FLOWERING PHENOLOGY AND POLLINATION BIOLOGY OF *Ceropegia thailandica* Meve AND *Ceropegia suddeei* Kidyoo

Mr. Chayanin Kraithep

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Botany
Department of Botany
Faculty of Science
Chulalongkorn University
Academic Year 2018

ชีพลักษณ์การออกดอกและชีววิทยาการถ่ายเรณูของหญ้าพันเกลียว *Ceropegia thailandica* Meve และหญ้าพันเกลียวสุดดี *Ceropegia suddeei* Kidyoo

นายชญานินทร์ ไกรเทพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	FLOWERING PHENOLOGY AND POLLINATION BIOLOGY OF *Ceropegia thailandica* Meve AND *Ceropegia suddeei* Kidyoo
By	Mr. Chayanin Kraithep
Field of Study	Botany
Thesis Advisor	Aroonrat Kidyoo, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Science
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Professor SUPACHITRA CHADCHAWAN, Ph.D.)

..... Thesis Advisor
(Aroonrat Kidyoo, Ph.D.)

..... Examiner
(Assistant Professor Kanogwan Seraypheap, Ph.D.)

..... External Examiner
(Santi Watthana, Ph.D.)

ชญานินทร์ ไกรเทพ : ชีพลักษณ์การออกดอกและชีววิทยาการถ่ายเรณูของหญ้าพันเกลียว *Ceropegia thailandica* Meve และหญ้าพันเกลียวสุดดี *Ceropegia suddeei* Kidyoo. (FLOWERING PHENOLOGY AND POLLINATION BIOLOGY OF *Ceropegia thailandica* Meve AND *Ceropegia suddeei* Kidyoo) อ.ที่ปรึกษาหลัก : ดร.อรุณรัตน์ คิดอยู่

ดอกของพืชสกุล *Ceropegia* มีโครงสร้างซับซ้อนซึ่งเกี่ยวข้องกับการถ่ายเรณู ได้แก่ เส้าเกสร (gynostegium) ชุดกลุ่มเรณู (pollinarium) และ กระบังรอบ (corona) นอกจากนี้ยังมีการเชื่อมติดกันของโคนกลีบดอกเป็นหลอดยาว และส่วนล่างของหลอดมักป่องออกเป็นกระเปาะ เมื่อดอกบานแฉกกลีบดอกจะแยกออกจากกันเฉพาะตรงโคน แต่ส่วนปลายยังคงจรดเชื่อมกันอยู่ เกิดเป็นโครงสร้างที่มีรูปร่างคล้ายกรงนก (cage-like structure) ซึ่งจะมีลักษณะที่เป็นกับดัก เมื่อแมลงพาหะถ่ายเรณูถูกดึงดูดให้เข้ามายังดอก จะถูกกักขังไว้ภายในดอกช่วงเวลาหนึ่ง จากการศึกษาที่ผ่านในประเทศไทยมีรายงานพบพืชสกุล *Ceropegia* จำนวนทั้งสิ้น 18 ชนิด เมื่อพิจารณาลักษณะสัณฐานวิทยาของดอกพืชสกุล *Ceropegia* ที่พบในประเทศไทย พบว่าส่วนใหญ่ดอกจะมีหลอดกลีบดอกยาว และส่วนล่างมักโป่งพองออกเป็นกระเปาะ อย่างไรก็ตามพบว่ามีบางชนิดที่หลอดกลีบดอกสั้น ได้แก่ หญ้าพันเกลียว (*Ceropegia thailandica* Meve) ซึ่งอาจส่งผลต่อประสิทธิภาพในการกักขังแมลงพาหะถ่ายเรณูไว้ภายในดอก หญ้าพันเกลียวมีลักษณะสัณฐานวิทยาหลายประการคล้ายกับหญ้าพันเกลียวสุดดี (*Ceropegia suddeei* Kidyoo) ทั้งสองชนิดเป็นพืชเฉพาะถิ่น (endemic species) ของประเทศไทย โดยหญ้าพันเกลียวพบการกระจายพันธุ์ในเขตรักษาพันธุ์สัตว์ป่าภูวัว จังหวัดบึงกาฬ ส่วนหญ้าพันเกลียวสุดดี พบในบริเวณอุทยานแห่งชาติภูพาน จังหวัดสกลนคร ทั้งหญ้าพันเกลียวและหญ้าพันเกลียวสุดดี เป็นพืชล้มลุกอายุหลายปี ลักษณะคล้ายหญ้า หัวใต้ดินมีลักษณะกลม มีใบรูปแถบ ช่อดอกมีก้านยาว มีดอกย่อยหนึ่งดอก โคนหลอดกลีบดอกมีเป็นกระเปาะ บริเวณส่วนปลายของกลีบดอกมีลักษณะเป็นหางเรียวยาวและบิดพันเป็นเกลียว อย่างไรก็ตาม พืชสองชนิดนี้มีลักษณะสัณฐานวิทยาของดอกบางประการที่แตกต่างกันอย่างเห็นได้ชัด คือ หญ้าพันเกลียวสุดดีมีหลอดกลีบดอกยาว ส่วนหญ้าพันเกลียวมีหลอดกลีบดอกสั้น จึงน่าสนใจที่จะศึกษากลไกการถ่ายเรณูเปรียบเทียบกัน ผลการศึกษาพบว่า ระยะเวลาเฉลี่ยในการพัฒนาของดอกหญ้าพันเกลียวคือ 26.2 วัน ซึ่งใกล้เคียงกับหญ้าพันเกลียวสุดดีที่มีระยะเวลา 25.05 วัน ดอกของพืชทั้งสองชนิดจะบานตั้งแต่เวลา 6:00-7:00 น. โดยจะบานอยู่นาน 1-2 วัน แมลงที่เป็นพาหะถ่ายเรณูของหญ้าพันเกลียวและหญ้าพันเกลียวสุดดี เป็นแมลงหวี่ในวงศ์ Chloropidae และ Millichiidae กลไกการถ่ายเรณูของหญ้าพันเกลียวและหญ้าพันเกลียวสุดดีแบ่งออกได้เป็น 5 ขั้นตอน คือ 1) ดอกปลดปล่อยกลิ่น เพื่อดึงดูดแมลงจากระยะไกลให้เข้ามายังดอก 2) แมลงลงเกาะบริเวณส่วนกลางของแฉกกลีบดอกและไต่ไปมาระหว่างแฉกกลีบดอก จากนั้นจะตกลงไปในหลอดกลีบดอกที่มีลักษณะเป็นกระเปาะ 3) ในขณะที่แมลงถูกกักขังอยู่ภายในดอก แมลงจะพยายามหาน้ำต้อยที่สะสมอยู่บริเวณ nectar cup ใกล้ ๆ กับ guide rails และ anther 4) และขณะที่แมลงหาน้ำต้อยนั้น อาจทำให้ชุดกลุ่มเรณู (pollinarium) ติดมากับปากของแมลง ในทางกลับกัน เมื่อแมลงที่มีชุดกลุ่มเรณูติดอยู่บริเวณปากเข้ามาภายในดอก ก็มีโอกาสที่กลุ่มเรณูจะถูกถ่ายลงระหว่าง guide rails ทำให้เกิดการถ่ายเรณู (pollinating) และ 5) ขั้นตอนสุดท้าย คือการปลดปล่อยแมลงออกจากดอก โดยหญ้าพันเกลียวและหญ้าพันเกลียวสุดดีมีกลไกการปลดปล่อยแมลงออกจากดอกแตกต่างกันอย่างเห็นได้ชัด หญ้าพันเกลียวสุดดีจะมีการโค้งลงของก้านดอกเพื่อปลดปล่อยแมลงออกจากดอก ซึ่งจะไม่พบกลไกเช่นนี้ในหญ้าพันเกลียว ซึ่งปลดปล่อยแมลงโดยการเหี่ยวของขนภายในหลอดดอกและส่วนที่เป็นกระเปาะ เมื่อพิจารณาค่าประสิทธิภาพการถ่ายเรณู โดยการคำนวณค่า Pollen Transfer Efficiency (PTE) พบว่าหญ้าพันเกลียวและหญ้าพันเกลียวสุดดี มีค่า 0.16 และ 0.21 ตามลำดับ ซึ่งเมื่อเทียบเคียงกับการ *Ceropegia* ชนิดที่มีการศึกษาก่อนหน้าที่มีการศึกษาในประเทศไทย พบว่ามีค่าที่แตกต่างกันพอสมควร จากการศึกษาชีพลักษณ์การออกดอกของหญ้าพันเกลียวและหญ้าพันเกลียวสุดดี ยังพบว่าดอกของพืชทั้งสองชนิดจะทยอยบานทีละดอก ทำให้ในแต่ละวันพืชแต่ละต้นจะมีดอกบานเพียง 1 ดอก ซึ่งจะช่วยป้องกันการผสมของดอกที่อยู่ในต้นเดียวกัน (geitonogamy) นอกจากนี้ยังพบว่า *C. thailandica* และ *C. suddeei* อาจมีกลไกการป้องกันผสมภายในดอกเดียวกัน (autogamy) โดยการเปลี่ยนระนาบของชุดกลุ่มเรณู

สาขาวิชา พฤกษศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5971930523 : MAJOR BOTANY
KEYWORD: endemic species; nectar; PTE; geitonogamy; autogamy

Chayanin Kraithep : FLOWERING PHENOLOGY AND POLLINATION BIOLOGY OF *Ceropegia thailandica* Meve AND *Ceropegia suddeei* Kidyoo. Advisor: Aroonrat Kidyoo, Ph.D.

