, 2018).

Z. barbatum has a long history of use as an anti-inflammatory and analgesic herbal remedy in the traditional medicine of Myanmar. It is used for treating gout and relieving muscle, bone, and joint pain. However, the composition of both volatile and non-volatile constituents of *Z. barbatum* has never been screened or reported on to date. Therefore, the objective of this study was the identification and characterization of the VOC composition in the rhizome of *Z. barbatum* landraces.

3.1.2 Gas chromatography-mass spectrometry (GC-MS) method used on the identification of VOCs

The hydrodistillation and solvent extraction methods are often used to collect VOCs from various plant matrices (Tholl *et al.*, 2006; Ormeño *et al.*, 2011; George *et al.*, 2018). However, these methods have several disadvantages associated with low recovery, destruction of sample matrix and the use of destructive organic solvents (George *et al.*, 2018). The headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) is non-destructive, efficient and solvent-free and is the most common approach used to collect VOCs from plants (Materić *et al.*, 2015; Kusano *et al.*, 2016).

In this study, the VOCs analyzed using solid-phase microextraction (SPME) methods following the protocol of Kusano *et al.* (2016), which can be directly analyzed using gas chromatography combined with time-of-flight-mass spectrometry (GC-TOF-MS).

3.2 Materials and Methods

3.2.1 Plant material and selection criteria

To achieve the objectives of this study, the rhizomes of six *Z. barbatum* accessions from the collection of the GRC UT (Tsukuba, Japan) were used as experimental material (Table 3.1). An individual rhizome of each candidate *Z. barbatum* accession was planted in a plastic pot of 30 x 50 cm and maintained in the open field of GRC UT from May to October of 2019. The candidate samples were selected based on geographic origins representing three different regions of Myanmar, and based on the reported detected genetic diversity in a former study (Wicaksana *et al.*, 2011). The study was conducted in November after the rhizomes were harvested.

3.2.2 Experimental design, sample collection and preparation of VOC extraction for GC-MS analysis

The experimental design included two biological replicates for each accession, and two analytical replications for each biological replicate, respectively.

Z. barbatum rhizomes were harvested, washed well and cut into small, 0.5 cm^2, pieces. Approximately 10 g of chopped pieces were cryohomogenized to a fine powder in Multi-beads Shocker MB2000 (Yasui Kikai Co., Ltd., Osaka, Japan) and used to prepare the aliquots in the working concentration of 500 µg/mL. The aliquots were stored at -30°C up to use and to quench metabolism. EPA524.2 fortification solution (fluorobenzene, 4-bromofluorobenzene, 1,2-dichlorobenzene-d4) was used as an internal standard solution. The standard *n*-alkane solution was used for the determination of RI (C8–C20).

3.2.3 GC-TOF-MS analysis

The extraction of VOCs and data processing of the current experiment was performed following the workflow scheme adjusted from Kusano *et al.* (2016). The collection of total VOCs in HS samples was carried out using preconditioned solid-phase microextraction (SPME) fiber of 50/30 µm DVB/CAR/PDMS (Supelco, Missouri, USA).

The analytes for VOC extraction were prepared in 20 mL HC vials (Supelco, Missouri, USA) consisting of1.0 mL sample solution (500 µg/mL), 1.0 mL of 100 mM EDTA (pH 7.5) and 10 µL EPA 524.2 fortification solution (20 µg/mL). Samples were incubated for 10 min at 80°C, and then

the VOCs were extracted for 20 min. Samples were introduced randomly through a CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland)

The samples were injected to Agilent 6890N (Agilent Technologies, Wilmington, USA) gas chromatograph equipped with a Rxi-5Sil MS column (30 m × 0.25 mmnID × 0.25 μm; RESTEK, Bellefonte, USA). The injection mode was splitless; helium carrier gas was used at a constant flow rate of 1 mL/min. The GC temperature program was as follows: initial column temperature was maintained at 55°C for 3 min, then increased to 150 °C at the rate of 15 °C/min, and to 200°C at the rate 3°C/min and finally maintained at 200 °C for 2 min. The back-inlet temperature was kept at 250°C.

Mass spectral analysis was performed on Pegasus III 4D TOF-MS (LECO, XX, USA). The MS ionization energy (voltage) was set at 70 eV. The ionization source temperature was 200°C; MS scan range (*m/z*) was 29-500 amu; acquisition rate: 30 spectra/second.

3.2.4 Peak detection, normalization and identification of VOCs

For the peak detection, the non-processed data obtained by GC-TOF-MS analysis were transformed to the NetCDF format using Leco ChromaTOF version 4.71.0.0 (LECO, St. Joseph, USA). All data-pretreatment procedures, including baseline correction, peak alignment, smoothing, time-window setting, and deconvolution by the hierarchical multi-curve resolution (H-MCR) method (Jonsson *et al*., 2006) were carried out using MATLAB 7.0 (Mathworks, Natick, USA).

Normalization of peaks was done by the cross-contribution compensating multiple standard normalization (CCMN) method (Kusano *et al.*, 2016) and by calculating an area of the selected mass spectral values for internal standards using MATLAB R2011b (Mathworks). The adjusted MS mass spectra obtained by the H-MCR method were matched against the reference mass spectra of different libraries (Stein *et al.*, 1999; Skogerson *et al.*, 2011; Adams, 2012; Terpenoids Library - MassFinder, 2020), using the NIST mass spectral search program (version 2.2) and the custom software for peak annotation developed by Kusano *et al*. (2016).

The similarity (≥850 or 900) and the RI difference (<30 units) were used to extract the same or very similar compounds from the referenced libraries and NIST05 (Kusano *et al.*, 2016). When the standard deviation (SD) of the absolute RI difference of these compounds was less than 8.8 units, the similarity of ≥800 with differences less than <20 units were applied, and the peaks were considered as putatively annotated compounds.

3.2.5 Statistical analysis

The multivariate analysis was done on SIMCA 14.0 software (Umetrics AB, Umeå, Sweden) and IBM SPSS software version 24.0 (IBM Corp, Armonk, NY, USA). The VOC profile data were log_2-transformed, and then statistically analyzed using the LIMMA package (Smyth, 2004), which includes FDR correction for multiple testing (Benjamini and Hochberg, 1995) in the R environment for statistical computing (version 3.5.0).

3.3 Results

The VOC profile of *Z. barbatum* was characterized by data of 24 samples (six samples in two biological x two analytical replicates) resulting in 362 extracted mass spectral peaks as a data matrix. The detected peaks were provisionally identified using an automated annotation pipeline, following Kusano *et al.* (2016). The mass spectra (MS) and retention index (RI) of each peak were matched against the reference reported in the libraries (Stein *et al.*, 1999; Skogerson *et al.*, 2011; Adams, 2012; Terpenoids Library-MassFinder, 2020) and used for the identification of VOCs.

3.3.1 Composition of identified VOCs

Out of 362 peaks, 81 detected peaks were considered putatively annotated compounds when their mass spectra matched a value greater than 800 with RIs of corresponding peaks of less than 20 units with those in the corresponding libraries. The molecular formula of each annotated peak, class of chemical compounds and the CAS registered number were investigated using the free open chemistry databases PubChem (Kim *et al.*, 2019) and ChemSpider (ChemSpider. Search and Share Chemistry, 2019).

The molecular formula of each annotated peak allowed the determination of the class and proportion of the main organic compounds after calculation. The proportion of chemical compounds by the class in the profile of *Z. barbatum* samples was calculated by counting the number of annotated compounds in each class as a total of 100%, and then each class was relatively calculated to the summary value. The results revealed that the main class of organic compounds in the HS of *Z. barbatum* species consists of monoterpenoids and sesquiterpenoids, followed by oxygenates (i.e. alcohols, aldehydes, ketones, and esters) and other hydrocarbons (Figure 3.1). Despite the fact, that the group of "Oxygenates" occupies a larger proportion in the graph (40%),

each class separately comprises a small fraction of the group (from the total 100%) and remains lower compared to the proportions of monoterpenes and sesquiterpenes (Figure 3.1).

3.3.2 Multivariate data analysis (MVDA)

The multivariate data analysis (MVDA) such as principal component analysis (PCA) and orthogonal partial least square projection to latent structures differential analysis (OPLS-DA) are used for data analysis and to draw conclusions from the obtained metabolomics dataset (Worley and Powers, 2013). The PCA and OPLS-DA were performed on SIMCA software (version 14.0, Umetrics AB, Umeå, Sweden) to provide a graphical overview and visualize the differences between complex classes of VOC data sets.

The OPLS-DA scores plot generated from the GC-TOF-MS data (n=24) indicated a clear separation among the assessed groups of samples (Wold *et al.*, 2001; Kettaneh *et al.*, 2005). The OPLS-DA allowed the group of ZO105 samples to gather in the center of the score scatter plot and therefore this accession was chosen as a provisional target control sample to perform future data analysis (Figure 3.2). The first two principle components of the PCA accounted for a variance of 13.2% (R2X[1]=0.132) and 8.4% (R2X[2]=0.0837).

A total of 81 determined volatile organic compounds were included in the PCA and hierarchical cluster analysis (HCA) on the Euclidean distance from the average value (two biological and two analytical replicates) of the data after normalization using SPSS software version 24 (IBM Corp, Armonk, NY, USA) (Figures 3.3 and 3.4).

The PCA analysis indicated a clear separation between groups and displayed two groups (Group I and II, Figure 3.3) that were situated in the positive quadrants of the generated PCA plot. The contribution of two first components, PC1 and PC2, towards variance was 81.6% and 11.9%, respectively, with a total cumulative contribution of 93.5% of variance. The accessions ZO63 and ZO160 from the Bago region were grouped together into Group I, whereas Group II was comprised of accessions ZO105, ZO191, ZO217 and ZO223 from the Mandalay region and Shan State.

The HCA dendrogram between groups indicated a cluster similarity and hierarchical relationship and generated a solution with two clusters (Figure 3.4). The number of clusters (I and II) was determined by using the rescaled distances in the dendrogram based on a cut-off point where the distances among combined clusters increase substantially as the between-group variability increases in terms of volatile composition. These clusters are formed in the same groups

in the generated PCA plot: the vertical axis correlated positively with Cluster I and the horizontal axis correlated positively with Cluster II.

3.3.3 Identified VOCs

The VOC changes of each *Z. barbatum* accession compared to the control (ZO105) were recorded by subtracting the average of the normalized responses of the annotated peaks (log_2-transformed value) and assessing the extent of significant difference (false discovery rate (FDR) <0.05), which was presented as the fold change value of a metabolite concentration normalized relative to the control.

The results show that two accessions, ZO191 and ZO223, had s VOC profile similar to that of the control accession ZO105. The level of the annotated VOCs between these accessions does not show any significant differences. The VOC profile of accession ZO217 was similar to the control ZO105, except for two compounds – terepinen-4-ol and 2,5-bornanediol. Two accessions ZO63 and ZO160 differed significantly (FDR<0.05) compared to the control accession ZO105. In particular, 11 compounds and 14 compounds in the profiles of accessions ZO63 and ZO160, respectively, were different compared to the control accession ZO105 (Table 3.2). The identified discriminative compounds were elemol acetate, β-farnesene, α-ylangene, α-zingiberene, germacrene A, β-bisabolene, β-sesquiphellandrene, valencene, cuparene, and selina-5,11-diene. Moreover, two compounds α-bergamotene and β-(*E*)-guaiene were not detected in the VOC profile of the control accession ZO105 (Table 3.3).

The study revealed that among 81 compounds, 24 compounds were significantly different in content between the six examined *Z. barbatum* accessions (Table 3.3). The compounds that showed significant differences between accessions were butyl pivalate, α-phellandrene, α-terpinene, β-phellandrene, 3-methyldecane, γ-terpinene, (4*E*)-7-methyl-4-decene, terpinolene, (E)-3-caren-2-ol, octyl acetate, terpinen-4-ol, (Z)-sabinene hydrate acetate, bornyl acetate, valeric acid, 2,7,10-trimethyldodecane, 2,5-bornanediol, α-ylangene, 12-chloro-5-dodecyne, β-farnesene, α-zingiberene, (*E*)-β-guaiene, β-bisabolene, β-sesquiphellandrene, 7-epi-α-selinene, and elemol acetate.

3.4 Discussion

The study was conducted in order to identify and characterize the VOC profile of *Z. barbatum* species of what has not been reported to date. The results show that VOC composition were

different among *Z. barbatum* individuals collected from different eco-geographical regions of Myanmar supporting the proposed hypothesis.

The major classes of identified VOCs in the profile of the *Z. barbatum* species were monoterpenes (21%) and sesquiterpenes (31%) (Figure 3.1). Most of the terpenoids possess pronounced biological activities, due to which wide applications for pharmacology, medicine and biotechnology are found. Monoterpenes are active biologically with strong antibacterial, anti-inflammatory and antitumor activities (Koziol *et al.*, 2014; Sobral *et al.*, 2014). The compounds belonging to the sesquiterpene class possess antimicrobial and anti-insecticidal agents and influence the regulation and prevention of oxidative damage and inflammation-mediated biological damage (Repetto and Boveris, 2010; Perveen, 2018). The combination of ambient temperature, vapor pressure, and size of the monoterpene pool in plant tissue are the most significant factors influencing the monoterpene emission, while the sesquiterpenes possess high reactivity and low vapor pressure (Materić *et al.*, 2015). In this regard, it can be suggested that the healing properties of a medicinal product from the milled rhizome of *Z. barbatum* can probably be due to the bioactivity of the monoterpene class compounds, which may activate during a steam-heating process.

The study revealed that four *Z. barbatum* accessions collected from Shan State and Mandalay region, i.e. ZO105, ZO190, ZO217, and ZO223, have similar VOC profiles (Table 3.3). Whereas, two accessions collected from the Bago region, i.e., ZO63 and ZO160, had variation in terms of the VOC composition compared to the four other accessions. In total, 24 compounds were significantly different between the examined samples. The multivariate analysis (PCA and HCA) has also supported this intraspecific discrimination in terms of VOC composition between the group of the assessed *Z. barbatum* accessions. The studies on genetic diversity using morphological and molecular markers have also revealed variation among *Z. barbatum* accessions dividing them into two morphotype groups with comparatively higher genetic diversity (Wicaksana *et al.*, 2011).