The flowers of the genus *Ceropegia* have complex structures that involve in pollination mechanism. These structres gynostegium, pollinarium and corona. Moreover, the corolla tube which is inflated at base and the corolla lobes are connate only at tip forming a cage-like structure functions as a trap. When pollinators are attracted to flower, they could be temporarily trapped inside the flower. From previous studies, there are about 18 species in Thailand. Most Thai species have flowers characterized by a long narrow corolla lobe with distinct inflated basal portion. However, some species have a short corolla tube, such as *C. thailandica* Meve. This characteristic may affect the efficiency of flowers in detaining their pollinators and a different mechanism may be adopted by flowers to achieve their pollination success. *Ceropegia thailandica* possesses several morphological characters that are similar to those of *C. suddeei* Kidyoo. Both species are endemic species, the former occurrs in Phu Wau Wildlife Sanctuary, Beung Kan Province, the latter in Phu Phan national park, Sakon Nakorn Province respectively. They are the grass-like perennial herbs with subglobose tubers, linear leaves, single-flowered inflorescences with long peduncle and the terminal part of corolla lobes draw out into long tails and twisted. However, they are a quite difference in the lenth of corolla tube, *C. thailandica* has short corolla tube while, *C. suddeei* has long corolla tube. Therefore, it is interesting to compare their pollination mechanism. The results show that the average period for flower development of *C. thailandica* was 26.2 days, while that of *C. suddeei* was 25.05 days. The flowers of the two species started blooming in the morning, about 6:00-7:00 A.M. and remained in anthesis until 1-2 days. The effective insect pollinators of *C. thailandica* and *C. suddeei* were small flies of the family Chloropidae and Millichiidae. The pollination mechanism of *C. thailandica* and *C. suddeei* can be divided into 5 processes: 1) The flowers emitted floral scents to attract their pollinators from long distance, 2) The insect pollinators landed on the middle part of corolla lobe, crawled around the corolla lobe, then fell into the inflated of the flower, 3) While the insect pollinators were detained in the flower, they tried to find nectar present in the nectar cups located near the guide rails and anther, 4) thereby the pollinarium could be attached to the insect's mounth parts. Likewise, if the insects entered the flower with a pollinarium, the pollinaria could be inserted between the guide rails, resulting in pollination success, 5) After anthesis, the insect pollinators were finally released from the flower. There was a great difference of untrapping mechanisms between *C. thailandica* and *C. suddee*. In *C. suddeei*, the flower reoriented from erect to horizontal positions, owing to bending of the pedicel. Then, the insect pollinators were released from the flowers. In a different way, the pedicel of *C. thailandica* did not bent down after anthesis, but the flower released the insect pollinators by wilting of the hairs inside the inflated portion of corolla tube. The Pollen Transfer Efficiency (PTE) of *C. thailandica* and *C. suddeei* are 0.16 and 0.21 respectively. These values were different from those reported in other Thai species. Interestingly, the flowering phenology prevented geitonogamy. Each individual plant of *C. thailandica* and *C. suddeei*, developed flowers in different stages, but each of these flowers bloomed one after another. Therefore, there was only a single flower at anthesis per plant per day. Lastly, it sees that *C. thailanddica* and *C. suddeei* could prevent autogamy by pollinarium reconfiguration.

Field of Study:	Botany	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to express my deep appreciation to my thesis advisor, Dr. Aroonrat Kidyoo for her encouragement, kindness and especially the valuable help thoughout this research.

Besides, I would like to appreciate to the thesis committee Professor Dr. Supachitra Chadchawan, Assistant Professor Dr. Kanogwan Sereypheap and Dr. Santi Wattatana for their valuable suggestions.

I appreciate all members of plant of Thailand research Unit, Department of Botany, Faculty of Science, Chulalongkorn University for their kindness help during my field and laboratory work.

Special thanks to Phu Wau Wildlife Sanctuary and Phu Phan National Park for their permission to collect the specimens and hospitality during my specimens collecting.

This research was supported by Development and Promotion of Science and Technology Talent Project (Royal Government of Thailand Scholarship)

Finally, I would like to express my deepest thanks to my parents, my sister, my brother and member of my family for their encouragement and supports.

Chayanin Kraithep

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii
ABSTRACT (ENGLISH) iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
CHAPTER I INTRODUCTION 1
CHAPTER II LITERATURE REVIEW 4
2.1 Development of the knowledge of plant pollination 4
2.2 Pollination studies in Asclepiadoideae (Apocynaceae) 6
2.3 Pollination studies of *Ceropegia* species 7
2.4 *Ceropegia thailandica* Meve and *Ceropegia suddeei* Kidyoo 11
CHAPTER III MATERIALS AND METHODS 13
3.1 Materials 13
3.1.1. Tools and equipment 13
3.1.2. Chemicals 14
3.2 Methods 15
3.2.1 Study and collecting relevant information 15
3.2.2 Field survey 15
3.2.3 Study of flowering phenology 16
3.2.4 Study of floral visitors 16
3.2.5 Morphological study of insect visitors 16
3.2.6 Study of floral traits associated with pollination mechanism 17
3.2.7 Structures responsible for production and/or emission/secretion of floral scents and nectar 18
3.2.8 Pollen transfer efficiency (PTE) 20

3.2.9 Data analyses 20
CHAPTER IV RESULTS 21
4.1. Study area 21
4.1.1 Phu Wau Wildlife Sanctuary 21
4.1.2 Phu Phan National Park 22
4.1.3 Natural habitats of *Ceropegia thailandica* Meve and *C. suddeei* Kidyoo 25
4.2. Flowering phenology 27
4.2.1 Flower development 27
4.2.2 Blooming phenology 32
4.3. Interaction of flower and pollinator 35
4.3.1 Insect visitation 35
4.3.2 Insect pollinators 38
4.4. Study of morphological characters of flowers associated with pollination 41
4.4.1 The floral structures that involve trapping in *Ceropegia thailandica* and *C. suddeei* 41
4.4.2 The quantitative characteristics of flower that involve in pollination mechanism 45
4.4.3 The morphlogical characteristics and size of pollinarium of *Ceropegia thailandica* and *C. suddeei* 47
4.4.4 Attractants 49
4.4.1.1 Floral scent 49
4.4.4.2 Osmophore 51
4.4.4.3 Nectar 54
4.4.4.4 Nectary 55
4.5. Visiting behavior of insect pollinators of *Ceropegia thailandica* and *C. suddeei* 58
4.6. Environmental factors in relation to blooming of flowers and behavior of insect pollinators 61
4.7. Pollen Transfer Efficiency (PTE) 65
CHAPTER V DISCUSSION 69

5.1. Natural habitats of *Ceropegia thailandica* and *C. suddeei* 69
5.2. Flowering phenology of *Ceropegia thailandica* and *C. suddeei* 69
5.3. Floral scent of *C. thailandica* and *C. suddeei* 70
5.4. Trapping pollination mechanism of *Ceropegia thailandica* and *C. suddeei* 71
5.5. Effective pollinator of *C. thailandica* and *C. suddeei* 74
5.6. Breeding system of *Ceropegia thailandica* and *C. suddeei* 74
5.7. Pollen Transfer Efficiency (PTE) 77
CHAPTER VI CONCLUSION 78
REFERENCES 80
APPENDIX 85
VITA 89

LIST OF TABLES

Page

Table 3.1 Treatments for detecting various cellular substances 19

Table 4.1 Flower development of *C. thailandica* and *C. suddeei* 27

Table 4.2 The insects visiting flowers of *Ceropegia thailandica*.....36

Table 4.3 The insects visiting flowers of *Ceropegia suddeei*.....37

Table 4.4 Morphological characters insect pollinators of *C. thailandica*: family Millichiidae and Chloropidae 39

Table 4.5 Morphological characters insect pollinators of *C. suddeei*: family Millichiidae and Chloropidae 40

Table 4.6 The size of the eight parts of flower of *Ceropegia thailandica* and *C. suddeei*.....45

Table 4.7 Volatile organic compounds (VOCs) identified in floral scents collected from the flowers of *Ceropegia thailandica* and *C. suddeei*.....50

Table 4.8 Blooming period, number of pollinaria that were removed from anthers and number of pollinia that were inserted between the guiderails in the flowers of *Ceropegia thailandica* 66