The *Z. barbatum* accessions used in this study had a pleasant camphor-citrus aroma which were different between accessions when olfactory tested. Most of the aroma-contributing volatiles are reported for monoterpenoids (Iijima *et al.*, 2014). Geraniol diphosphate (GDP) is a universal monoterpenoid precursor to producing geraniol by geraniol synthase in plants (Dong *et al.*, 2013). The geraniol and its derivatives geranial, geranyl acetate, geraniol and citronellol are the major aroma-contributing compounds reported for the *Z. officinale* rhizome, which is characterized by a pleasant fresh citrus aroma and was reported as the most olfactory aroma-active compound

(Nishimura, 1995). Even though the geraniol-related compounds are structurally similar, they differ in aroma properties and the composition of compounds and can be variable due to the cultivation environment and maturity of the rhizome (Iijima *et al.*, 2014). Seventeen monoterpenoids have been identified in the VOC profile of *Z. barbatum*, which might contribute to a light lemon-mint or lemon-camphor aroma in this species.

Plants maintain the memory of any stress event they have experienced and VOCs are one of the factors able to shape the plant's stress memory because of their volatility allowing the plant to quickly reach distant plant parts (Brilli *et al.*, 2019). In this study, all accessions were grown in uniform (i.e., in pots in the field of GRC UT) at the same altitude and under the same ecological conditions, due to which the four *Z. barbatum* accessions (ZO190, ZO217, ZO223 and ZO150) collected from different eco-geographical regions of Myanmar possess similar VOC profiles. However, the variation of VOCs observed in accessions ZO63 and ZO160 is probably related to the influence of geographical and ecological (abiotic, biotic) factors on the production of VOCs in plants referring to the "plant memory" (Brilli *et al.*, 2019). Demasi *et al.* (2018) reported on qualitative and quantitative intra-specific variations in secondary metabolites of *Lavandula angustifolia* Mill. due to altitude influence; sesquiterpenes were present in higher amounts of the low-altitude populations. Negative correlations of secondary metabolites with latitude and positive correlations with temperature were reported by Guo *et al.* (2013) for *Scutellaria baicalensis*.

VOCs play a prevailing role in the evolutionary process as a response to biotic and abiotic stresses and the adaptation of the plant in its environment (Dicke and Loreto, 2010; Loreto and Schnitzler, 2010; Vivaldo *et al.*, 2017). VOCs such as monoterpenes (camphene and pinene) actively participate in the mechanisms leading to systemic acquired resistance (SAR). For instance, bergamotenes serve as pheromones for some insects, thus plants defend themselves by attracting the predators of herbivorous pests by producing such natural pheromones; the green leaf volatiles (GLVs) such as *Z*-3-hexenyl acetate, can induce the resistance of plants to fungal pathogens or reduce cold-stress damage (Kessler and Baldwin, 2001; Schnee *et al.*, 2006; Brilli *et al.*, 2019). Given the aforesaid, it can be assumed that the similarities and differences of VOCs observed within *Z. barbatum* might be related to the plant memory, i.e., the place of origin where they were collected. The four accessions ZO190, ZO217, ZO223 and ZO105 with similar VOC profiles might be more tolerant of abiotic and biotic stresses, while accessions ZO63 and ZO160 are more sensitive to the stresses in a natural environment, due to which their VOC profiles were different compared to the control ZO105.

3.5 Practical value and application

The results demonstrate that the GC-TOF-MS approach is suitable for the identification of VOCs and detection of the chemical variation in unknown species for comparative analysis. Untargeted GC-TOF-MS analysis can be used for the general assessment and characterization of VOCs. In perspective, the targeted (quantitative) analysis is needed to reveal chemical diversity of the specific compound of interest according to proposed objectives.

The current study is the first report on the characterization of VOC composition in underexploited medicinal *Z. barbatum* species, which has not been reported to date. The information from this study could be useful for ethnobotanical and pharmacological studies. The results can serve as a base for investigating the potential therapeutic effects of *Z. barbatum* as a medicinal species and to validate its traditional use as a healing remedy.

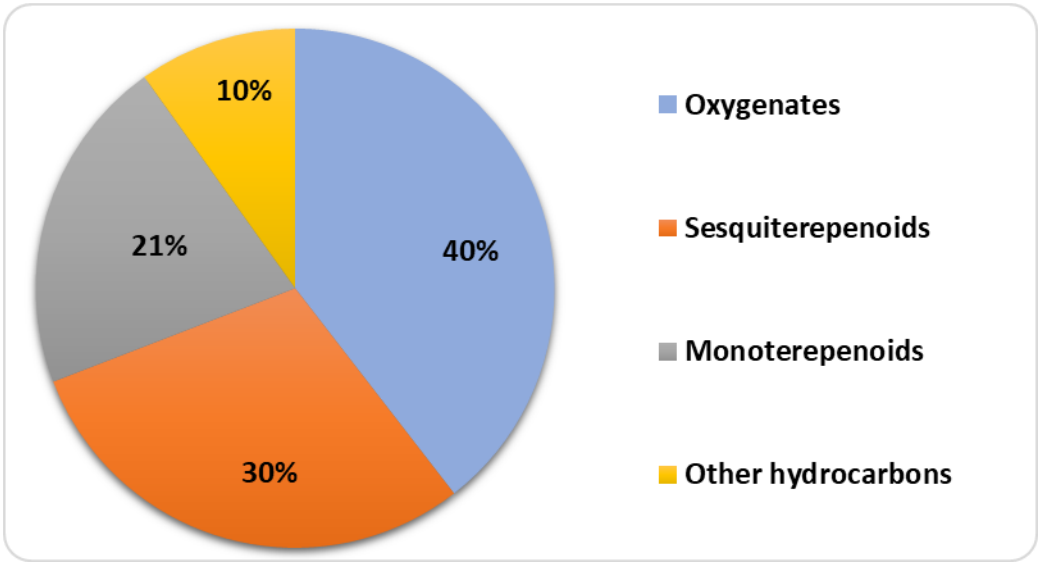

Figure 3.1 Proportion chart of the identified classes of organic compounds from 81 putatively annotated peaks in the headspace of six *Zingiber barbatum* accessions. Pie chart "Oxygenates" consists of the combined classes of alcohols, aldehydes, ketones, and esters.

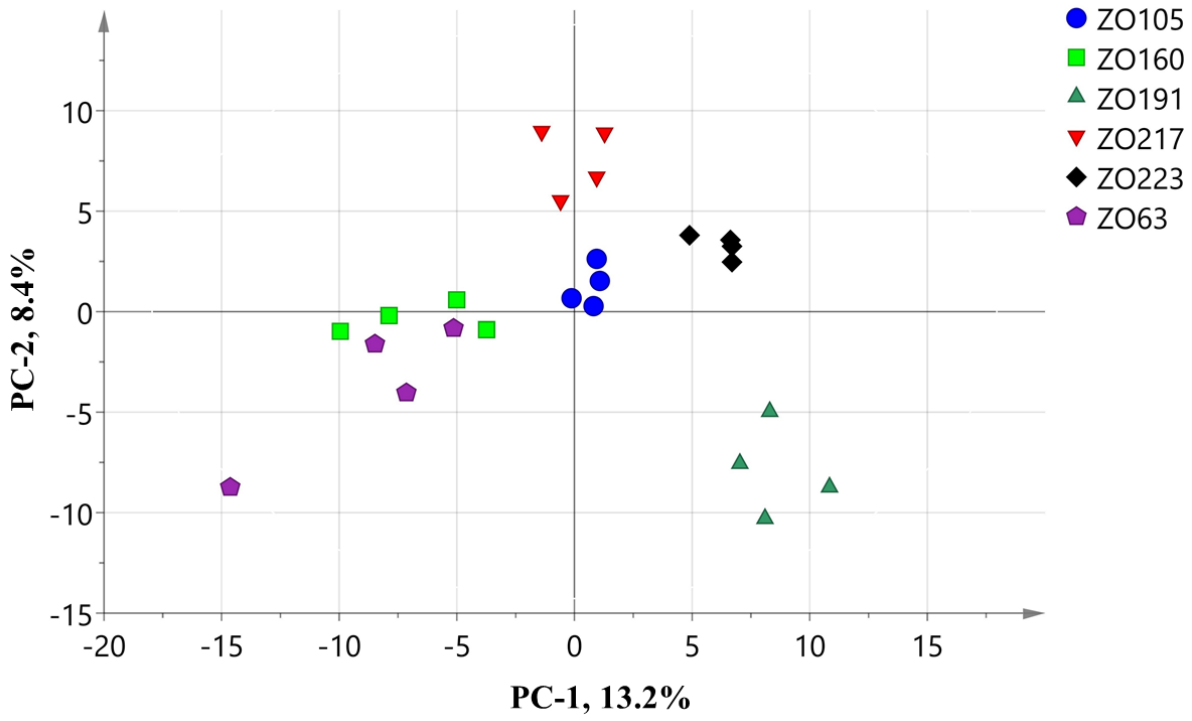

Figure 3.2 The OPLS-DA score plot generated from the GC-TOF-MS data (*n*=24) of six *Zingiber barbatum* accessions. The first two principle components of the PCA accounted for a total 21.6% of variances.

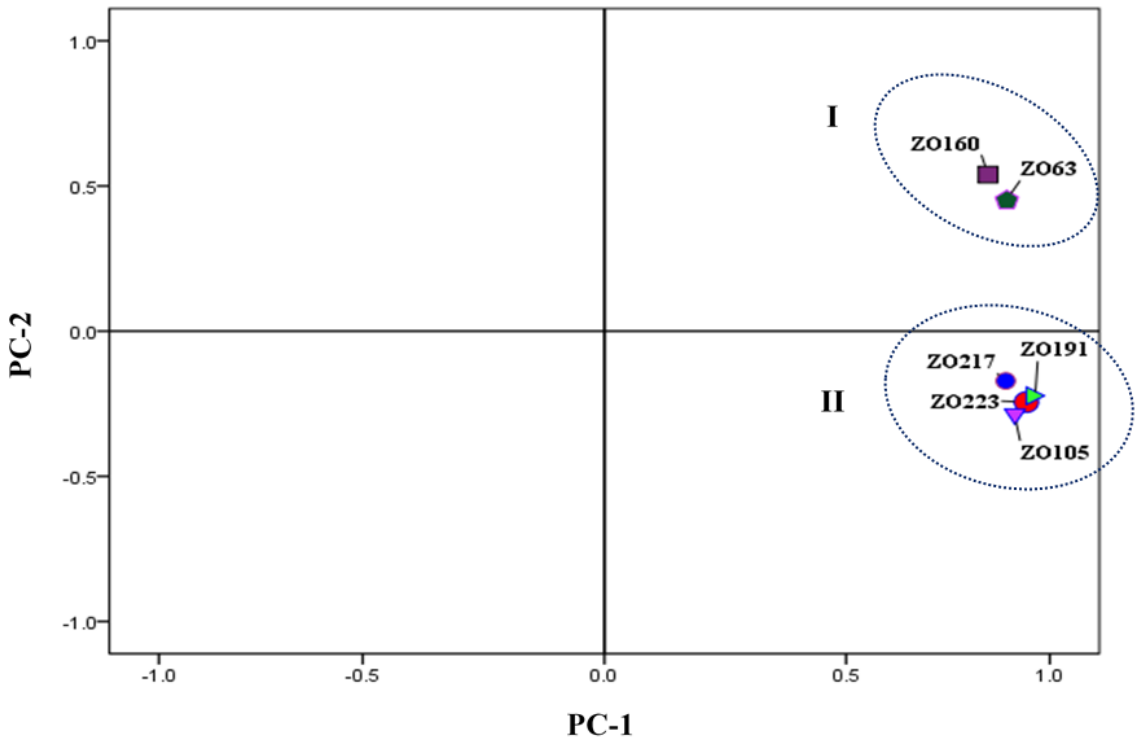

Figure 3.3 Principal component analysis (PCA) based on identified VOCs in the profile of six *Zingiber barbatum* accessions. PCA score plot grouped the examined *Z. barbatum* accessions into Groups I and II. The contributions of the first and second principal components were 81.6% and 11.9%, respectively.

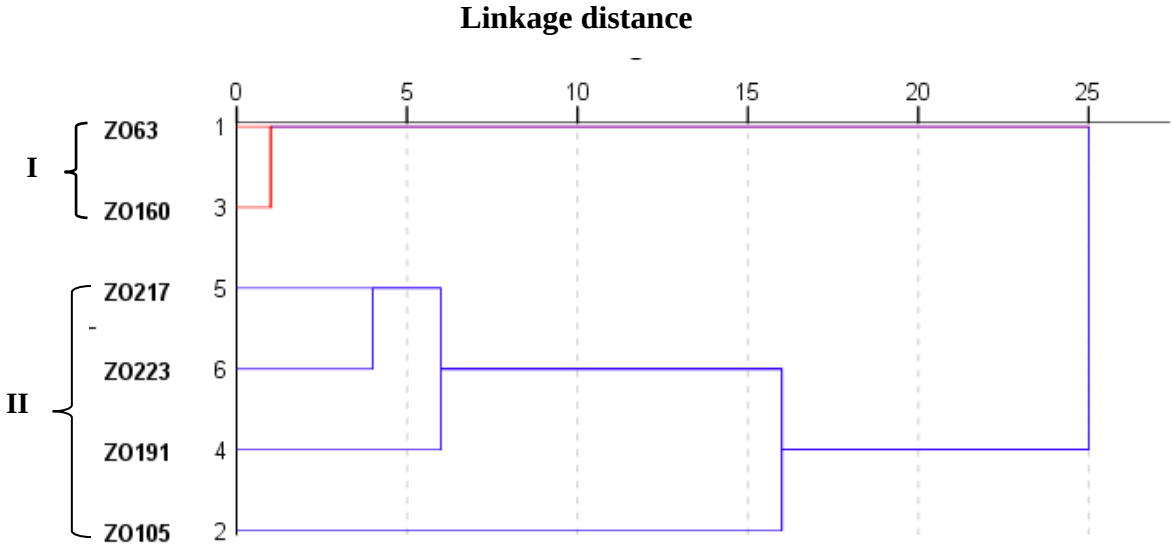

Figure 3.4 Dendrogram obtained by hierarchical cluster analysis (HCA) based on the Euclidean distance between groups of six *Zingiber barbatum* accessions.