Table 4.9 Blooming period, number of pollinaria that were removed from anthers and number of pollinia that were inserted between the guiderails in the flowers of *Ceropegia suddeei* 67

LIST OF FIGURES

Page

Figure 4.1 Average monthly temperature, quantity of rainfall and relative humidity in Phu Wau Wildlife Sanctuary in 2017 22

Figure 4.2 Average monthly temperature, quantity of rainfall and relative humidity in Phu Phan National Park 23

Figure 4.3 Vegetations of the study areas 24

Figure 4.4 Habitats of study species 26

Figure 4.5 The flowers of *Ceropegia thailandica* in different development stages 28

Figure 4.6 The average and standard deviation (SD) of flower development of *Ceropegia thailandica* stages 1-9 29

Figure 4.7 The average and standard deviation (SD) of flower development of *Ceropegia suddeei* 30

Figure 4.8 The average duration and standard deviation (SD) of flower development of *Ceropegia suddeei* from stages 1-9 31

Figure 4.9 Anthesis period of flowers of *C. thailandica* 32

Figure 4.10 Anthesis period of flowers of *C. suddeei* 33

Figure 4.11 Withered flower of *Ceropegia thailandica* compared to *C. suddeei* 34

Figure 4.12 Flower morphology *Ceropegia thailandica* and *C. suddeei* 43

Figure 4.13 The internal structure of flower of *Ceropegia thailandica* and *C. suddeei* 44

Figure 4.14 The eight parts of flower of which the size were measured 46

Figure 4.15 The pollinaria of *Ceropegia thailandica* and *C. suddeei* 48

Figure 4.16 Transverse sections of the corolla lobe of *Ceropegia thailandica* stained with different dyes 52

Figure 4.17 Transverse sections of the corolla lobe of *Ceropegia thailandica* stained with different dyes 53

Figure 4.18 The major sugar composition of nectar of *Ceropegia thailandica* and *C. suddeei* 54

Figure 4.19 SEM (A) and LM photographs (B-C) showing guide rails, nectaries and nectariferous tissue of *Ceropegia thailandica* 56

Figure 4.20 SEM (A) and LM photographs (B-C) showing guide rails, nectaries and nectariferous tissue of *C. suddeei* 57

Figure 4.21 Visiting behaviors of insect pollinators while remained inside the flower of *Ceropegia thailandica* and *C. suddeei* 59

Figure 4.22 The attaching pollinia on the mouthpart of pollinators 60

Figure 4.23 Average hourly relative humidity and temperature at Phu Wau Wildlife Sanctuary (May to October 2017) 61

Figure 4.24 Average hourly light intensity of Phu Wau Wildlife Sanctuary (May to October 2017) 62

Figure 4.25 The average number of insect pollinators entering flower of *Ceropegia thailandica* for each hour (May to July 2017) 63

Figure 4.26 Average hourly relative humidity and temperature at Phu Phan National Park (May to October 2017) 64

Figure 4.27 Average hourly light intensity at Phu Phan National Park (May to October 2017) 64

Figure 4.28 The average number of insect pollinators entering flower of *Ceropegia suddeei* (May to July 2017).....65

Figure 4.29 The sections of gynostegium where the pollinarium was removed and a pollinium was inserted between the guide rails 68

Figure 5.1 Diagram showing pollination mechanism of *Ceropegia thailandica* and *C. suddeei*.....73

Figure 5.2 Pollinarium configuration of *Ceropegia thailandica* (A, B and C) and *C. suddeei* (D, E and F) 76

CHAPTER I
INTRODUCTION

Pollen grain is male gametophyte of flowering plant producing male gamete or sperm to fertilize with egg cell. However, pollen grains are non-motile, the pollen vectors removing pollen grains from the anthers and deposit them on the stigmatic surface are thus required. These pollen vectors may be abiotic agents or biotic agents. Wind, water and gravity are abiotic agents by which the transfer of the pollen grains is non-directional. Thus, a very large number of pollen grains must be produced to ensure successful pollination (Abrol, 2011). Accordingly, angiosperms have evolved a more efficient pollination by various biotic agents such as insects, birds, and some mammals. Most angiosperms are insect-pollinated (Pellmyr, 1992). Insects are often attracted by shape, color, nectar and scents of flowers (Irwin *et al.*, 2004). Floral adaptations in relation to different plant-pollinators relationships can lead to an evolutionary shift of pollination system from generalized to specialized (Johnson and Steiner, 2000).

In Asclepiadoideae (Apocynaceae), the plants species have developed the elaborate floral structures. Their flowers are characterized by 1) one or more whorls of corona, 2) a gynostegium formed by post genital fusion of androecium and gynoedium, and 3) a pollinarium comprising two pollinia and accessory structures, i.e. corpusculum and translator arms, for attachment to the pollinator in order to be transported during pollination (Rahman and Wilcock, 1992; Wiemer *et al.*, 2012; Demarco, 2017). A single pollinium contains masses of pollen grains packed together. This ensures that large pollen loads are deposited on stigmatic surface (Lipow and Wyatt, 2000). Pollinaria are located in the anther locules, which are spatially separated from stigma (herkogamy). This arrangement prevents autogamous selfing and pollen vectors are thus necessary for pollination (Medrano *et al.*, 2005).

The genus *Ceropegia* L. (Asclepiadoideae, Apocynaceae) comprises more than 200 species of perennial herbs occurring naturally in Southeast Asia, India, Madagascar, Africa, tropical Arabia, the Canary Islands, New Guinea and northern Australia (Masinde, 2004a; Heiduk *et al.*, 2010; Kullayiswamy *et al.*, 2013). Most

Ceropegia species can be immediately recognized by their unique floral morphology. The corolla tube is inflated at base. The inflated basal portion can vary in length and diameter. The corolla lobes are apically connate to form a cage-like structure (Meve, 2009; Kidyoo, 2014a; 2015). These structures allow flower to temporarily imprison the pollinating flies inside (Heiduk *et al.*, 2015; Heiduk *et al.*, 2017; Auttama *et al.*, 2018). Thereby, the *Ceropegia* flowers are generally known as pitfall flower. In *C. dolichophylla*, *C. sandersonii* and *C. thaithongae*, the mechanism of pitfall flower to deceive their pollinator starts when the flower attracts pollinator by visual cue and floral scent. After that the insect pollinators come to the flower, they crawl around cage-like structure and finally slide into inflated base. Escaping is restricted by the presence of the hair. The pollinators are detained for about several hours then, the hair and inflated base collapse allowing pollinators to escape (Heiduk *et al.*, 2010; Heiduk *et al.*, 2015; Auttama *et al.*, 2018). Previous studies in several species of *Ceropegia* such as *C. aristolochioides*, *C. imbricata*, *C. inornata*, *C. meyerijohannis*, *C. dolichophylla* and *C. sandersonii* (Masinde, 2004a; Heiduk *et al.*, 2010; Heiduk *et al.*, 2016) reported that pollinators are small flies of the families Ceratopogonidae, Chloropidae, Milichiidae, and Phoridae. The floral volatiles play a key role in pollinator attraction in *Ceropegia*. The scent perceptible to the human nose can vary from sweet, fruity to slightly or very pungent or putrid (Heiduk *et al.*, 2010; Auttama *et al.*, 2018). Heiduk, *et al*. (2010; 2015; 2016) suggested that *Ceropegia* species have a specialized pollination mechanism. The plants emit the scents that mimic the odor of the food source of their pollinators.

In Thailand, there are about 18 species, mostly found in the eastern and northeastern floristic regions (Meve, 2009; Kidyoo, 2014a; 2015; Kidyoo, 2018). Most Thai species have flowers characterized by a long narrow corolla lobe with distinct inflated basal portion. However, certain species have a quite short corolla tube, such as *C. thailandica* Meve and *C. acicularis* Kidyoo (Meve, 2009; Kidyoo, 2014a). This characteristic may affect the efficiency of flowers in detaining their pollinators and a different mechanism may be adopted by flowers to achieve their pollination success. Therefore, it is interesting to carry out a study to compare the pollination mechanism of these two species.

Ceropegia suddeei Kidyoo is morphologically similar to *C. thailandica,* they are the grass-like perennial herb with subglabose tuber, linear leaves, single-flowered inflorescences with long peduncle and the terminal part of corolla lobe is long tail and twisted. Both of them are found in exposed area in deciduous dipterocarp forests (Forest and Plant Conservation Research Office, 2015). *Ceropegia thailandica* occurs in Phuwua-Phulanka forests in Nakhon Phanom province, while *C. suddeei* can be found in Phu Phan national park. Their flowers bloom during rainy season in May. Distinct contrasting floral morphological feature between these two *Ceropegia* species is the length of the corolla tube. *Ceropegia suddeei* has long corolla tube of 2.8 – 4.1 cm, whereas *C. thailandica* has short corolla tube of 1.5 – 2.3 cm that may be less efficient in detaining pollinators (Meve, 2009; Heiduk *et al.*, 2010; Kidyoo, 2014a; Auttama *et al.*, 2018). Both species are classified as critically endangered because of threats from habitat loss, land-use change, tourism, deforestation and road construction (Forest and Plant Conservation Research Office, 2015). All these threats can potentially lead both species to the extinction. It is thus necessary to study the pollination biology of both species, because it is an important process to ensure pollination success that lead to the understanding of their reproductive biology for preparing the *ex situ* conversation in the future. This study is not only going to reveal the pollination mechanism of these species, but this information can also reveal the cause of species reduction in their natural habitats leading to the solution for maintaining the populations of both species in the future.