Table 3.1 The origin background of *Zingiber barbatum* accessions used for characterization of the VOC composition.

No	Accession (GRC UT)	Species	Collection site		Elevation (m a.s.l.)	Year of acquisition by SMTA
			Nearest city	Region/State		
1	ZO63	*Z. barbatum*	Nattalin	Bago	4	2004
2	ZO105	*Z. barbatum*	Pyon oo lwin	Mandalay	1,070	2004
3	ZO160	*Z. barbatum*	Thayarwaddy	Bago	15	2007
4	ZO191	*Z. barbatum*	Pin Da Ya	Shan	1,164	2008
5	ZO217	*Z. barbatum*	Aung Ban	Shan	1,367	2009
6	ZO223	*Z. barbatum*	Kyauk Pa Daung	Mandalay	595	2009

Table 3.2 The VOCs that significantly differed in accessions ZO63 and ZO160 compared to the control accession ZO105 based on Log_2 transformed data.

ID	Compound	RI	Molecular formula	ZO105/ZO63			ZO105/ZO160		
				log_2FC	***p*-value**	**FDR**	**log_2FC**	***p*-value**	**FDR**
ID197	2,5-bornanediol	1339.4	$C_{10}H_{18}O_2$	-6.93	0.00	0.06	-8.40	0.00	0.03
ID214	α-Ylangene	1385.7	$C_{15}H_{24}$	7.09	0.00	0.02	7.17	0.00	0.01
ID236	12-Chloro-5-dodecyne	1445.2	$C_{12}H_{21}Cl$	4.24	0.00	0.04	4.53	0.00	0.00
ID238	β-Farnesene	1450.2	$C_{15}H_{24}$	7.61	0.00	0.00	7.16	0.00	0.01
ID240	Selina-5,11-diene	1455.6	$C_{15}H_{24}$	13.93	0.00	0.00	13.97	0.00	0.00
ID257	Cuparene	1484.2	$C_{15}H_{22}$	3.49	0.00	0.06	3.54	0.00	0.03
ID261	γ-Amorphene	1491.3	$C_{15}H_{24}$	3.55	0.01	0.08	7.36	0.00	0.03
ID262	Germacrene A	1495.1	$C_{15}H_{24}$	11.87	0.00	0.00	11.99	0.00	0.00
ID263	α-Zingiberene	1498.8	$C_{15}H_{24}$	4.54	0.00	0.03	4.55	0.00	0.02
ID265	Valencene	1503.5	$C_{15}H_{24}$	5.77	0.00	0.00	5.79	0.00	0.00
ID269	β-Bisabolene	1511.0	$C_{15}H_{24}$	4.04	0.00	0.02	3.86	0.00	0.03
ID274	β-Sesquiphellandrene	1526.8	$C_{15}H_{24}$	3.45	0.00	0.02	3.57	0.00	0.02
ID276	7-epi-α-Selinene	1529.5	$C_{15}H_{24}$	9.05	0.00	0.00	8.97	0.00	0.00
ID315	Elemol acetate	1671.2	$C_{17}H_{28}O_2$	10.97	0.00	0.00	9.59	0.00	0.00

The log_2-fold change (log_2FC) was calculated using the LIMMA package. The analysis includes two biological and two analytical replicates of each *Z. barbatum* accession. The false discovery rate: FDR< 0.05. Abbreviations: RI - retention index.

Table 3.3 The total identified VOCs in the profile of six *Zingiber barbatum* accessions and the detected significant differences between them.

Compounds	ZO63	ZO105	ZO160	ZO191	ZO217	ZO223
1,3,5-Cycloheptatriene	0.0037 ± 0.00^{a}	0.0030 ± 0.00^{a}	0.0026 ± 0.00^{a}	0.0032 ± 0.00^{a}	0.0016 ± 0.00^{a}	0.0016 ± 0.00^{a}
Hexanal	0.0039 ± 0.00^{a}	0.0053 ± 0.00^{a}	0.0054 ± 0.00^{a}	0.0053 ± 0.00^{a}	0.0026 ± 0.00^{a}	0.0019 ± 0.00^{a}
2-Methylbutan-2-yl acetate	0.0017 ± 0.00^{a}	0.0015 ± 0.00^{a}	0.0025 ± 0.00^{a}	0.0003 ± 0.00^{a}	0.0032 ± 0.00^{a}	0.0000 ± 0.00^{a}
(*Z*)-3-Octene	0.0052 ± 0.00^{a}	0.0051 ± 0.00^{a}	0.0060 ± 0.00^{a}	0.0042 ± 0.00^{a}	0.0044 ± 0.00^{a}	0.0016 ± 0.00^{a}
Heptanal	0.0020 ± 0.00^{a}	0.0016 ± 0.00^{a}	0.0013 ± 0.00^{a}	0.0029 ± 0.00^{a}	0.0004 ± 0.00^{a}	0.0004 ± 0.00^{a}
γ-Butyrolactone	0.0005 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0010 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0020 ± 0.00^{a}	0.0013 ± 0.00^{a}
α-Thujene	0.3026 ± 0.09^{a}	0.5561 ± 0.34^{a}	0.2202 ± 0.07^{a}	0.4575 ± 0.28^{a}	0.1506 ± 0.14^{a}	0.1610 ± 0.09^{a}
2-Octanone	0.0073 ± 0.00^{a}	0.0057 ± 0.00^{a}	0.0038 ± 0.00^{a}	0.0041 ± 0.00^{a}	0.0028 ± 0.00^{a}	0.0010 ± 0.00^{a}
Sabinene	0.2518 ± 0.13^{a}	0.3244 ± 0.15^{a}	0.2460 ± 0.28^{a}	0.5253 ± 0.32^{a}	0.0995 ± 0.04^{a}	0.1621 ± 0.16^{a}
Butyl pivalate	0.0756 ± 0.04^{ab}	0.0596 ± 0.03^{abc}	0.0583 ± 0.03^{abc}	0.0159 ± 0.01^{bc}	0.0867 ± 0.03^{a}	0.0093 ± 0.00^{c}
Decane	0.0822 ± 0.01^{a}	0.0788 ± 0.01^{a}	0.0790 ± 0.01^{a}	0.0878 ± 0.01^{a}	0.0786 ± 0.01^{a}	0.0831 ± 0.01^{a}
Octanal	0.0121 ± 0.00^{a}	0.0106 ± 0.00^{a}	0.0125 ± 0.01^{a}	0.0125 ± 0.00^{a}	0.0086 ± 0.01^{a}	0.0060 ± 0.00^{a}
α-Phellandrene	0.1163 ± 0.02^{ab}	0.2078 ± 0.13^{a}	0.0780 ± 0.01^{ab}	0.1233 ± 0.06^{ab}	0.0352 ± 0.01^{b}	0.0657 ± 0.02^{b}
1,4-Dichlorobenzene	0.0432 ± 0.00^{a}	0.0397 ± 0.01^{a}	0.0420 ± 0.01^{a}	0.0486 ± 0.00^{a}	0.0369 ± 0.01^{a}	0.0384 ± 0.00^{a}
α-Terpinene	0.3295 ± 0.08^{ab}	0.6362 ± 0.43^{a}	0.2094 ± 0.06^{ab}	0.3550 ± 0.19^{ab}	0.0849 ± 0.05^{b}	0.1691 ± 0.08^{ab}
o-Cymene	0.7593 ± 0.29^{a}	1.5060 ± 0.90^{a}	0.6516 ± 0.14^{a}	0.7904 ± 0.49^{a}	0.5244 ± 0.04^{a}	0.5888 ± 0.15^{a}
Limonene	0.0205 ± 0.01^{a}	0.0299 ± 0.02^{a}	0.0130 ± 0.00^{a}	0.0169 ± 0.01^{a}	0.0064 ± 0.00^{a}	0.0085 ± 0.01^{a}
β-Phellandrene	0.1163 ± 0.03^{ab}	0.2334 ± 0.13^{a}	0.0852 ± 0.02^{b}	0.1389 ± 0.07^{ab}	0.0344 ± 0.03^{b}	0.0720 ± 0.03^{b}
3-Methyldecane	0.0012 ± 0.00^{a}	0.0011 ± 0.00^{a}	0.0008 ± 0.00^{ab}	0.0004 ± 0.00^{b}	0.0012 ± 0.00^{a}	0.0003 ± 0.00^{b}

Table 3.3 Continued

Compounds	ZO63	ZO105	ZO160	ZO191	ZO217	ZO223
2-Methyldecane	0.0224 ± 0.01^{a}	0.0217 ± 0.00^{a}	0.0248 ± 0.01^{a}	0.0334 ± 0.01^{a}	0.0209 ± 0.00^{a}	0.0326 ± 0.01^{a}
γ-Terpinene	0.4753 ± 0.12^{ab}	0.9370 ± 0.68^{a}	0.2969 ± 0.10^{ab}	0.5496 ± 0.30^{ab}	0.1251 ± 0.07^{b}	0.2317 ± 0.12^{ab}
1-Octanol	0.0076 ±0.00^{a}	0.0067 ± 0.00^{a}	0.0071 ± 0.00^{a}	0.0080 ± 0.00^{a}	0.0071 ± 0.01^{a}	0.0075 ± 0.00^{a}
(*Z*)-Sabinenhydrate	0.0162 ± 0.02^{a}	0.0077 ± 0.01^{a}	0.0060 ± 0.01^{a}	0.0207 ± 0.01^{a}	0.0043 ± 0.01^{a}	0.0081 ± 0.01^{a}
(*E*)-Sabinenhydrate	0.0663 ± 0.07^{a}	0.0386 ± 0.05^{a}	0.0274 ± 0.04^{a}	0.0807 ± 0.05^{a}	0.0215 ± 0.02^{a}	0.0325 ± 0.03^{a}
(4*E*)-7-Methyl-4-decene	0.0063 ± 0.00^{ab}	0.0057 ± 0.00^{ab}	0.0056 ± 0.00^{ab}	0.0083 ± 0.00^{a}	0.0053 ± 0.00^{b}	0.0069 ± 0.00^{b}
4-Tolualdehyde	0.0022 ± 0.00^{a}	0.0021 ± 0.00^{a}	0.0016 ± 0.00^{a}	0.0008 ± 0.00^{a}	0.0025 ± 0.00^{a}	0.0008 ± 0.00^{a}
Terpinolene	0.1097 ± 0.03^{ab}	0.2114 ± 0.15^{a}	0.0692 ± 0.02^{ab}	0.1185 ± 0.07^{ab}	0.0289 ± 0.02^{b}	0.0544 ± 0.03^{ab}
P-Cymenene	0.0039 ± 0.00^{a}	0.0098 ± 0.01^{a}	0.0029 ± 0.00^{a}	0.0035 ± 0.00^{a}	0.0014 ± 0.00^{a}	0.0024 ± 0.00^{a}
Decane	0.0518 ± 0.01^{a}	0.0569 ± 0.01^{a}	0.0529 ± 0.01^{a}	0.0692 ± 0.01^{a}	0.0499 ± 0.00^{a}	0.0639 ± 0.02^{a}
Nonanal	0.0302 ± 0.03^{a}	0.0571 ± 0.05^{a}	0.0409 ± 0.02^{a}	0.0371 ± 0.05^{a}	0.0513 ± 0.02^{a}	0.0320 ± 0.02^{a}
2,4,6-Trimethyldecane	0.0064 ± 0.00^{a}	0.0070 ± 0.00^{a}	0.0069 ± 0.00^{a}	0.0095 ± 0.00^{a}	0.0057 ± 0.00^{a}	0.0090 ± 0.01^{a}
(*E*)-3-Caren-2-ol	0.0005 ± 0.00^{ab}	0.0012 ± 0.00^{a}	0.0004 ± 0.00^{ab}	0.0008 ± 0.00^{ab}	0.0002 ± 0.00^{b}	0.0004 ± 0.00^{ab}
Octyl acetate	0.0104 ± 0.00^{a}	0.0066 ± 0.00^{ab}	0.0098 ± 0.00^{ab}	0.0079 ± 0.00^{ab}	0.0070 ± 0.00^{ab}	0.0061 ± 0.00^{b}
L-Camphor	0.0043 ± 0.00^{a}	0.0041 ± 0.00^{a}	0.0054 ± 0.00^{a}	0.0063 ± 0.00^{a}	0.0061 ± 0.00^{a}	0.0056 ± 0.00^{a}
1-Nonanol	0.0022 ± 0.00^{a}	0.0013 ± 0.00^{a}	0.0017 ± 0.00^{a}	0.0033 ± 0.00^{a}	0.0025 ± 0.00^{a}	0.0036 ± 0.00^{a}
Tetrahydrolinalyl acetate	0.0009 ± 0.00^{a}	0.0011 ± 0.00^{a}	0.0009 ± 0.00^{a}	0.0010 ± 0.00^{a}	0.0012 ± 0.00^{a}	0.0007 ± 0.00^{a}
Terpinen-4-ol	0.7246 ± 0.04^{b}	1.2493 ± 0.21^{a}	0.6297 ± 0.10^{ac}	0.7903 ± 0.09^{b}	0.2650 ± 0.06^{c}	0.8691 ± 0.32^{b}
Naphthalene	0.0040 ± 0.00^{a}	0.0037 ± 0.00^{a}	0.0040 ± 0.00^{a}	0.0046 ± 0.00^{a}	0.0036 ± 0.00^{a}	0.0043 ± 0.00^{a}