Objective

To study and compare flowering phenology and pollination biology of *Ceropegia suddeei* and *C. thailandica.*

CHAPTER II
LITERATURE REVIEW

2.1 Development of the knowledge of plant pollination

The first report of pollen tube germination was provided by Giovanni Amici in 1840. He observed the stigmas of *Portulaca oleracea* and focused on a single pollen grain attached to the stigmatic hairs. While studying, he saw a tubular gut suddenly emerge from a side of the pollen grain. For three hours, he watched the tube grew down through the side of the hair and disappear into the tissue of stigmas. This was the first study revealing pollen tube germination of flowering plant (Abrol, 2011).

Charles Darwin conducted a great study of pollination orchids are fertilized by insects". This book was his first detailed demonstration of the power of natural selection, and explained how complex ecological relationships resulted the coevolution of orchids and insects. It opened up the new study areas of pollination ecology, directly related to Darwin's ideas on evolution, and supported his view that natural selection led to a variety of forms through the important benefits achieved by cross-fertilization. His study also pointed to the unique flowers in family Orchidaceae, Araceae, Aristolochaiceae and Apocynaceae: Asclepiadoideae (Abrol, 2011).

Delpino was an Italian botanist who made early observations on floral biology, particularly the pollination of flowers by insects. He introduced a very broad view of plant ecology and was the first to suggest pollination syndromes, sets of traits associated with specific kinds of pollinators. He wrote *Pennsieri sulla Biologia Vegetale* (Thoughts on Plant Biology) in 1867. He initiated the study of the interaction of the plants with their environments, regarding this as being a normal function of plant (Abrol, 2011).

Although the pollen tube germination had been revealed by Giovanni Amici in1830, the process of plant fertilization was not completely revealed until the development of microtome and paraffin embedding technique. Double fertilization was discovered more than a century ago by Sergei Nawaschin in Kiev, Russian

Empire, and Léon Guignard in France. Each made the discovery independently of the other. However, due to the limitation of the light microscope, there were many unanswered questions regarding the process of double fertilization. Until the development of the electron microscope, many of these questions were answered and the pollination-fertilization process could be described as following: The pollen tube extends down toward the ovary through the style. The tip of the pollen tube then enters the ovary and penetrates through the micropyle opening in the ovule. The pollen tube proceeds to release the two sperm in the megagametophyte. One sperm fertilizes the egg cell and the other sperm combines with the two polar nuclei of the large central cell of the megagametophyte. The haploid sperm and haploid egg combine to form a diploid zygote, while the other sperm and the two haploid polar nuclei of the large central cell of the megagametophyte form a triploid nucleus (triple fusion) (Abrol, 2011).

The understanding of the trap pollination mechanism was elucidated 40 years later by experimental studies of the pollination of *Arum maculatum* and *A. nigrum* (Araceae) (Abrol, 2011). Insects are mainly attracted by the scent, and visual cues of the spadix contrast against the spathe. Heat production also involved in the phase of insect attraction and enhanced the volatilisation of the odoriferous volatile compounds. Once attracted, the insects fall or enter into the floral chamber where they were trapped. The upper hairs, which were modified sterile male flowers, located at the entrance of the floral chamber, prevented the insects from escaping out, and/or to reduce light entrance. Large dung flies have been observed to alight on the spadix of *A. maculatum* and *A. nigrum* (Dormer, 1960). The study of pollination mechanism in *A. maculatum* and *A. nigrum* were the starting point of trap flower study and many species were studied after *A. maculatum* and *A. nigrum*, especially *Ceropegia* species (Asclepiadoideae, Apocynaceae) (Abrol, 2011).

2.2 Pollination studies in Asclepiadoideae (Apocynaceae)

Plants in the subfamily Asclepiadoideae, commonly known as asclepiads, have various floral morphological characters and attracted the attention of botanists by exceptional floral arrangements (Brown, 1833; Corry, 1884; Endress, 1994). Generally, in the asclepiads, the unique features of this subfamily are: (1) the gynostegium that are formed by the fusion of gynoecium and androecium. It can be divided into five sectors, (2) The corona, a sterile organ that occurs besides the corolla, and (3) the pollinarium which is formed by two pollinia from adjacent anthers. All of these features make the subfamily outstanding among other subfamilies of Apocynaceae (Wiemer *et al.*, 2012). In the stapeliads, the parts of corolla usually fuse forming an annulus. The pollinaria are located on the stylar head and varies among species by size and shape. When the insects visit flower, their body parts are trapped in the anther wings leading to pollination process (Meve and Liede, 1994).

The pollination biology in Asclepiadoideae has been intensively studied in 1990s. Ollerton and Liede (1997) studied pollination systems in the Asclepiadoideae: a survey and preliminary analysis, they found that in the Asclepiadoideae, at least eight pollination systems can be identified, utilizing distinct pollen vectors and with more or less specialized floral attributes. These systems, together with examples of genera which contain representative species, are listed below:

1. Generalized insect pollination: e.g. *Asclepias*
2. Large Hymenoptera pollination: e.g. *Asclepias*, *Calotropis* and *Gomphocarpus*
3. Wasp pollination: e.g. *Cynanchum* and *Morrenia*
4. Butterfly pollination: e.g. *Asclepias*
5. Night-flying moths pollination: e.g. *Telosma* and *Cryptostegia*
6. Open-access fly pollination: e.g. *Marsdenia*, *Matelea*, *Gonolobus*, and *Vincetoxicum*.
7. Decay-attraction fly pollination: e.g. *Stapelia* and *Caralluma*

8. Trap-flower fly pollination: e.g. *Ceropegia*

It is not surprising because the flowers of Asclepiadoideae are extremely variable, resulting in distinctly different pollination system. Among these, one of the most interesting cases is trap-flower fly pollination found in *Ceropegia* species. They have pitfall flowers that can temporarily detain the pollinators inside to ensure pollination success (Kunze, 1991).

2.3 Pollination studies of *Ceropegia* species

Ceropegia species have received increasing attention from taxonomists and pollination biologists in the past decades owing to their unique flowers, well known as 'trap flowers' or 'pitfall flowers'. The genus comprises more than 200 species of perennial herbs occurring naturally in Southeast Asia, India, Madagascar, Africa, tropical Arabia, the Canary Islands, New Guinea and northern Australia. Most *Ceropegia* species can be immediately recognized by their flowers, of which the corolla tubes vary in length, diameter and size of inflated base, and the corolla lobes apically connate to form cage-like structure. The flowers emit scent to attract their pollinators (Heiduk *et al.*, 2010) and exude nectar which plays an important role in reorientation of insects to the male and female reproductive structures (Karuppusamy and Pullaiah, 2009). When the pollinators land on the tip of flower and crawl into the slippery tube, they can slide into the inflated base. Escape from flower is prevented by the presence of the hairs and/or length and shape of the inflated base of the corolla tube. The insects are generally detained within the flower for about 24 hours, then the hairs or corolla tube collapse allowing pollinators to escape (Heiduk *et al.*, 2010).

The flowers emit scent to attract their pollinators (Heiduk, 2010) and exude nectar which plays an important role in reorientation of insects to the male and female reproductive structures (Karuppusamy and Pullaiah, 2009).

Heiduk *et al.* suggested that *Ceropegia* species have specialized pollination. They emit floral volatiles that mimic the scent of the food sources or oviposition sites of the pollinating flies. Many previous studies revealed that the flies were attracted by

rotting scent released from the tip of flower (Heiduk *et al.*, 2010; Heiduk *et al.*, 2015; Heiduk *et al.*, 2016).

Ali (1994) investigated the pollinaria removal-insertion in *Ceropegia bulbosa* Roxb. His study revealed that after entering into the flower, the insects were trapped in the flower for 18-24 hours. They usually stayed on the stigma head and tried to suck the nectar from the open base of the stigmatic chamber and also from the coronal cups below the stigmatic chambers. During this process, the lower portion of the mouth parts came in contact with the corpusculum presented at the apex of anther slit (stigmatic chamber). Thus, the corpusculum attached to the mouth parts and as insect lifted, pollinarium due to attach corpusculum also removed from the flower. Conversely, when insect with attached pollinarium visited flower, the pollinia insertion began when the two pollinium of the pollinarium attached to insect's mouth parts tilted downward and became in hanging position so that the external appendage of each pollinium can catch inserted into the anther slit like a hook. As insect pulled its head, break occur at the translator arm and a pollinium is left in the stigmatic chamber.