Table 3.3 Continued

Compounds	ZO63	ZO105	ZO160	ZO191	ZO217	ZO223
5,6-Dimethylundecane	0.0180 ± 0.00^{a}	0.0143 ± 0.00^{a}	0.0185 ±0.00^{a}	0.0192 ± 0.00^{a}	0.0176 ± 0.00^{a}	0.0175 ± 0.00^{a}
(*Z*)-Sabinene hydrate acetate	0.0150 ± 0.00^{ab}	0.0255 ± 0.01^{a}	0.0119 ± 0.00^{b}	0.0174 ± 0.01^{ab}	0.0091 ± 0.00^{b}	0.0120 ± 0.01^{b}
1,3-Di-tert-butylbenzene	0.0303 ± 0.01^{a}	0.0318 ± 0.01^{a}	0.0313 ± 0.01^{a}	0.0254 ± 0.01^{a}	0.0340 ± 0.02^{a}	0.0227 ± 0.01^{a}
4,6-Dimethyldodecane	0.0022 ±0.00^{a}	0.0030 ± 0.00^{a}	0.0024 ± 0.00^{a}	0.0040 ± 0.00^{a}	0.0032 ± 0.00^{a}	0.0041 ± 0.00^{a}
Bornyl acetate	0.1514 ±0.04^{a}	0.0481 ± 0.02^{b}	0.1204 ± 0.03^{a}	0.0277 ± 0.01^{b}	0.0265 ± 0.01^{b}	0.0165 ± 0.01^{b}
Terpinen-4-ol acetate	0.0206 ± 0.01^{a}	0.0275 ± 0.01^{a}	0.0133 ± 0.01^{a}	0.0275 ± 0.01^{a}	0.0092 ± 0.00^{a}	0.0168 ± 0.01^{a}
Tridecane	0.0037 ± 0.00^{a}	0.0034 ± 0.00^{a}	0.0034 ± 0.00^{a}	0.0041 ± 0.00^{a}	0.0033 ± 0.00^{a}	0.0027 ± 0.00^{a}
Valeric acid	0.0061 ± 0.00^{a}	0.0014 ± 0.00^{b}	0.0030 ± 0.00^{ab}	0.0011 ± 0.00^{b}	0.0003 ± 0.00^{b}	0.0002 ± 0.00^{b}
2,7,10-Trimethyldodecane	0.0036 ± 0.00^{a}	0.0044 ± 0.00^{a}	0.0027 ± 0.00^{a}	0.0065 ± 0.00^{a}	0.0037 ± 0.00^{a}	0.0043 ± 0.00^{a}
2,5-Bornanediol	0.0001 ± 0.00^{b}	0.0040 ± 0.00^{a}	0.0001 ± 0.00^{b}	0.0032 ± 0.00^{a}	0.0000 ± 0.00^{b}	0.0027 ± 0.00^{a}
β-Terpinyl acetate	0.0923 ± 0.02^{a}	0.0979 ± 0.04^{a}	0.0670 ± 0.04^{ab}	0.0652 ± 0.02^{ab}	0.0265 ± 0.01^{b}	0.0354 ± 0.02^{ab}
Propanoic acid	0.0286 ± 0.00^{a}	0.0265 ± 0.00^{a}	0.0293 ± 0.00^{a}	0.0311 ± 0.00^{a}	0.0317 ± 0.00^{a}	0.0270 ± 0.01^{a}
α-Ylangene	0.0075 ± 0.01^{a}	0.0001 ± 0.00^{b}	0.0063 ± 0.00^{ab}	0.0000 ± 0.00^{b}	0.0000 ± 0.00^{b}	0.0000 ± 0.00^{b}
β-Elemene	0.0040 ± 0.00^{a}	0.0011 ± 0.00^{a}	0.0049 ± 0.00^{a}	0.0001 ± 0.00^{a}	0.0001 ± 0.00^{a}	0.0001 ± 0.00^{a}
Tetradecane	0.0355 ± 0.01^{a}	0.0290 ± 0.01^{a}	0.0344 ± 0.01^{a}	0.0445 ± 0.01^{a}	0.0354 ± 0.00^{a}	0.0343 ± 0.01^{a}
γ-Elemene	0.0138 ± 0.02^{a}	0.0015 ± 0.00^{a}	0.0119 ± 0.01^{a}	0.0002 ± 0.00^{a}	0.0004 ± 0.00^{a}	0.0001 ± 0.00^{a}
α-Bergamotene	0.0021 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0018 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}
12-Chloro-5-dodecyne	0.0046 ± 0.00^{a}	0.0002 ± 0.00^{b}	0.0039 ± 0.00^{ab}	0.0004 ± 0.00^{ab}	0.0001 ± 0.00^{b}	0.0001 ± 0.00^{b}
Dihydrocurcumene	0.0070 ± 0.01^{a}	0.0007 ± 0.00^{a}	0.0064 ± 0.00^{a}	0.0006 ± 0.00^{a}	0.0007 ± 0.00^{a}	0.0005 ± 0.00^{a}

Table 3.3 Continued

Compounds	ZO63	ZO105	ZO160	ZO191	ZO217	ZO223
β-Farnesene	0.0304 ± 0.01^{a}	0.0002 ± 0.00^{b}	0.0244 ± 0.01^{a}	0.0004 ± 0.00^{b}	0.0001 ± 0.00^{b}	0.0003 ± 0.00^{b}
Selina-5,11-diene	0.0165 ± 0.02^{a}	0.0000 ± 0.00^{a}	0.0122 ± 0.01^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}
3,11-Acoradiene	0.0213 ± 0.02^{a}	0.0045 ± 0.00^{a}	0.0212 ± 0.02^{a}	0.0037 ± 0.00^{a}	0.0026 ± 0.00^{a}	0.0025 ± 0.00^{a}
α-Curcumene	0.0023 ± 0.00^{a}	0.0003 ± 0.00^{a}	0.0019 ± 0.00^{a}	0.0002 ± 0.00^{a}	0.0002 ± 0.00^{a}	0.0000 ± 0.00^{a}
γ-Curcumene	0.0369 ± 0.04^{a}	0.0025 ± 0.00^{a}	0.0316 ± 0.02^{a}	0.0015 ± 0.00^{a}	0.0008 ± 0.00^{a}	0.0006 ± 0.00^{a}
Cuparene	0.2146 ± 0.20^{a}	0.0178 ± 0.01^{a}	0.1867 ± 0.11^{a}	0.0268 ± 0.02^{a}	0.0126 ± 0.01^{a}	0.0190 ± 0.01^{a}
γ-Amorphene	0.0001 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0007 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}
Germacrene A	0.4349 ± 0.43^{a}	0.0001 ± 0.00^{a}	0.3900 ± 0.22^{a}	0.0001 ± 0.00^{a}	0.0001 ± 0.00^{a}	0.0001 ± 0.00^{a}
α-Zingiberene	0.3821 ± 0.34^{a}	0.0191 ± 0.02^{b}	0.3373 ± 0.17^{ab}	0.0206 ± 0.02^{b}	0.0116 ± 0.01^{b}	0.0110 ± 0.00^{b}
Valencene	0.0501 ± 0.05^{a}	0.0007 ± 0.00^{a}	0.0459 ± 0.03^{a}	0.0007 ± 0.00^{a}	0.0005 ± 0.00^{a}	0.0006 ± 0.00^{a}
2,4-Di-tert-butylphenol	0.7761 ± 0.08^{a}	0.6983 ± 0.14^{a}	0.8000 ± 0.10^{a}	0.7950 ± 0.11^{a}	0.8134 ± 0.13^{a}	0.6575 ± 0.19^{a}
(*E*)-β-Guaiene	0.1372 ± 0.11^{a}	0.0000 ± 0.00^{b}	0.1087 ± 0.06^{ab}	0.0000 ± 0.00^{b}	0.0000 ± 0.00^{b}	0.0000 ± 0.00^{b}
β-Bisabolene	0.0519 ± 0.04^{a}	0.0036 ± 0.00^{bc}	0.0448 ± 0.02^{ab}	0.0075 ± 0.00^{bc}	0.0021 ± 0.00^{c}	0.0038 ± 0.00^{bc}
σ-Cadinene	0.0043 ± 0.00^{a}	0.0008 ± 0.00^{a}	0.0029 ± 0.00^{a}	0.0006 ± 0.00^{a}	0.0005 ±0.00^{a}	0.0004 ± 0.00^{a}
β-Sesquiphellandrene	1.1945 ± 0.80^{a}	0.1111 ± 0.07^{b}	1.3167 ± 0.68^{a}	0.1737 ± 0.11^{b}	0.0875 ± 0.03^{b}	0.0855 ± 0.05^{b}
7-epi-α-Selinene	0.0815 ± 0.07^{a}	0.0002 ± 0.00^{b}	0.0657 ± 0.03^{ab}	0.0003 ± 0.00^{b}	0.0001 ± 0.00^{b}	0.0001 ± 0.00^{b}
1-Iodoundecane	0.0008 ± 0.00^{a}	0.0010 ± 0.00^{a}	0.0007 ± 0.00^{a}	0.0014 ± 0.00^{a}	0.0010 ± 0.00^{a}	0.0012 ± 0.00^{a}
(*E*)-γ-Macrocarpene	0.0081 ± 0.01^{a}	0.0010 ± 0.00^{a}	0.0063 ± 0.00^{a}	0.0001 ± 0.00^{a}	0.0008 ± 0.00^{a}	0.0001 ± 0.00^{a}
Germacrene B	0.0081 ± 0.01^{a}	0.0015 ± 0.00^{a}	0.0076 ± 0.01^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}	0.0000 ± 0.00^{a}

Table 3.3 Continued

Compounds	ZO63	ZO105	ZO160	ZO191	ZO217	ZO223
Trimethyl pentanyl diisobutyrate	0.0164 ± 0.00[a]	0.0169 ± 0.00[a]	0.0163 ± 0.01[a]	0.0186 ± 0.01[a]	0.0183 ± 0.01[a]	0.0126 ± 0.00[a]
2-Allyl-1,4-dimethoxy-3-methylbenzene	0.0190 ± 0.01[a]	0.0112 ± 0.00[a]	0.0148 ± 0.01[a]	0.0349 ± 0.02[a]	0.0315 ± 0.02[a]	0.0214 ± 0.01[a]
Hexadecane	0.0106 ± 0.00[a]	0.0089 ± 0.00[a]	0.0115 ± 0.01[a]	0.0178 ± 0.01[a]	0.0112 ± 0.00[a]	0.0117 ± 0.01[a]
Elemol acetate	0.0493 ± 0.01[a]	0.0000 ±0.00[c]	0.0230 ± 0.02[b]	0.0001 ± 0.00[c]	0.0001 ± 0.00[c]	0.0000 ± 0.00[c]

Compounds with the same letter are not significantly different (Tukey's HCD test, $\alpha = 0.05$). Each value shows the mean ± standard deviation (std) of four replications (two biological and two analytical replications) for each examined accession of *Z. barbatum*.

CHAPTER 4

Estimation of the Relative Nuclear DNA Content, Genome Size, and Inferred Ploidy Level in *Zingiber barbatum* Wall.

4.1 Introduction

Polyploidy is ubiquitous among angiosperms and suggests that ancient whole-genome duplication (WGD) incident in basal angiosperm lineages was a major force of evolution in this clade (Cui *et al.*, 2006; Soltis *et al.*, 2009; Wendel *et al.*, 2016).

Monocotyledons comprise about 25% of all angiosperms (Leitch *et al.*, 2010) in connection with which the estimation and the analysis of the genomic characteristics are keynotes for understanding the evolutionary and phylogenetic relationships in this order. One of the notable features of monocots is wide genomic diversity, which consists of different genomic features such as the number of genomes characterizing the ploidy level of a species; the amount of DNA per genome, known as a genome size; a range of chromosome number and its organization; the genomic nucleotide composition expressed as the proportion of guanine and cytosine bases in the DNA molecule (GC content), and so on (Leitch *et al.*, 2010; Šmarda *et al.*, 2014).

Recent genomic studies (e.g. reconstruction and characterization of the ancestral genome size) in monocotyledons revealed that all monocots' ancestral genome size was small (1C = 1.9 pg) with several major increases and decreases during their evolution (Leitch *et al.*, 2010). Analysis of the genomic diversity in monocots revealed that 70-80% of species are estimated to be polyploids, and their number continues to increase (Goldblatt, 1980; Soltis *et al.*, 2009; Leitch *et al.*, 2010; Paterson *et al.*, 2012; Wendel *et al.*, 2016). Different cytological mechanisms influence the genomic variation most of which is due to the action of the transposable elements (TEs) (De Storme and Mason, 2014; Michael, 2014; Vitte *et al.*, 2014; Wendel *et al.*, 2016).

4.1.1 Mechanisms contributing to genome size reduction and increment

Different mechanisms can influence the genome size increment or reduction. Gene duplication, ploidy alterations (polyploidization), and insertion can contribute to the genome size increment; spontaneous mutations or recombination contributes to both genome gain or decrement, whereas deletion contributes to the genome reduction.

The existence of polyploidy has played an important role not only during the evolution, but also during the domestication processes of the plant species (Zhang *et al.*, 2019). Polyploidy events have been studied in plants extensively (Ruprecht *et al.*, 2017). The WGDs or polyploidy have occurred among all angiosperms at least once, contributed plants adaptation toward the rapidly fluctuating environment (Adams and Wendel, 2005; Chen, 2007).

During the diploidization process, i.e. when the genes are lost or modified resulting in chromosomal rearrangement, unnecessary paralogous regions in euchromatin could reduce, whereas species-specific regions in heterochromatin could increase (Leitch and Bennett, 2004). It has been proposed that the involvement of transposable elements (TEs) are the main sources of mechanisms related to genome size increment during WGD (Bennetzen *et al.*, 2005; Grover and Wendel, 2010).

TEs are segments of DNA that can mobilize from their initial location and (re)insert into a new position in the genome, resulting in new genetic variation within a species (Hirsch and Springer, 2017; Sahebi *et al.*, 2018). The lineage-specific amplification and/or deletion of TEs is common in plants both in close and distant related species and comprises up to 80% of many plant genomes (Vitte *et al.*, 2014; Wendel *et al.*, 2016).

TEs impact the genome size through the involvement in the heterochromatin increment during WGD (Vicient and Casacuberta, 2017). Two classes of TEs have been discovered based on the transposition mechanism: Class I, known as retrotransposons, functions via reverse transcription, i.e., it utilizes a "copy-and-paste" mechanism to amplify DNA sequences; and Class II, DNA transposons encode the protein transposase, which they require for insertion and excision (Ivics and Izsvák, 2005; Wicker *et al.*, 2007; Pray, 2008).

Many species display genome reduction when there is no longer any need on subsets of their genes. This mostly happens when the species adapt to a symbiotic lifestyle and/or loses its photosynthetic function. The species reconfigure plastomes attributable to convergent losses of photosynthesis and functionality of housekeeping genes, providing a good system for studying genome evolution under relaxed selective pressures (Wicke *et al.*, 2013). The loss of photosynthesis betrays the chromosomal architecture in that recombinogenic factors accumulate, resulting in large-scale chromosomal rearrangements as functional reduction proceeds.

While the higher abundance of retrotransposons impacts the plant genome size increment, the illegitimate recombination (IR) and unequal intra-strand homologous recombination (UR) mechanisms involved in a recombination-based type of deletion event result in the reduction of the

genome size (Bennetzen *et al.*, 2005; Grover and Wendel, 2010). The IR and UR influence the decrement of the genome size differently and depending on the plant species (Vitte and Bennetzen, 2006).

4.1.2 Cytological studies in species of the genus *Zingiber*

Members of *Zingiber* display a wide range of inter- and intrageneric variation in their reproductive habits and ploidy levels (Sadhu *et al.*, 2016). Despite the importance of wide use as a vegetable and a medicinal crop, the cytology of *Zingiber* species is studied only to a limited extent. The recently updated Plant DNA C-values database (Leitch *et al.*, 2019) displays records only for *Z. officinale* (Šmarda *et al.*, 2014) and *Z. mioga* (Zhang *et al.*, 2013).

Polyploidy and chromosomal alteration have played a significant role in the evolution and diversification of Zingiberaceae species resulting in a blurring of morphological boundaries between different taxa (Jatoi *et al.*, 2007; Leong-Skornickova *et al.*, 2007). Cytological parameters such as the chromosomal number and genome sizes were used for studying evolutionary aspects in Zingiberaceae; however, many of these studies are old and disagreement still exists among different reports. The extent of polyploidy depends on the basic chromosome number (Jatoi *et al.*, 2007). Frequently, closely related species belonging to the same genus constantly save the basic number of chromosomes. The basic chromosome number reported for members of *Zingiber* is x=11 or x=12 (Jatoi *et al.*, 2007; Nirmal Babu *et al.*, 2012; Šmarda *et al.*, 2014). The complex chromosome structural changes during evolution ensued to increase or decrease the chromosome numbers and the ploidy level in the order Zingiberales (Jatoi *et al.*, 2007). The variation of genome size has significant consequences at the cellular, tissue and organismal levels leading to influence phenological and ecological behaviours of the plants (Leong-Skornickova *et al.*, 2007).

The genome size is characterized in terms of its C-value in picograms (or Mbp) of DNA present in an unreplicated haploid or gametic nucleus (Swift, 1950) and provides important information for genome biodiversity (Zhang *et al.*, 2013). A literature review regarding cytological studies for *Z. barbatum* species is absent. Assessment of *Z. barbatum* genetic resources from Myanmar revealed inter- and intraspecific variability and high genetic diversity (Wicaksana, 2012). Moreover, high intra- and interspecific variation has led to ambiguity concerning species concept, due to the fact that this species is often known by a local name in Myanmar, and the same name is used also for *Z. montanum* (cassumunar ginger).

Hence, due to reported diversity, both genetic and morphological, and the growing interest in the study and conservation of the wild, medicinal and under-exploited species in Myanmar, this cytological study was proposed. The objective was designated in order to evaluate the relative nuclear content, genome size and inferred ploidy level of *Z. barbatum* to characterize its cytological status.

4.2 Materials and Methods

4.2.1 Plant materials

The plant material consisted of twenty *Z. barbatum* accessions from the collection of the GRC UT (Tsukuba, Japan) (Table 4.1). All plant materials used in the current study were maintained in the field of GRC UT under uniform conditions. The cytological evaluation was done on *Z. barbatum* accessions obtained during the field study on the exploration of plant genetic resources in Myanmar under a Grand-in-Aid for Overseas Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Watanabe *et al.*, 2006; Kawase *et al.*, 2011).

4.2.2 Experimental design and plant nuclei isolation

The experiment was carried out in three independent biological replications in order to check and validate experimental reproducibility and reliability. Experimental plants were grown to a size of 50-60 cm with 6-8 fully developed leaves. Fresh young leaves, approximately 4.0 cm in length, were collected between 4:00 to 6:00 AM (JST), and were used as experimental materials to conduct the flow cytometry analysis.

Ready to use Cystain® UV Precise P kit (05-5002 Partec, GmhH, Münster, Germany) direction based on DAPI (4'-6-diamino-2-phenylindole) staining was used for extraction and staining of relative nuclear DNA from plant tissues. Cell nuclei of leaf tissue were isolated by mechanical chopping according to the manufacturer's protocol. Approximately 1 cm^2 of leaf tissue was chopped with a sharp razor blade in a Petri dish (60 mm x 15 mm) containing 400 µL extraction buffer. Suspension was filtered through a 50 µm disposable nylon filter (Sysmex, Partec, GmhH, Goerlitz, Germany) into a 2.0 mL round-bottom sample tube and incubated for 30-60 sec on ice in the dark. Isolated nuclei suspension was stained by adding 1,600 µL staining buffer and immediately analyzed using flow cytometry ploidy analyzer (Partec PA, GmbH, Münster,

Germany). The referenced standard sample (*Z. officinale*) was used to adjust the gain of the flow cytometer before starting analysis, then all other samples were analyzed relatively.

4.2.3 Estimation of nuclear DNA content, genome size and ploidy level

Estimation of the nuclear DNA content of a sample of interest requires a reference standard with known genome size. *Z. officinale*, 2C-value of 3.23 pg (Šmarda *et al.*, 2014), was used as an external reference standard to conduct the experiment and estimation of the relative nuclear content among the examined *Z. barbatum* accessions. The average of the peak value of all standard samples in each replication, obtained during the experiment, was calculated and accepted as an absolute unit for the standard peak mean and used in future estimations/calculations. The relative nuclear DNA content (2C-value) and ploidy level of unknown samples were calculated following Doležel *et al*. (2007). Estimated genome size (EGS) based on 2C-value was computed using 1 pg = 978 Mbp (Dolezel *et al.*, 2003). The following formulas were used for calculation 2C-value, ploidy level and genome size:

a. Estimation of 2C DNA content:

$$Sample\ 2C\ value : Reference\ 2C\ value\ \times \frac{Sample\ 2C\ mean\ peak\ position}{Reference\ 2C\ mean\ peak\ position}$$

b. Estimation of genome size:

$$Sample\ genome\ size\ (unknown) = Sample\ 2C\ value \times \frac{978}{1}$$

c. Estimation of ploidy level:

$$Samp.\ ploidy\ (integer) = Refer.\ ploidy \times \frac{Mean\ position\ of\ G1\ sample\ peak}{Mean\ position\ of\ G1\ reference\ peak}$$

4.2.4 Statistical analysis

Data analysis was conducted using IBM SPSS software version 24.0 (IBM Corp, Armonk, NY, USA). The reproducibility of the experiment was tested using the one-way analysis of variance (ANOVA) in order to determine whether there are any statistically significant differences between the three independent repeated measurements for each sample (three biological replications).

Additionally, the Pearson correlation coefficient (PCC) was applied to support data analysis and to measure the strength of the relationship between two independent repeated measurements. Tukey's HSD test was performed to find the level of significant difference between the assessed *Z. barbatum* accessions based on 2C-value.

4.3 Results

4.3.1 Reproducibility and accuracy assay of experiment

The quality of the generated flow cytometry data based on three independent measurements for each assessed sample were tested using ANOVA and PCC. ANOVA performance revealed no significant differences among the three independent measurements (Table 4.2) and PCC analysis showed that overall data has a positive relationship and is correlated (Figure 4.1) between each other. The certainty values of data analysis allowed the assessment of the reliability of experimental performance.

Chopping the young leaf of *Z. barbatum* accessions in ready to use Cystain buffer based on DAPI released high numbers of intact nuclei with an average of 2,366 nuclei isolated from 1 cm^2 of leaf tissue. Most nuclei form high-resolution histograms forming a single peak respective to each sample channel, corresponding to the nuclei being in the G0/G1 phase of the cell cycle.

The quality of fluorescent analysis in *Z. barbatum* accessions was assessed based on suggested flow cytometry data characteristics for a proper nuclear DNA content assessment, i.e. minimal amount of debris, symmetrical G1-peaks, exhibiting in a low coefficient of variation (CV%). CV-value varied between 2.68-5.40%, with an average value of 3.65% for all examined *Z. barbatum* accessions throughout of this experiment. CV-value varied from 2.75 to 4.93% with an average value of 3.51% for the reference standard *Z. officinale*. The results are shown in Table 4.3.

4.3.2 Estimated relative nuclear DNA content, genome size and ploidy level

Estimation of the relative DNA content, GS and inferred ploidy level of *Z. barbatum* are summarized and the results are presented in Table 4.3.

A low intraspecific variation was detected between the twenty examined *Z. barbatum* accessions regarding DNA content in picograms. The 2C-value of between assessed *Z barbatum* accessions ranged from 2.90 ± 0.26 pg (accession ZO208) up to 5.98 ± 0.05 pg (accession ZO189), with an

average value of 5.04 ± 0.22 pg. Tukey's HSD test based on 2C-value revealed significant differences only among three accessions: ZO189, ZO213 and ZO208 (Table 4.3).

The results reveal that eighteen examined *Z. barbatum* accessions out of twenty are triploid, and only two accessions are characterized as diploid (ZO208) and tetraploid (ZO189) species (Table 4.3), reflecting its intraspecific variation.

The mean value for genome size (1 pg = 978 Mbp, Dolezel *et al*., 2003) between assessed groups of samples was 2.52 pg, ranging between values of 1.45 pg in ZO208 to 2.84 pg in ZO189. The estimation of genome size suggests that *Z. barbatum* has a relatively small genome size (1C<3.5 pg) according to classification by Leitch *et al*. (1998).

4.4 Discussion

Structural changes in the genome both at the ploidy and chromosomal levels, as well as the variability of the overall genome size, significantly influence the plant diversification and speciation (De Storme and Mason, 2014; Wendel *et al.*, 2016) resulting in a long-lasting process of plant adaptation during evolution.

The diversification of the Zingiberaceae took place about 26 million years ago while the evolution reckons about 65 million years, which was verified by the fossil Zingiberopsis of the Upper Cretaceous or Lower Eocene of North America (Kress and Specht, 2006). Genome size, as reflected in C-values, represents a key for plant biodiversity characterization. The variation in genome size is simultaneously reflected in genotype and phenotype (Xiao-Ming *et al.*, 2017). Significant associations between the genome size variation and geographical distribution, taxonomy, life history, and evolutionary affiliation which were suggested to be determined by a selective force, have been reported (Grime and Mowforth, 1982; Leitch *et al.*, 1998; Bennetzen *et al.*, 2005; Vesely *et al.*, 2012).

The current study is the first report on the characterization of the genome size, nuclear DNA content and inferred ploidy level in the *Z. barbatum* species with no previously reported C-value. The cytological studies reported only on *Z. officinale* and *Z mioga* (Zhang *et al.*, 2013; Šmarda *et al.*, 2014).

Estimation of the relative DNA content and genome size was done using *Z. officinale* as an external reference standard with a known 2C-value, obtained from the Plant DNA C-values Database (Leitch *et al.*, 2019). The reported 2C-value for diploid *Z. officinale* is 3.23 pg (1C = 1.61 pg) (Šmarda *et al.*, 2014; Leitch *et al.*, 2019). Assessment of the relative nuclear DNA content revealed a small intraspecific variation with no clear gap in nuclear DNA C-values between the examined *Z. barbatum*

accessions. The actual gap was observed only in accession ZO208, considered a diploid, compared with all other accessions (Figure 4.2), although phenotypical differences between these accessions were not observed (personal observation). The 2C-value of diploid and tetraploid *Z. barbatum* accessions differed by almost two-fold (2C = 2.90-5.68 pg). It has been reported that DNA content alone is often insufficient to distinguish between plants with different ploidy levels the FCM measurements alone cannot be considered an accurate indicator for this purpose (Leong-Skornickova *et al.*, 2007), and therefore FCM measurements should always be accompanied by chromosome counts.

The coefficient of variation (CV) is the attribute of the histogram peak describing its width and is expressed in percentage. It fundamentally affects the resolution of flow cytometry acquisition and thus a reasonably low threshold needs to be kept guaranteeing the reliability of the results. CV is defined as the standard deviation divided by the mean of a series of fluorescence values (CV% = SD of the peak/mean channel position of the peak x 100) and allows a comparison of the quality of the detected peaks located in different fluorescence channels. In the current study, an arbitrary threshold of 3% was not exceeded in 60% of the assessed accessions and was up to 5% in 40% of the assessed *Z. barbatum* accessions. The instrument instability, secondary metabolites, oxidation or sample processing could be reasons for the between-repetition fluctuation of FCM measurements resulting in a slightly higher CV value than the accepted arbitrary threshold for the analysis. It should be noted that during the experiment sample oxidation occurred during staining steps, which was inherent to almost all of the *Z. barbatum* accessions (to a lesser or greater degree). Therefore, the samples were prepared in ice and analyzed almost immediately (incubated up to 1 min). Conversely, CV values below 3% is considered good (i.e. low threshold) and values of up to 5% are acceptable when the 2C-value of a new species is under analysis (Doležel *et al.*, 2007).

All assessed *Z. barbatum* genotypes are characterized as having a relatively small genome size based on Leitch *et al.* (1998) classification. Soltis *et al.* (2003) reported that a small genome size is reconstructed as an ancestral through much of the monocot. The report is also correlated with findings of Leitch *et al.* (1998) that extant basal angiosperms are characterized by very small 1C-values. Generally, no significant differences based on 2C-value and inferred ploidy level were observed in the relationships among all *Z. barbatum* genotypes, with the exception of the ZO208 and ZO189 accessions. However, future analyses are necessary to confirm these inferences..

The potential role of chromosomal events in the evolution of Zingiberaceae has been reported as a factor of naturalization and diversification (Sadhu *et al.*, 2016). Natural selection directly

impacts phenotypes; more genetic variation within a population usually enables more phenotypic variation. However, the evaluated *Z. barbatum* genotypes displayed phenotypic similarities, despite its nuclear DNA content and ploidy level, which is assumed to influence the phenotypic appearance. Murray (2005) reported that blurred phenotypic boundaries between individuals of the same species can be observed when the species undergo "incipient" or "ongoing" speciation and diversification

4.5 Practical value and application

The results of the present study show that the flow cytometry method can be used as a rapid and precise method to analyze nuclear DNA content in *Z. barbatum* species. The method was optimized and could be used in further experiments in *Zingiber* species.