Vogel (1990) studied the osmophore structure in *Ceropegia elegant* Decne. The osmophore was formed by the dome-shaped papillose epidermal cells with central protruding located on the corolla lobes. The papillose epidermal cells contained a medium-sized vacuole in the basal part, where the enlarge nucleus was located. In the papillar tip, which was full of plasma, there are 1 to 2 very small vacuoles. Below the epidermis there were 1 to 2 layers of parenchyma cells, which were characterized by relatively small, spherical and several dot like microvacuoles. The next few layers had distinct intercellular spaces. Besides, he revealed that the number of osmophore cell layers differ from species to species.

Masinde (2004a) studied trap-flower fly pollination in African *Ceropegia* species. This study found that the pollinators belonged to three families of Dipter: Millichideae, Chloropideae and Ceratopogonidae. The size of pollinators was 1–2.5 mm long. The majority of flies visiting the flower regardless of flower species, habitat, temporal variation or geographical location, were female with a ratio of

females to males at 14:1. Moreover, the pollinaria were found on the insect's mouth parts, almost always on the proboscis.

Base on the pollination studies in *Ceropegia* species in 1990s the botanists had tried to elucidate pollination ecology in trap-flower including pollinarium removal, pollinium insertion and morphological characters of flowers related to pollination mechanism. Moreover, some studies investigated osmophore and nectary, the area producing odor and nectar that act as attractants to pollinators. Nevertheless, the pollination mechanism was mostly deduced based on morphological features of flowers.

Until 2000s the pollination studies of *Ceropegia* were mostly conducted in laboratory (*ex situ* grown plant). Karuppusamy and Pullaiah (2009) investigated pollination system and *ex situ* fruit set in *C. juncea* Wight. Their reported small, mostly female Dipteran flies which carried pollinaria on the proboscis as pollinators. The complex and diverse floral morphology of tubular corolla and their disposition as well as other commonly occurring features namely vibratile corolla lobes and hairs, specialized hairs on corona and sliding zone and differential lighting within the flower are important mechanisms for attracting insect pollinators (Karuppusamy and Pullaiah, 2009).

Murthy *et al.* (2012) reported the check-list and conservation strategies of the genus *Ceropegia* in India. They noted the growth of pollen tubes while pollinia were still attached to gynostegium in some stapeliads, thus implying the possibility of self-pollination. It was also demonstrated by artificial pollination experiments that self-pollination was possible in some stapeliads species. Moreover, they observed single follicle development due to self-pollination in *Ceropegia spiralis* Wight. However, they also revealed that cross-pollination was usually more successful.

Coombs *et al.* (2011) studied the role of trapping hairs in pollen export and receipt in *Ceropegia ampliata* E. Mey. They found the small flies were trapped within a bulbous base of flower after moving through an elongated corolla tube that is frequently lined with stiff hairs. When these hairs wilted after several days, insects

held in the bulbous chamber at the base of corolla tube were released. Futhermore, they investigated nectary of *C. ampliata*. Although, there was evidence suggesting that the region of nectar production occurred in the tissue behind the guiderails, they found no staining in this area, which could be because of either the viscosity of the dye or the presence of viscous nectar preventing dye from coming into contact with nectaries.

Heiduk *et al.* (2010) studied scent chemistry and pollinator attraction in deceptive trap flowers of *Ceropegia dolichophylla* Schltr. This study investigated the native pollinators of *C. dolichophylla*, the scent compounds emitted by the flowers, the temporal and spatial scent emission and the effectiveness of scent for attracting flies in the non-native range. They found that two species of insect acted as pollinators in natural habitat, namely *Desmometopa m-nigum* (Milichidae) and *Neophyllomyza* sp. (Milichidae). Moreover, two species of insect found in greenhouse in Bayreuth Germany, namely *Desmometopa* sp. and *D. sordida*. The Milichiid flies had the noteworthy traits of kleptoparasitism (stealing food from other animals). They were known to feed on the prey (haemolymph or other secretion) of predatory arthropod e.g. spiders. *Ceropegia dolichophylla* mimicked the odor of the food sources of the pollinating flies, i.e. dead insects. Scent was emitted by the tip of the flower (tip of corolla lobe). During the period of measurement, the total amount of scent emission was likely to depend on daytime. However variation among individual flower was high and no significant differences in the scent emission among difference times were found. The flower emitted a nitrogen bearing compound (N-3-methylbutylacctamind), spiroacetal, aliphatics, an irregular terpene and a few compounds of unknown class. The effectiveness of scent for attraction of flies in the non-native range could be investigated using bioassay, a flower scent sample (in acetone) of *C. dolichophylla* attracted 15 flies and no fly individual was attracted by the acetone control. In 2015, Heiduk conducted an electrophysiological analysis of pollinator using gaschromatography- electroantennographic detection (GC-EAD). For measurements, the head of a flies was cut off at the base of the thorax, mounted between two electrodes to detect antennal responses of female flies. The results revealed only 3 (*Desmometopa* sp., *D. sordida* and *D. varipalpis*) of the 14 flies from

China and three of the five female *D. sordida* from Bayreuth gave obvious antennal signals. Antennae of the other flies had too much noise in their signals and, thus, were not included in the analysis. The antenna of *D. varipalpis* responded to 19 compounds, most strongly to N-(3-methylbutyl) acetamide. All three female of *D. sordida* from Bayreuth responded to the same seven compounds. Five of them were also active on flies from China and scent samples from plants collected in the field (Heiduk *et al.*, 2010; Heiduk *et al.*, 2015).

Heiduk *et al.* (2016) studied *Ceropegia sandersonii* whose floral scent mimicked the odor of attacked Honey bee. Such odor attracted the kleptoparasitic flies of the genus *Desmometopa* (Milichiidae) for pollination. These flies were well known to feed on honey bees that were eaten by spider. The interesting information of this study was the electrophysiological analysis of fly pollinator and the attacked honey bee compared to the electrophysiological analysis of fly pollinator and floral scent of *C. sandersonii*. Mamy compounds detected in floral scent of *C. sandersonii* were similar to those detected in the odor of attacked honey bee. They concluded that *C. sandersonii* was specialized on the kleptoparasitic fly pollinators by deploying volatiles linked to the flies' food source. This study described a new example of how a plant could achieve pollination through chemical mimicry of the food sources of adult carnivorous animal.

2.4 *Ceropegia thailandica* Meve and *Ceropegia suddeei* Kidyoo

Ceropegia suddeei Kidyoo (Kidyoo, 2014a) is an endemic species found in Phu Phan National Park in Sakon Nakorn province, Thailand. It is a perennial herb bearing one-flowered cyme . The flower is greenish-brown or reddish-brown with tubular-urceolate corolla tube and narrowly linear-lanceolate corolla lobes which is drawn out into long tails. The five stamens are united with the stigma to form gynostegium. Five lateral stigmatic surfaces are covered by the adjacent anther wingsguide rails (Liede and Kunze, 1993). Two whorls of corona are present, i.e .the shallowly cup-shaped outer corona and the long erect inner corona (Kidyoo, 2014a) . Each anther has two pollen sacs, each of which contains a pollinium. Two pollinia are

joined by two translator arms, which in turn are joined to a corpusculum to form a pollinarium located above the guide rail (Masinde, 2004a).

Ceropegia thailandica Meve (Meve, 2009) is a species found in Phu Wau Wildlife Sanctuary in Beung Kan province. It is a grass like perennial herb with one-flowered cyme. Its flower is brown or purple-brown with awl-like corolla lobes and ovoid or subglobose corolla tube. *Ceropegia thailandica* is related to *C. suddeei*. Most of the morphological characters of these two species are similar, i.e. perennial herb, grass-like species with subglobose tuber, non-twining, linear leaves, single-flowered inflorescences, long peduncles and corolla lobes drawn out into long tails and twisted to the right. However, there are some different traits, especially for the length and shape of corolla tube. Besides, these species usually grow in open areas in dry dipterocarp forests in the Upper Northeast Region and are often found growing intermingled with grasses on rocky plains at about 300–500 m elevations.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1. Tools and equipment

3.1.1.1 Data logger

3.1.1.2 Altimeter

3.1.1.3 GPS tracker

3.1.1.4 Digital camera

3.1.1.5 Video camera

3.1.1.6 Power Bank

3.1.1.7 Computer

3.1.1.8 Notebook

3.1.1.9 Nylon mesh bags

3.1.1.10 Dynamic headspace extraction system

3.1.1.11 30 mg Porapak Q adsorbent tube

3.1.1.12 Vials, inserts and screw caps

3.1.1.13 1.5 and 2 ml Microcentrifuge tube

3.1.1.14 Nalophan bag

3.1.1.15 Borosilicate glass bottle

3.1.1.16 Stereo light microscope

3.1.1.17 Compound light microscope

3.1.1.18 Scanning electron microscrope (SEM)

3.1.1.19 Refrigerator

3.1.1.20 Razor blades

3.1.1.21 Laminar air-flow cabinet

3.1.1.22 Hot air oven (40 °C)

3.1.1.23 Vacuum pump

3.1.1.24 Paraffin embedding plate

3.1.1.25 Rotary microtome

3.1.1.26 Microtome blades

3.1.1.27 Microscopic slides and cover slips

3.1.1.28 Slide warming plate

3.1.1.29 Alcohol burner

3.1.1.30 Aluminium foil

3.1.1.31 Needle

3.1.2. Chemicals

3.1.2.1 Distilled water

3.1.2.2 Ethyl alcohol

3.1.2.3 Dehydrant series

3.1.2.4 Xylene

3.1.2.5 Clove oil

3.1.2.6 Safranin O

3.1.2.7 Fast green

3.1.2.8 Neutral red

3.1.2.9 Sudan IV

3.1.2.10 Sudan black

3.1.2.11 Iodine

3.1.2.12 NADI reagent

3.1.2.13 3% Formalin

3.1.2.14 Paraffin oil

3.1.2.15 Paraffin

3.1.2.16 Formalin-acetic acid-alcohol (FAA) (37% formalin- glacial acetic acid-95% ethanol [2:1:10])

3.1.2.17 Haupt's adhesive

3.1.2.18 Permount mounting medium

3.1.2.19 Hexane

3.1.2.20 Toluene

3.2 Methods

3.2.1 Study and collecting relevant information

3.2.2 Field survey

The climatic data of the study sites were gathered. The altitude above the mean sea level were measured using an altimeter. The HOBO pendant temp/relative light two-channel data loggers (Onset Computer Corporation, Bourne, MA, USA) were installed for recording the light intensity and hourly air temperature during the study period (September 2017 to August 2018). Moreover, the mean daily temperature, relative humidity and monthly rainfall were obtained from the nearest local meteorology station. The habitats and population sizes of the study species were investigated and the maps demonstrating the approximate locations of the plant individuals in the population were made.

3.2.3 Study of flowering phenology

Growth and development, from the smallest flower bud which could be seen with naked eye to the early fruit-set stage, of at least 20 flowers per species (each from different plant individual) for each study were observed.

3.2.4 Study of floral visitors

Study of insects that visited the flowers at the anthesis stage and their visiting behaviors were made on at least ten flowers from different plants of each species. Continuous video recording were made during day time (6 a.m. to 6 p.m.) using the NV-GS75 digital cameras (Panasonic, Matsushita Electric Industrial Co., Ltd., Japan) from the first day of anthesis until the flower wilted.

Before opening of flowers, fully developed flower buds were tagged and covered with nylon mesh bags to protect them from insect visitation before video recording. On the first day of anthesis, the nylon mesh bags were removed from flowers and the insect visitation were recorded. At the end of the day (6 p.m.), the flowers that had not yet withered were re-covered with nylon mesh bags (to catch the insects that emerged from the flowers that bent down during 6 p.m. to 6 a.m.).

On the second day of anthesis, the insects detained in nylon mesh bag were collected and preserved in 70% ethanol for later examination. Then, the nylon mesh bags were removed and the video recording was re-made. This procedure was repeated until the monitored flowers were wilted.

The number and behavior of insects visiting flowers were subsequently checked from the recorded video files.

3.2.5 Morphological study of insect visitors

All the preserved floral visiting insects obtained from the study 2.4 were observed in laboratory using stereo light microscope. The morphological characters of

insect were studied. The insects were then classified into morphospecies and each was identified to at least family level.

Moreover, all the insect visitors were checked for presence of pollinaria on their body parts, particularly the mouthparts and legs to consider whether they were potential pollinators.

A part of insect samples was also prepared for observation under scanning electron microscope (SEM). Firstly, the samples were dehydrated in a graded ethanol series. After that, they were subjected to critical-point drying using liquid CO_2, sputter-coated with gold and photographed under a JEOL JSM-6610LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 15 kV.

3.2.6 Study of floral traits associated with pollination mechanism

3.2.6.1 Morphological characters of at least 10 flowers at anthesis stage for each species were examined qualitatively and quantitatively. The qualitative characters studied were colors and shapes of different parts of flower, i.e. sepals, corolla tube, corolla lobes, window (an opening located between the basal region of two adjacent corolla lobes), hairs (present inside the corolla tube), gynostegium, pollinarium (pollinia, corpusculum and translator arms) and corona. The *quantitative characters* measured were sizes of different floral parts, i.e. width and length of corolla tube, width and length of windows, width and length of inflated tube and width and height of gynostegium.

3.2.6.2 Attractants produced by flowers

3.2.6.2.1 Floral scent

Volatile organic compounds (VOCs) emitted by the flowers of each *Ceropegia* species were collected using dynamic headspace technique (Meekijjaroenroj *et al.*, 2007). At least three samples were collected from the flowers picked up from a plant on the first day of anthesis and other three samples from second-day flowers. Each sample corresponded to 1-2 flowers (depending on the strength of the smell). A control (air around the extraction area) were collected

simultaneously with each sample and all samplings were conducted under the same condition. The VOCs adsorbed in the Porapak Q adsorbent (ARS Inc., Gainesville, Florida, USA) were then eluted with hexane in laboratory and stored at -20 °C until analysis by Gas chromatography/Mass spectrometry machine (GC/MS). The volatile compositions emitted from the first day-flowers were compared with those of the second day-flowers.

3.2.6.2.2 Nectar

One day before anthesis, at least 5 fully developed flower buds for each species were covered with nylon mesh bags for preventing visitation of insects. When the flowers opened, the nylon mesh bags were removed and these flowers were cut lengthwise to inspect the presence of nectar. After that, the gynostegium was removed and put in a 1.5 ml microcentrifuge tube containing 0.5 ml of distilled water. The nectar was dissolved in water and the eluant was then kept at low temperature until analysis by High Performance Liquid Chromatography (HPLC) to detect glucose, fructose and sucrose.

3.2.7 Structures responsible for production and/or emission/secretion of floral scents and nectar

Anatomical features of the scent producing/emitting structure, termed osmophore, and the nectar producing/secreting structure, termed nectary, were investigated. Based on the previous studies on several *Ceropegia* speccies (Vogel and Renner, 1990), the osmophore was found located on the adaxial side of the corolla lobes. Therefore, the corolla lobes of *C. thailandica* and *C. suddeei* were examined using a free-hand section method. The fresh materials of corolla lobes were cut in transvers section and stained with different solution to detect various cellular substances. The six treatments were done including water (control), iodine solution, neutral red, Sudan IV, Sudan black and Nadi reagent as shown in the Table 1, and all stained sections were studied and photographed using a Nikon Eclipse E200 photomicroscope.

Table 3.1 Treatments for detecting various cellular substances.

Treatment	Detection
Iodine	Staining black to blue in presence of starchs grains (Ruzin, 1999)
Neutral red	The living cells incorporate neutral red into their vacuoles, indicating the high metabolic activity which could represent secretory cells. The positive result will show the vacuole stained red in cells(Ruzin, 1999; Francisco and Ascensao, 2013).
Sudan IV	Detecting lipids, triglycerides and lipoproteins that are commonly involved in biosynthesis of volatile organic compounds (VOCs) . Sudan dyes would be solubilized and become orange stain (Ruzin, 1999)
Sudan black	Staining blue-black in presence of neutral triglycerides and lipids (Ruzin, 1999).
Nadi reagent	Staining blue in presence of terpenes (Ruzin, 1999; Ibanez *et al.*, 2010).

The anatomical structure of nectary were investigated by means of paraffin embedding method. On the first day of anthesis, the gynostegia were removed from flowers, then fixed in FAA solution (37 %formalin - glacial acetic acid - 95 % ethanol [2:1:10]). The fixed materials were then dehydrated in a graded ethanol series. Then, the samples were embedded in paraffin (Ruzin, 1999) and sectioned at 14 μm using rotary microtome HM 340 E with cool-cut. The obtained sections were double strained with safranin O and fast green (Johansen, 1940) and photographed using Nikon Eclipse E200 photomicroscope.

To study the surface of the secretory structures, the samples of corolla lobes and gynostegia were prepared for observation under SEM (see 2.5.2 for preparation method).

3.2.8 Pollen transfer efficiency (PTE)

In 15 preserved flowers of *Ceropegia suddeei* and 15 flowers of *C. thailandica* obtained from the study 2.6.1, the number of pollinaria removed from the anthers and the number of pollinia inserted between the guide rails (covering the stigmatic surfaces) were counted under stereo light microscope. The PTE for each population were calculated using the equation below:

$$\text{PTE} = \frac{\text{Pi}}{2\text{xPr}}$$

Pi = inserted pollinia

Pr = removed pollinia

3.2.9 Data analyses

The results obtained from all these studies were analyzed. The pollination biology of *Ceropegia suddeei* and *C. thailandica* were discussed and compared to each other.