The nuclear DNA content (C-value) and EGS of *Z. barbatum* characterized in this study provides a valuable reference for better understanding the taxonomic and phylogenetic relation of *Z. barbatum* with close relatives in *Zingiber* from the cytological point of view. The genome size and C-value may have a practical value for taxonomical and phenotypical characterization or provide beneficial information for phylogenetic and molecular studies.

In fact, and as a future perspective, the cytological findings of this study need to be confirmed and confronted with a sequenced genome analysis that allows understanding of the mechanisms involved in genome size increment and evolution, as well as to understand factors affected by this parameter.

Table 4.1 List of *Zingiber barbatum* accessions used in the current study for the cytological assessment.

No.	Accession number	Species	State/Region	Country	Year of acquisition with SMTA
1	ZO63	*Z. barbatum*	Bago	Myanmar	2004
2	ZO105	*Z. barbatum*	Mandalay	Myanmar	2004
3	ZO113	*Z. barbatum*	Mandalay	Myanmar	2004
4	ZO116	*Z. barbatum*	Mandalay	Myanmar	2004
5	ZO154	*Z. barbatum*	Shan State	Myanmar	2007
6	ZO155	*Z. barbatum*	Shan State	Myanmar	2007
7	ZO156	*Z. barbatum*	Mandalay	Myanmar	2007
8	ZO157	*Z. barbatum*	Mandalay	Myanmar	2007
9	ZO160	*Z. barbatum*	Bago	Myanmar	2007
10	ZO189	*Z. barbatum*	Shan	Myanmar	2008
11	ZO190	*Z. barbatum*	Shan	Myanmar	2008
12	ZO191	*Z. barbatum*	Shan	Myanmar	2008
13	ZO208	*Z. barbatum*	Shan	Myanmar	2009
14	ZO213	*Z. barbatum*	Shan	Myanmar	2009
15	ZO214	*Z. barbatum*	Shan	Myanmar	2009
16	ZO216	*Z. barbatum*	Shan	Myanmar	2009
17	ZO217	*Z. barbatum*	Shan	Myanmar	2009
18	ZO223	*Z. barbatum*	Mandalay	Myanmar	2009
19	ZO224	*Z. barbatum*	Mandalay	Myanmar	2009
20	ZO231	*Z. barbatum*	Shan	Myanmar	2009

Table 4.2 Analysis of variance (ANOVA) of the obtained flow cytometry data between and within three replications.

Source	SS	df	MS	F	*P*-value	F crit.
Between replications	1 139.3	2.0	569.6	0.18	0.84	3.10
Within replications	290 744.6	90.0	3 230.5			
Total	291 883.9	92.0				

SS, sum of squares; df, degree of freedom; MS, mean square; F and F critical, F statistic value. The *p*-value is significant at $\alpha<0.05$. F value is lower than F critical, $0.18<3.10$, therefore null hypothesis (H0: $\mu1 = \mu2 = \mu3$) is accepted, i.e., means of three independent measurements are equal.

Table 4.3 Summary of results of the flow cytometry analysis among twenty assessed *Zingiber barbatum* accessions. The data includes results of calculated 2C nuclear DNA content with standard deviation, 1C-value expressed in DNA picograms and megabase pairs (1 pg = 978 Mbp), inferred ploidy level, coefficient of variation (CV%), number of nuclei.

Accession number	2C-value (pg) ± std*	1C-value (pg)	1C-value (Mbp)	Number of nuclei	CV† (%)	Ploidy level
ZO208	2.90 ± 0.26^{c}	1.45	1 418	1 476	3.03	2
ZO63	5.38 ± 0.31^{ab}	2.69	2 629	2 184	3.61	3
ZO105	5.30 ± 0.09^{ab}	2.65	2 591	2 824	3.33	3
ZO113	5.05 ± 0.44^{ab}	2.53	2 471	2 281	3.74	3
ZO116	4.93 ± 0.33^{ab}	2.47	2 413	2 366	5.40	3
ZO154	4.97 ± 0.21^{ab}	2.49	2 431	2 888	4.11	3
ZO155	5.31 ± 0.22^{ab}	2.66	2 597	2 235	2.82	3
ZO156	5.08 ± 0.24^{ab}	2.54	2 483	2 447	3.75	3
ZO157	5.25 ± 0.07^{ab}	2.63	2 570	2 262	3.03	3
ZO160	5.11 ± 0.24^{ab}	2.55	2 497	2 676	3.43	3
ZO190	5.23 ± 0.11^{ab}	2.61	2 555	1 447	3.93	3
ZO191	5.16 ± 0.47^{ab}	2.58	2 525	2 817	3.25	3
ZO213	4.65 ± 0.04^{b}	2.33	2 276	2 889	5.08	3
ZO214	5.36 ± 0.15^{ab}	2.68	2 619	2 263	3.35	3
ZO216	5.29 ± 0.06^{ab}	2.65	2588	2 389	2.68	3
ZO217	5.26 ± 0.20^{ab}	2.63	2 573	2 032	3.36	3
ZO223	4.95 ± 0.16^{ab}	2.48	2 421	2 914	3.49	3
ZO224	4.94 ± 0.56^{ab}	2.47	2 414	2 652	3.45	3
ZO231	4.97 ± 0.13^{ab}	2.48	2 428	2 363	3.48	3
ZO189	5.68 ± 0.05^{a}	2.84	2 779	1 907	4.20	4
Mean	**5.04 ± 0.22**	**2.52**	**2464**	**2366**	**3.65**	3

*Compounds with the same letter are not significantly different (Tukey's HSD test, α=0.05). Each value shows an average of 2C-value ± std (standard deviation).

† CV%, Coefficient of variation expressed in percentage.

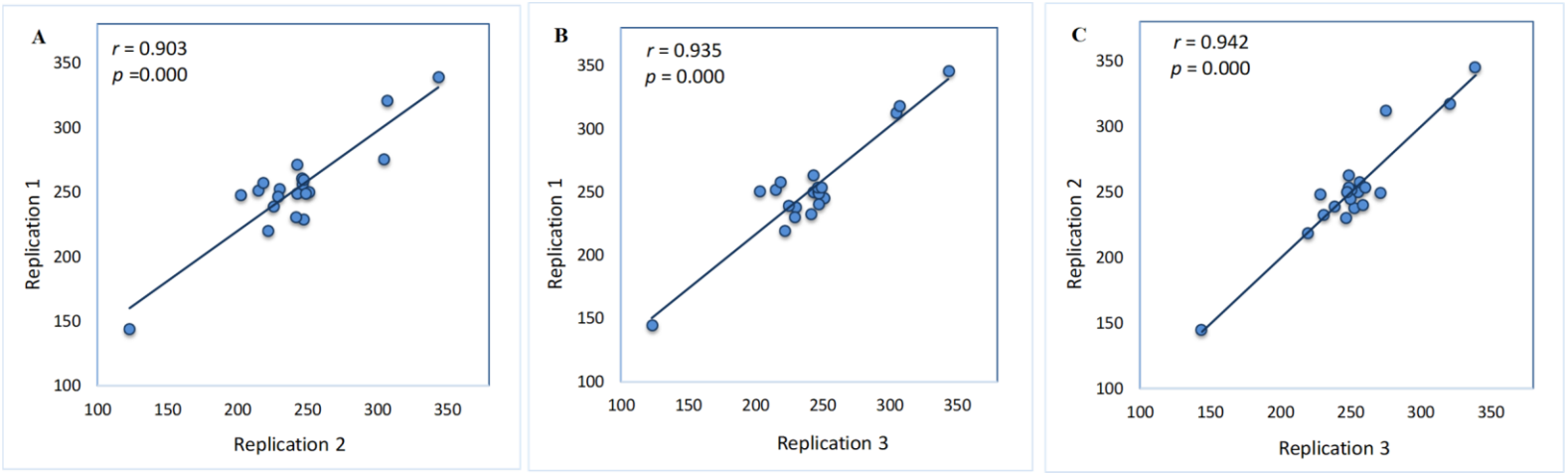

Figure 4.1 Pearson correlation coefficient (PCC) scatter plot between three independent replications of the flow cytometry analysis. A, PCC scatter plot between replications 1 and 2; B, PCC scatter plot between replications 1 and 3; C, PCC scatter plot between replications 2 and 3. PCC scatter plots demonstrate a strong positive correlation between replications demonstrating the confidence and reliability of the performed experiment.

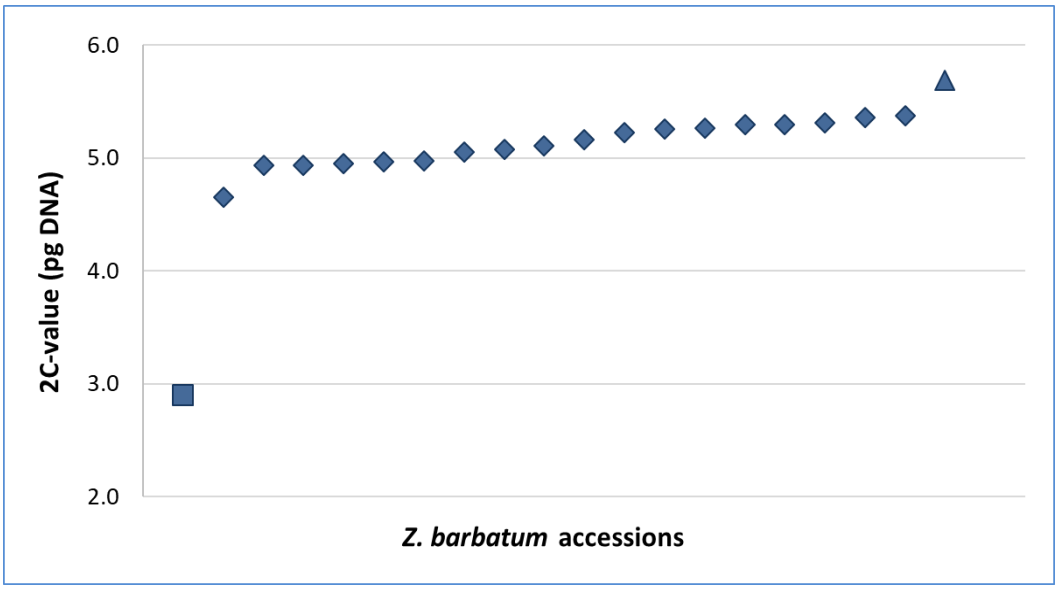

Figure 4.2 Distribution of 2C-values (DNA pg average) investigated for twenty *Zingiber barbatum* accessions. Symbol explanation: square, accession ZO208 (2C = 2.90 pg, diploid); diamonds, 18 accessions considered as triploid (2C-value range 4.65-5.38 pg, Table 4.3); triangle, accession ZO189 (2C = 5.68 pg, tetraploid).

Figure 4.3 General view of three *Zingiber barbatum* genotypes in comparison that were characterized as diploid, triploid and tetraploid accessions. A – accession ZO208, characterized as a diploid; B – accession ZO191, characterized as a triploid; C – accession ZO189, characterized as a tetraploid.

CHAPTER 5

General Discussion, Conclusion and Recommendations

5.1 Discussion

Myanmar is a country in South-East Asia and located in an Indo-Burma Biodiversity hotspot (Myers *et al.*, 2000). Being situated in a biodiversity hotspot Myanmar comprises an abundant number of unique species of flora and fauna of the region. However, still little is known about plant genetic diversity in Myanmar due to a lack of research on its vegetation compared to other Southeast Asian countries, where abundant flora is well investigated and known (Tanaka, 2012).

In Myanmar, the plant diversity investigation proceeds very slowly. Recent revision and wide inventory studies of the flora of Myanmar led to the discovery of many new species, new records and noteworthy plant collections reported by many scientists over the world during the last decades (Kress *et al.*, 2003; Watanabe *et al.*, 2006; Kawase *et al.*, 2011; Tanaka, 2012; Aung *et al.*, 2017; DeFilipps and Krupnick, 2018). On the basis of a comprehensive compilation done by DeFillipps and Krupnik (2018), 123 families, 367 genera and 472 species are utilized as a medicinal plant in Myanmar.

The plant-based products are widely used not only in traditional medicine in Myanmar, but also as a food, in aromatherapy and cosmetics (oils and essences) and as a colouring agent. Thus, it promotes the development of trade at the local, as well as at the international level. There is a huge demand for medicinal plant utilization, because they are often cheaper than their chemical alternatives, locally available, and easy to consume raw or as simple, prepared medicine (Dhanik *et al.*, 2017).

Most of the Myanmar population lives in rural areas and agriculture plays an essential role in developing local and national economies. The food and agricultural sectors are most vulnerable considering current global as well as local issues, such as global warming, species extinction, migration, growth of population, uneven distribution of resources, and low investment in major sectors of the economy. Innovative and prompt actions are needed to ensure the sustainability of the food supply and of the agricultural sector in Myanmar. Contributions towards conservation measures should be intercorrelated with the country's program on sustainable utilization and development of plant resources, which would be attractive from an economic point of view and have benefits as a source of income at national and local levels.

Z. barbatum is valuable as an herbal remedy in Myanmar and local farmers have grown this plant in small places, such as near fences or levees, more for personal consumption as a medicine than for commercial production or for food, and there is low awareness about this species. Limited information led to neglecting this species and could threaten its subsequent existence. The underexploited medicinal species may undergo extinction due to a lack of comprehensive studies and the unknown current status in natural habitats. Characterization of and assessment of underexploited species are essential in genetic diversity studies and is a basis for sustainable crop improvement, sustainable utilization, and development of conservation programs.

The ambiguity that occurs in the description of *Z. barbatum* is due to the misuses of the traditional specific epithet "Meik-tha-lin" for both *Z. barbatum* and *Z. montanum* by the local farmers in Myanmar. This brings confusion about the characterization of these two different taxa for biological purposes. A study of the interspecific relationship between these two species by using two gene and three intergenic spacer regions of the cp-DNA showed that *Z. barbatum* and *Z. montanum* are in fact two different species (Wicaksana, 2012). However, the study also revealed that *Z. barbatum* genotypes collected from Myanmar were in fact mixed species. The morphological characterization of *Z. barbatum* genotypes in the collection of the GRC UT divided them into two groups, Group 1 and Group 2, respectively (Wicaksana, 2012). Further phylogenetic analysis of the five cp-DNA regions resulted in 100% similarity with *Z. montanum* haplotype of those *Z. barbatum* genotypes that belong to Group 1.