CHAPTER IV
RESULTS

4.1. Study area

4.1.1 Phu Wau Wildlife Sanctuary

Phu Wau Wildlife Sanctuary is located in the northeastern part of Thailand, Bueng Kan province, at 18 degrees, 15 minutes, 58.68 seconds north of the equator, and 104 degrees, 0 minutes and 22.33 seconds west of the Prime Meridian. It is only about 2 km from the border and two sides parallel to the Mekong River. The area is at 160-448 m elevation and characterized by sandstone and steep cliffs in the east to the west. The highest point is Phu Wua, Lung Tum Soong, at about 448 m elevation. The soil is mostly sandy. On the side of the Chanan Waterfall, there are some areas of clay loam. The area behind it is mostly sandstone. Phu Wau Wildlife Sanctuary is mostly covered with dry dipterocarp forest. The dominant plant species are *Shorea roxburghii* G. Don, *S. siamensis* Miq. and *Dipterocarpus obtusifolius* Teijsm. ex Miq. Dry evergreen forests can be found in some areas with *Dipterocarpus alatus* Roxb. ex G.Don, *Hopea odorata* Roxb. and *H. ferrea* Laness. as dominant species (Figure 4.3A).

In 2017, the average temperature throughout the year in the sanctuary is 27.1 degree Celsius. The lowest temperature is 22.8 degree Celsius in December while, the highest temperature is 29 degree Celsius in April (Figure 4.1). The average relative humidity throughout the year is 73.6%. The highest relative humidity is 87% in July and the lowest relative humidity is 60% in February. Furthermore, the average rainfall throughout the year is 123 mm. The highest rainfall is 295 mm in August while, the lowest rainfall is 4.4 mm in December (Figure 4.1).

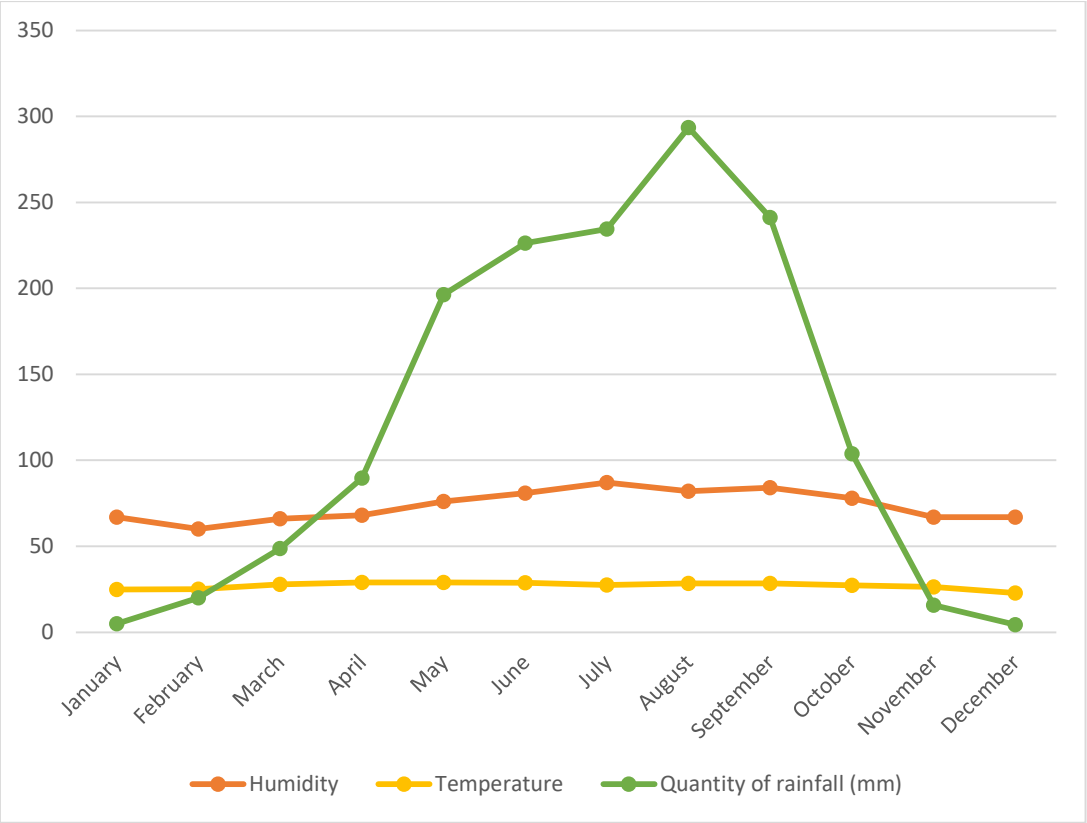

Figure 4.1 Average monthly temperature, quantity of rainfall and relative humidity in Phu Wau Wildlife Sanctuary in 2017.

4.1.2 Phu Phan National Park

Phu Phan National Park is located in the northeastern part of Thailand, Sakon Nakorn province, at 17 degrees, 3 minutes, 41.55 seconds north of the equator, and 103 degrees 38 minutes, and 22.37 seconds west of the Prime Meridian. Most area of the national park is at about 567 m elevation, characterized by sandstone mountains and covered by forest that is origin of the important rivers in Sakon Nakorn province, i.e. Aoon river and Poong river. The soil is mostly sandy like Phu Wau Wildlife Sanctuary and there are some areas of clay loam. Plant community in Phu Phan National Park can be divided into three types: (1) dry dipterocarp forest, found in middle part of the national park. The dominant plant species are *Dipterocarpus terboculatus* Roxb., *Shorea roxburghii* G. Don. and *S. siamensis* Miq., (2) dry evergreen forest, found in northern and southern part, (3) mixed deciduous forest,

found in the ecotone between dry dipterocarp forest and mixed deciduous forest (Figure 4.3B).

In 2017, the average temperature of the area throughout the year is 26.3 degree Celsius. The lowest temperature is 22.1 degree Celsius in December and the highest temperature is 28.4 degree Celsius in June (Figure 4.2). The average relative humidity throughout the year is 76.4%, with the highest relative humidity of 92.6% in July and the lowest relative humidity of 62% in February. The average rainfall throughout the year is 137 mm, with the highest rainfall of 357 mm in August and the lowest rainfall of 4.7 mm in January (Figure 4.2).

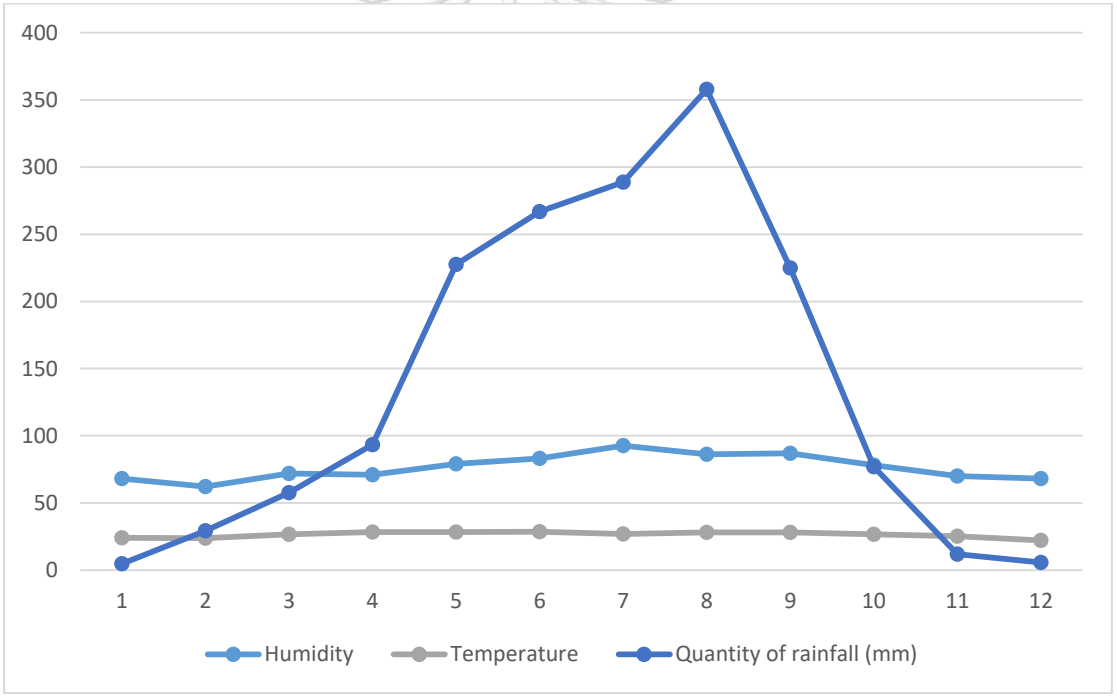

Figure 4.2 Average monthly temperature, quantity of rainfall and relative humidity in Phu Phan National Park.