Given the aforesaid, the substantial part of this study was focused on assessment and characterization of reproductive morphology, metabolite profiling and cytological investigation of *Z. barbatum* species from Myanmar. The current study also refers to a cycle of studies conducted on characterization of an existing genetic diversity and variability among *Z. barbatum* genotypes from Myanmar.

The study was conducted based on developed hypotheses that variation might be observed among *Z. barbatum* individuals collected from different eco-geographical regions of Myanmar resulting in its diversification and differentiation. The objectives were set to prove the proposed hypotheses. The objectives were aimed to assess and characterize the flower morphology, volatile metabolites composition, and the relative DNA content, as well as to estimate the genome size and determine inferred ploidy levels between different accessions of *Z. barbatum* species. The conducted studies fulfilled the proposed hypotheses and objectives.

5.1.1 Characterization of the reproductive morphology

Flower morphology of the genus *Zingiber* remains relevant to classical taxonomic studies insomuch as most species of the genus are phenotypically similar and difficult to distinguish at non-flowering stages. Limited studies are available on the description of morphological features of inflorescence and flower biology in *Zingiber barbatum* Wall. The present study is a first attempt to revise and characterize the phenotypic features of inflorescence and flowers in the underutilized medicinal species *Z. barbatum* based on the living collection of *Z. barbatum* genotypes in the GRC UT, which originated from Myanmar.

The conducted study revealed notable phenotypic variations of inflorescence shape and shape of the central labellum of the flower among two examined *Z. barbatum* genotypes, but quantitative parameters did not, in essence, differ. The observed variation within *Z. barbatum* might be the influences of collected mutations through the continuous asexual mode of propagation which has resulted in increasing genetic divergence of individuals in the population (Bengtsson, 2003; Olden, 2006). From another perspective, it could be either a genetically determined feature when the activity and position of the shoot apical meristem are determining the degree of the inflorescence architecture (Benlloch *et al.*, 2015) or a result of phenotypic plasticity when, due to various environmental conditions, variation of the same traits among species of the same taxa could be induced (Thompson, 1991).

The flowered *Z. barbatum* genotypes did not form seeds during the experimental observation. Several factors probably influenced the absence of seeds in *Z. barbatum.* One of the reasons could be due to the heterostyly of flowers which may be a contributing factor of sterility of *Zingiber* flowers. *Z. barbatum* flowers belong to longistylous morphotype in which anthers are situated below the slender longer style that protrudes out of the flower parts, and hence the pollen grains cannot reach the stigma. The other reason is due to self-incompatibility, when the growth of the pollen tube can be enzymatically inhibited in the style to prevent inbreeding (De Nettancourt, 2001). *Z. barbatum* flowers are monoclinous, a dichogamy (protandry or protogyny) can be another reason that influences the absence of seeds. The protandry as one type of breeding system has been reported in Zingiberaceae (Jatoi *et al.*, 2007); however, if more than two flowers of an individual asynchronously bloom, geitonogamy can occur (Gao *et al.*, 2004). To answer this question, it would be necessary to extend studies on palynology and pollination biology to understand the mechanism of the breeding system in this species.

Assessment and characterization of reproductive morphology using a morphological descriptive method based on qualitative and quantitative measurement is easy, low budget, and does not require high-tech equipment. Therefore, the descriptive morphology can be useful as a first step in the screening and characterization of underexploited or unknown species. The detailed description along with colour photographs of inflorescences and flowers provided in this study can be considered useful visual and informative material for future systematic, ethnobotanic, taxonomic, and genetic diversity studies on *Z. barbatum*.

5.1.2 Characterization of the volatile metabolite profile

Underutilized wild species, especially medicinal plants are valued as a source of useful bioactive constituents possessing medicinal properties. Of the entire variety of metabolites produced and emitted by plants, only a minority of them are involved in primary metabolic pathways in the life of the plant. Other secondary metabolites account for a dominant part of VOCs. Based on their biosynthetic origin and chemical structure the major classes of plant VOCs are volatile terpenes (i.e., monoterpenes, sesquiterpenes, diterpenes and isoprenes), oxygenates (i.e., aldehydes, alcohols, ketones and esters), phenylpropanoids, derivatives of fatty acids and amino acids, moderate volatiles such as furanocoumarins and their derivatives (D'Alessandro and Turlings, 2006; Agrawal, 2011; Bennaoum and Benhassaini, 2019).

The confined distribution of secondary metabolites makes them valuable taxonomic markers and has been widely used in assessing systematic relationships in angiosperms (Gershenzon and Mabry, 1983; Salatino *et al.*, 2000). From the chemotaxonomic point of view, the mono- and sesquiterpenes are the major classes of VOCs characteristic for the species in the genus *Zingiber* and in combination with the non-volatile phenolic compounds (gingerols and shogaols) provide their unique flavor, aroma and the bioactive properties (Vernin and Parkanyi, 2005; Wohlmuth *et al.*, 2006; Sharifi-Rad *et al.*, 2017).

The practical application of combined separative and spectroscopic techniques such as GC-TOF-MS provides a detailed analytical profile of the sample that can be analyzed by different methods (Liberto *et al.*, 2019). The volatile metabolite profiling of *Z. barbatum* species was carried out by GC-TOF-MS non-targeted analysis. As it was hypothesized, *Z. barbatum* genotypes collected from three eco-geographical regions of Myanmar displayed variations regarding the identified VOCs. Out of a total of 81 identified compounds, 24 compounds were different among the six *Z. barbatum* accessions that were discussed in detail in Chapter 3. The PCA and HCA

analysis resulted in the formation of two clusters based on identified VOCs. Cluster I was comprised of accessions ZO63 and ZO160 from the Bago region, while the Cluster II comprised accessions ZO191, ZO105, ZO217, and ZO223 collected from the Mandalay region and the Shan State of Myanmar. The first two components, PC1 and PC2, contributed a variance of 81.6% and 11.9%, respectively, with a total cumulative contribution to the variance of 93.5%.

The study has also revealed that the major class of identified compounds were monoterpenoids and sesquiterpenoids, which are characteristic classes of volatile compounds for the genus *Zingiber* (Kurobayashi *et al.*, 1991; Vernin and Parkanyi, 2005; Sukatta *et al.*, 2009; Tan *et al.*, 2018). It has been assumed that the healing properties of *Z. barbatum* may be due to the bioactivity of the monoterpenes, due to most of the monoterpenoids exhibiting biological activity, including antibacterial, anti-inflammatory, and antitumor activity (Koziol *et al.*, 2014; Sobral *et al.*, 2014).

It is known that VOCs play an important role in the evolutionary process as a response to biotic and abiotic stresses and in plant adaptation to its environment (Dicke and Loreto, 2010; Loreto and Schnitzler, 2010; Vivaldo *et al.*, 2017). Plants maintain the memory of any stress event they have experienced. Due to this, even in the studied *Z. barbatum* genotypes, which were grown under uniform conditions the observed similarities and differences between VOCs might be related to plant memory (i.e., their place of origin). The four accessions, ZO190, ZO217, ZO223, and ZO105, which were found to have similar VOC profiles, might be more tolerant to abiotic and biotic stresses in a natural environment. The two *Z. barbatum* genotypes, designated as accessions ZO63 and ZO16, might be more sensitive to the stresses in a natural environment, due to which their VOC profiles were different in comparison with the other four examined *Z. barbatum* genotypes.

Natural selection through abiotic (soil composition, drought stress and climatic factors) and biotic (pests, pollinators and diseases resistance, etc.) factors could influence the chemical variation among same taxa grown in different environments. However, the chemical diversity could be also correlated with altitude and longitude (Guo *et al.*, 2013; Şanli and Karadoğan, 2017; Demasi *et al.*, 2018).

5.1.3 Cytological characterization

Cytological characterization comprises a report regarding relative nuclear DNA content, estimated genome size, and inferred ploidy level in twenty *Z. barbatum* genotypes collected from three geographical regions of Myanmar, i.e., the Bago region, the Mandalay region and the Shan State. As it was reported above, *Z. barbatum* is a genetically and morphologically very variable

species and thus more reproducible and stable characters are needed to characterize and classify this species. The basic approach to the characterization of unknown or new species, with different or similar phenotypic features, is an estimation of the nuclear DNA content and the genome size.

Relative DNA content (2C-values) was reported to be a reproducible value in cytological studies for species identification (Čížková *et al.*, 2015). The study focused on the cytological characterization of a set of twenty *Z. barbatum* genotypes introduced into the collection of GRC UT during 2004-2009 that has not been reported on to date. Significant differences in nuclear DNA content were observed only in two *Z. barbatum* genotypes, designated as accessions ZO208 and ZO189. Assessment of the inferred ploidy level revealed that all evaluated *Z. barbatum* genotypes were triploid, except two accessions, which were diploid, ZO208, and tetraploid, ZO189. A clear gap was observed in the 2C-values of accession ZO208 (diploid) in comparison with all other assessed *Z. barbatum* genotypes, but no clear gap was observed in ZO189, characterized as triploid. Therefore the DNA content alone is often insufficient to distinguish between plants with different ploidy levels and the flow cytometry analysis alone cannot be considered an accurate indicator for this purpose (Leong-Skornickova *et al.*, 2007). Estimation of the genome size showed that all assessed *Z. barbatum* genotyped had small genome sizes, which is, in general characteristic for the family Zingiberaceae (Šmarda *et al*., 2014). It has been reported that small genome size is reconstructed as ancestral through most monocots (Soltis *et al*., 2003).

5.2. Conclusion

Wild species and relatives of many crops are rich in a wide genetic base, variability, and more tolerance to abiotic/biotic stresses. These species are a source of valuable genetic material for crop improvement programs. Generally, the genetic variation, often called genetic diversity, refers to the differences of genes (i.e., alleles) or the DNA segments between individuals. Genetic variation is a measure of the existing genetic differences within a population, and the presence of the high genetic variation observed within species or individuals of the same population depends on the harboring of many different alleles at a single chromosome locus. The newly formed alleles may increase the ability of an organism to survive and reproduce.

Compared to species with sexual reproduction, where the exchange of genetic material happens naturally, species with asexual reproduction have a low genetic variation due to limited gene flow. However, asexual species often harbor an abundance of variation coming from new mutations caused by allelic variation, genotypic variation, balancing selection, or continuous re-

creation of the asexual form (Bengtsson, 2003). These variations often lead to an increase in the homogeneity of individuals in the population or to increased divergency (Olden, 2006). The asexual mode of reproduction is also a characteristic of *Z. barbatum* species and, as it has been reported in former studies, *Z. barbatum* genotypes from Myanmar possess a high variability at morphological and genetic levels.

Even with a low amount of investigated plant materials, the current study shows the existence of variation between *Z. barbatum* genotypes from Myanmar, referring to its flower biology and volatile metabolite composition. The reproductive morphology characterization found in this study could supplement previous studies on the general characterization of *Z. barbatum* morphology. The results can be used as a key in ethnobotanical, systematic and taxonomic differentiation between *Z. barbatum* and *Z. montanum*. The present study is a novel study in the assessment and characterization of VOC composition and cytological status of *Z. barbatum* species. The results of metabolite characterization can be used in further exploration of the therapeutic properties of *Z. barbatum* species and are informative for the biomedical and pharmacological industries. The results of the cytological analysis can be used to continue future studies on determination cytotype structure based on geographical distribution. Chromosomal counts need to be done to support and validate the obtained results. In perspective, it will allow the determination of useful cytological markers for the characterization of this taxon.

Z. barbatum is a taxonomically challenging taxon with reported morphological variability (Wicaksana, 2012; Aung, 2016). Natural selection directly influences phenotypes, and more genetic variation within a population usually enables more phenotypic variation. Environmental factors that have adaptive value, human pressure (different agricultural approaches used by different ethnic communities) or when a species is going through an ongoing speciation and diversification processes might be possible reasons for the observed variation. Genetically diverse resources constitute the raw materials potentially contributing useful traits of interest for genetic crop improvement and breeding programs.

Although the current status of the existence of this species is not yet well known, this study could be informative for future ethnobotanical and taxonomical studies. It can be applied for developing PGR conservation and sustainable utilization programs regarding this indigenous toMyanmar species, which reflects the Global Plan of Action for the Conservation and Sustainable Utilization of the Plant Genetic Resources (FAO) postulating the importance of the biological basis

for food security aims for developing new sources for industrial, pharmacological and crop improvement purposes.

5.3 Recommendations and future promotion

1. To validate the detected variation regarding morphological features regarding the inflorescences and flowers, future comparative studies using different *Z. barbatum* genotypes, both growing in the wild and in landraces, are needed. Due to the rarity of flowering when growing under "artificial" conditions, such studies can be conducted at the regional and national levels, where the species can be found in natural habitats. Further detailed studies on pollination ecology, palynology, and mechanisms of a breeding system in *Z. barbatum* are required to elucidate questions related to the absence of seeds.
2. Further studies would be suitable to investigate both volatile (targeted) and non-volatile constituents, which may be useful in biochemistry and biomedical studies. Such studies can be extended to the national level which will cover more individuals of *Z. barbatum* from natural habitats. It could be assumed that the species growing in the wild will be more variable in its VOC profile. Moreover, descriptions of the medicinal properties of the major identified VOCs can be applied in perspective to assay their pharmacological properties. A comprehensive investigation of pharmacological and bioactive properties would be useful to increase the economic value of this underexploited medicinal species.
3. Although low intraspecific variation regarding the DNA content was observed, the cytological studies need to be continued, which will lead to validation of the results obtained during this study. Chromosomal counts need to be done to support the obtained results regarding the inferred ploidy levels. In perspective, a sequenced genome analysis would be suitable to confirm and confront the cytological findings.
4. Myanmar is considered a diversity center for *Z. barbatum*; however, very little is known about the real status of this species in nature. Is *Z. barbatum* a rare or already endangered species? It is still unknown. In order to preserve *Z. barbatum*'s genetic diversity in Myanmar, conservation measures and programs need to be developed both at local (regional) and at national levels for the better sustainability of its utilization.

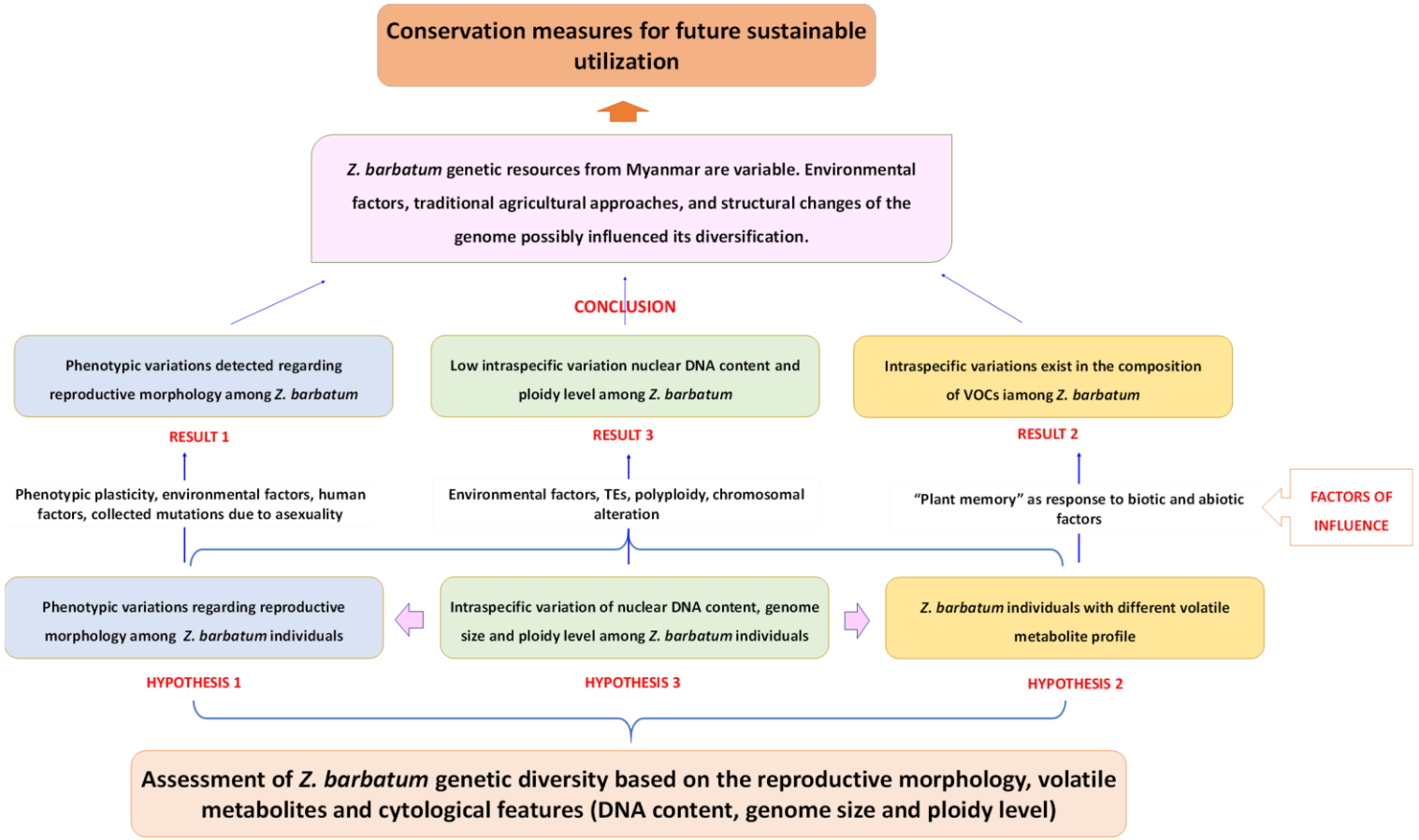

Figure 5.1 The general schematic chart represents the correlation of the hypothesis and results with a conclusion and proposed future application which was set for the current study.

概要

ミャンマーは幅広い国土、多種多様な気候条件及び民族的な多様性を持っており、そこには豊かな生物多様性が存在している。ミャンマーの変化に富む気候条件と地形は、温帯、熱帯、亜熱帯の多くの種の成長を可能にしている。独自の文化、伝統、生活のために利用する植物へのこだわりを持つ民族部族が数多く存在することも、植物の栽培方法や生産体系に影響を与えている。ミャンマーに存在する植物遺伝資源については、その探索と保全の取り組みへの予算投入が少ないため、今なお調査が終わっていない。本研究では、ミャンマーに存在するショウガ科の一種で、現在あまり研究が進んでいない *Zingiber barbatum* 種の評価に関する情報を提供する。

ショウガ科は多年生の単子葉植物であり、主に根茎を使う薬草である。ショウガ科は香料植物及び薬味用作物としての価値を持ち、また薬効も併せ持つ。ショウガ科の植物は、その乾燥物と生の植物個体の両方において治癒（又は癒し）の効果があることから、ミャンマーの伝統医療において使用されている。ショウガ属は 144 の種で構成され、そのうちの 37 種はミャンマーから報告されている。ショウガ属は、分類学上、花序の状態により 4 つの節に分類される。ほとんどのショウガ種には、その生物活性から薬効がある。しかし、食品、香辛料、栄養補助食品、及び伝統的な治療薬として広く使用されているにもかかわらず、研究が進んでいるのはわずか数種にとどまっている。

先行研究では、十分に活用されていない薬用種の *Zingiber barbatum* Wall. の評価と特性評価が焦点となっている。この種はミャンマー固有種であり、ミャンマーの伝統的な薬草療法として広く使用されている。*Z. barbatum* はショウガ属の *Cryptanthium* Horan. 節に含まれる。この種はミャンマー原産の薬用種であり、ミャンマー特有の固有種である。ミャンマーはその多様化の中心と考えられている。根茎は植物の重要な部分であり、ミャンマーの伝統医療においては、抗炎症鎮痛薬として使用できることを見出してきた。形態学的および遺伝的多様性の特性評価に関する限られた研究では、ミャンマー由来である *Z. barbatum* の様々な遺伝子型の間で高度な多様性が明らかにされている。この種

は、非常に多様な形態学的特徴を持っているため、最も細心の注意を要する分類群の 1 つとして報告されている。

包括的研究が不足しているため、*Z. barbatum* の特性評価には今なお不明確な部分が残っている。*Z. barbatum* の細胞学的および植物化学的研究は行われていない。花の生態の特性評価については、限られた数の、しかも決して包括的とは言えない古い論文が存在するのみである。現時点でのショウガ科の植物標本は、花の生態を研究するには不十分と言わざるを得ない。なぜならば、ショウガの花は繊細で短命なため、それらの特性評価は手の込んだものとなるのに加え、苞葉内で乾燥していく間に朽ち落ちてしまうからである。この事実を踏まえ、本研究の実質的な部分を、生殖形態の評価と特性評価、細胞学、および *Z. barbatum* の揮発性有機化合物の特性評価とした。本研究は、筑波大学遺伝子実験センター（GRC UT）（日本、つくば市）が所有する *Z. barbatum* のコレクションを用いて行われた。研究材料となる植物個体は、ミャンマーでの植物遺伝資源の探索中に採取された。コレクションに加えられた *Z. barbatum* の遺伝子型を持つ植物個体群は、適切な識別コード番号を付した系統として記録され、GRC UT の温室において栽培で保存されてきた。

Z. barbatum の 2 系統（ZO113 および ZO223）は、生殖形態を特徴づけるために評価された。ZO113 はミャンマーのネピドー地域から、ZO223 はマンダレー地域から入手した。本研究は、花序の状態や花についての観察、評価、および形態学的記述を、標準的な方法で実施した。評価と特性評価には、最小限の定量的及び定性的パラメーターを用いた。花の形態学的な特徴を調べて記述するため、花を解剖した。本研究には、顕花植物ごとの複製が存在しないため、統計分析は含まれていない。 2 つの定性的パラメーターは、*Z. barbatum* 遺伝子型を持つ 2 つの個体間で異なっていた。これらの差異は、発育し個体が大きく成長する過程における花序の形状に表われる表現型の変化も含まれていた。ZO113 の系統は、花序の出現段階において先端の尖った円錐形の頂部を持つ花序を形成した。その花序は、開花段階で徐々に鈍角の頂部を持つ狭卵形に変化し、開花の最終段階においては、尖った頂部を持つ幅広の紡錘形へと変化した。ZO223 の系統は短い

花柄に楕円形の花序を形成したが、開花段階では徐々に鈍角の頂部を持つ狭卵形に変化し、開花の最終段階では尖った頂部を持つ紡錘形へと変化した。

ZO113 では、中央の唇弁において様々な形状の裂片が観察された。それは唇弁の中央部で分岐したり、唇弁基部にピンク色の点が表れたりした。 *Z. barbatum* 遺伝子型を持つ 2 つの個体間で観察された表現型の差異は、生殖隔離によって引き起こされた遺伝的分岐、または継続的な無性型改造による突然変異の結果であると考えられる。

ミャンマー由来の 6 つの *Z. barbatum* 系統の根茎における揮発性有機化合物（VOC）を評価し特徴づけるため、ノンターゲットメソッドを用いた。 VOC は、TOF 質量分析法と組み合わせたガスクロマトグラフィー（GC-TOF-MS）を用いて特定した。階層解像度多変量曲線法（H-MCR）で得られた調整済み質量スペクトルは、ピークのアノテーションと同定を行うための異なるライブラリにおいて、参照質量スペクトルと一致した。全体として、*Z. barbatum* のプロファイルにおいて、主にモノテルペン（21%）とセスキテルペン（30%）の炭化水素で構成される 81 種類の VOC が確認された。その内の 24 の VOC では、 Tukey's の HSD 検定にかけた結果、6 つの *Z. barbatum* 系統間で有意に異なっていた。階層クラスター分析の結果では、81 の VOC が明確な群間分離を示し、2 つのクラスターを形成していることがわかった。クラスターⅠはバゴー地域から採取した ZO63 と ZO160 の系統で構成され、一方でマンダレー地域から採取した ZO105、ZO223 とシャン州から採取した ZO191、ZO217 を合わせて、クラスターⅡとされた。シャン州（ZO191 および ZO217）とマンダレー地域（ZO105 および ZO223）から採取された 4 つの系統は、相対的に同様の VOC プロファイルを示した。バゴー地域から採取された 2 つの系統（ZO63 および ZO160）の VOC 組成は、他の 4 つの系統と比較した結果、14 の化合物で異なっていた。評価されたすべての系統は、GRC UT において同じ高さ、同一の生態学的条件下で生育したが、VOC 組成にばらつきが認められた。この化学的な相違は、恐らく、自然環境下で植物に課せられた生物的および非生物的ストレスによって刻まれた「植物の記憶」に起因するものと思われる。

Z. barbatum 種の DNA 含量、ゲノムサイズ、および倍数性レベルについての最新情報は報告されていない。細胞学的評価は、*Z. barbatum* の 20 の系統及び、参照基準として *Z. officinale* の 1 つの系統を用いて実施した。核の DNA 含量（2C 値）の推定にはフローサイトメトリー法を用いた。 2 つの系統（ZO208 および ZO189）では、Tukey の HSD 検定の結果、核の DNA 含量に有意差を認めた。評価された *Z barbatum* の系統間の 2C 値は、2.90±0.26 pg（ZO208）から 5.98±0.05 pg（ZO189）までの範囲に及び、平均値は 5.04±0.22 pg だった。ゲノムサイズの推定においては、*Z. barbatum* の系統では比較的小さいゲノムサイズ（1C <3.5 pg）が確認された。これはショウガ科の一般的な特徴でもある。推定倍数性レベルの評価では、評価された系統全体としては 3 倍体であることが示された。ただし、2 つの系統において、それぞれ 2 倍体（ZO208）、4 倍体（ZO189）と特定された。今後は、*Z. barbatum* のゲノムサイズの増加及び進化に関与するメカニズムをよりよく理解するために細胞学的知見を確認し比較するには、配列が決定されたゲノムの解析が適していると思われる。

特定の目的のために限られた数の系統を比較しただけにもかかわらず、ミャンマーの *Z. barbatum* の複数の遺伝子型が、表現型で様々な変化を見せることが分かった。最近の研究により、*Z. barbatum* 種における花の生態、細胞学的状態、及び揮発性代謝産物の組成に関する包括的な情報が提供されている。*Z. barbatum* 種の各研究目的のもとに観察された多数の変化は、ミャンマーの経済的に重要な他の作物で報告されている、人間の影響、自然淘汰、無性生殖による長期にわたる改造、及び伝統的な栽培方法によるものかも知れない。将来的には、ミャンマーでの探索と利用を目的とした *Z. barbatum* の遺伝的多様性研究を強化する必要がある。さらには、ミャンマーにおける *Z. barbatum* の遺伝資源の持続可能な利用のためには、地域レベルと国レベルの両方で保全のための対策及びプログラムを策定する必要もある。本研究で得た知見は、民族植物学的及び薬理学的価値を持つミャンマー固有の *Z. barbatum* 種の保全や持続可能な利用のためのプログラムにおいて、考慮され得る情報となるであろう。本研究は、十分に活用されていないこの種の分類学的、体系的、植物化学的、遺伝的多様性の研究に有用であると思われる。

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LIST OF PUBLICATIONS

Original research articles

1. Musavvara Kh. Shukurova, Daisy Myint, Syed A. Gilani and Kazuo N. Watanabe. 2020. Description of flower biology of under-exploited species, *Zingiber barbatum* (Wall.) from Myanmar. *American Journal of Plant Sciences* **2020**, 11, 1031-1048. https://doi.org/10.4236/ajps.2020.117074

2. Musavvara Kh. Shukurova, Yonathan Asikin, Yanhang Chen, Miyako Kusano and Kazuo N. Watanabe. Profiling of Volatile Organic Compounds in Wild Indigenous Medicinal Ginger (*Zingiber barbatum* Wall.) from Myanmar. *Metabolites* **2020**, 10(6), 248. https://doi.org/10.3390/metabo10060248

Original research article – co-authored

1. Yanhang Chen, Yonathan Asikin, Musavvara Kh. Shukurova, Miyako Kusano and Kazuo N. Watanabe. Identification of Volatile Organic Compounds Relevant to Mango Aroma in *Curcuma amada* Roxb. from Myanmar. (in preparation).

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