Figure 4.3 Vegetations of the study areas. A: Phu Wau Wildlife Sanctuary. B: Phu Phan National Park.

4.1.3 Natural habitats of *Ceropegia thailandica* Meve and *C. suddeei* Kidyoo

Ceropegia thailandica and *C. suddeei* occur in moist grasslands of deciduous dipterocarp forests. They usually grow on sandy soil in open area. *Ceropegia thailandica* occurs in Phu Wau Wildlife Sanctuary, Beung Kan Province (Figure 4.4A), while *C. suddeei* occurs in Phu Phan national park, Sakon Nakorn Province (Figure 4.4B). Their habitats are extremely threatened by human activities and wildfire. In addition, the two species suffer from long dry season and soil erosion.

Figure 4.4 Habitats of study species. A: Habitat of *C. thailandica*, Phu Wau Wildlife Sanctuary. B: Habitat of *C. suddeei*, Phu Phan National Park.

4.2. Flowering phenology

4.2.1 Flower development

The flower development can be divided into nine stages.

Table 4.1 Flower development of *C. thailandica* and *C. suddeei.*

Stage	Characters
1	Young flower bud, the corolla were entirely covered by calyx.
2	The corolla was longer than calyx. The length of the corolla was less than that of calyx. The corolla lobe could not be discerned from the corolla tube.
3	The corolla got longer. Its length was more than twice the length of calyx. The corolla lobe could be clearly discerned from the corolla tube, but the corolla tube was still shorter than calyx.
4	The corolla tube became longer than calyx. The flower had not yet grown to full size.
5	The flower grew to full size, but the corolla lobes had not yet been separated.
6	Flower was at anthesis. The corolla lobes were separated throughout their length. The small openings could be seen at the base of the corolla lobes, but the distal part of the corolla lobes were still tightly twisted.
7	Flower was withered, dried out (*C. thailandica*) or curl down (*C. suddeei*).

8	The corolla was fallen, left only calyx and ovaries on the receptacle.
9	Ovaries developed into young fruits.

The average period for floral development of *C. thailandica* from the first stage to the eighth stage (Figure 4.5) was 26.2 days. The first stage took 8.05 ± 0.83 days. The second, third and fourth stages took consecutively 7.55 ± 0.69, 2.10 ± 0.30 and 3.85 ± 0.67 days. The fifth stage took only 1 day and sixth stage 1.40 ± 0.50 days. The seventh as well as eighth stages took only 1 day, after that the ovary either developed into young fruit in pollinated flower or fell out in non-pollinated flower (Figure 4.6).

Figure 4.5 The flowers of *Ceropegia thailandica* in different development stages.

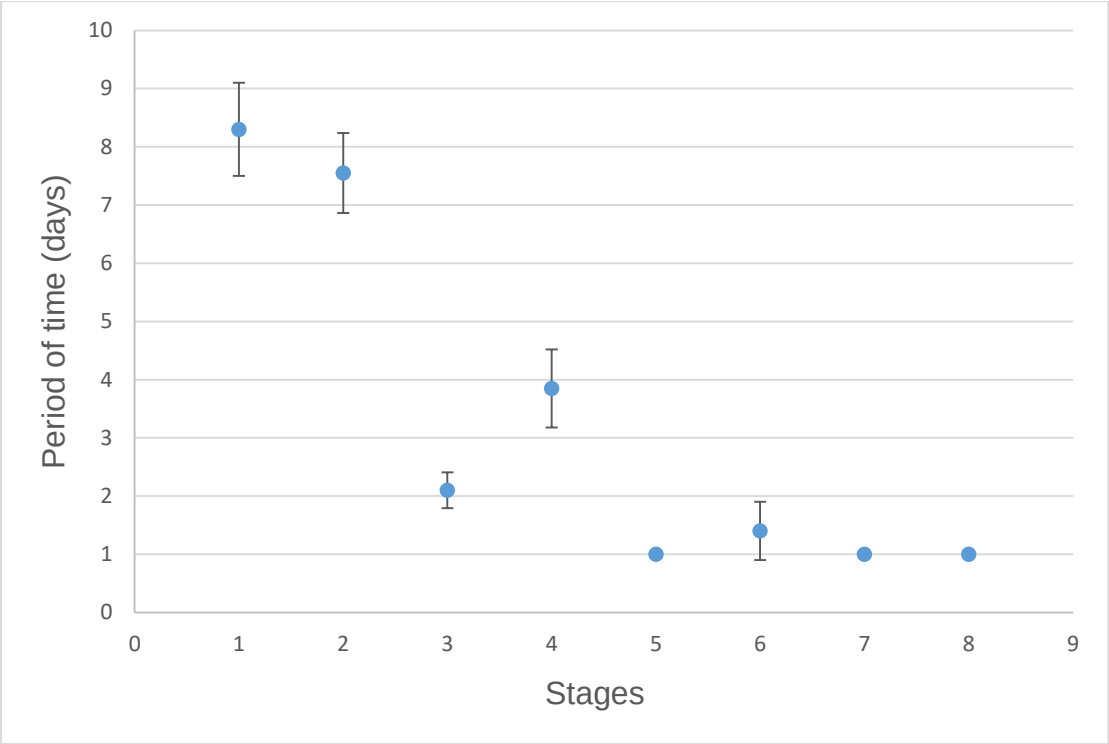

Figure 4.6 The average and standard deviation (SD) of flower development of *Ceropegia thailandica* stages 1-9.

The average period of flower development of *C. suddeei* from the first stage to the eighth stage was 25.05 days (Figure 4.7). The first stage took 8.05 ± 0.80 days. The second, third and fourth stages took respectively 8.10 ± 0.79, 2.4 ± 0.50 and 2.45 ± 0.60 days. The fifth stage took only 1 day and the sixth stage 1.20 ± 0.44 days. The seventh and eight stages took 1 day before young fruit appeared in pollinated flower or the ovary was shred in non-pollinated flower (Figure 4.8).

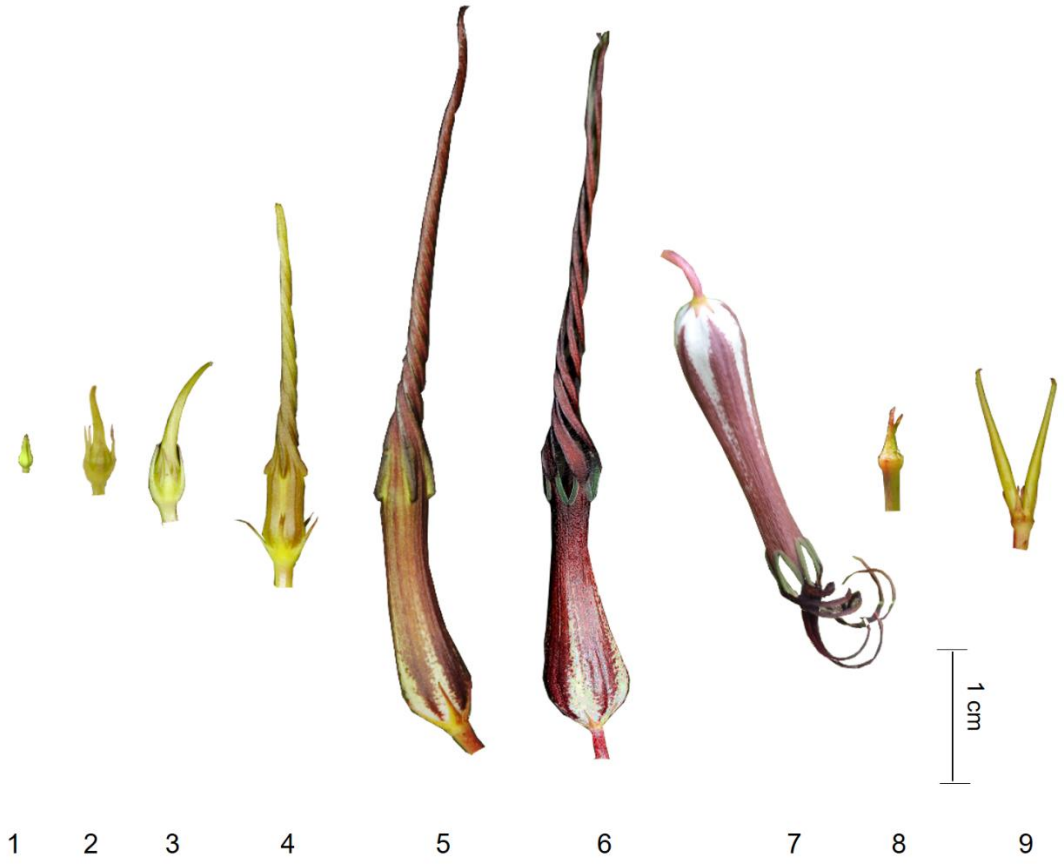

Figure 4.7 The average and standard deviation (SD) of flower development of *Ceropegia suddeei*.

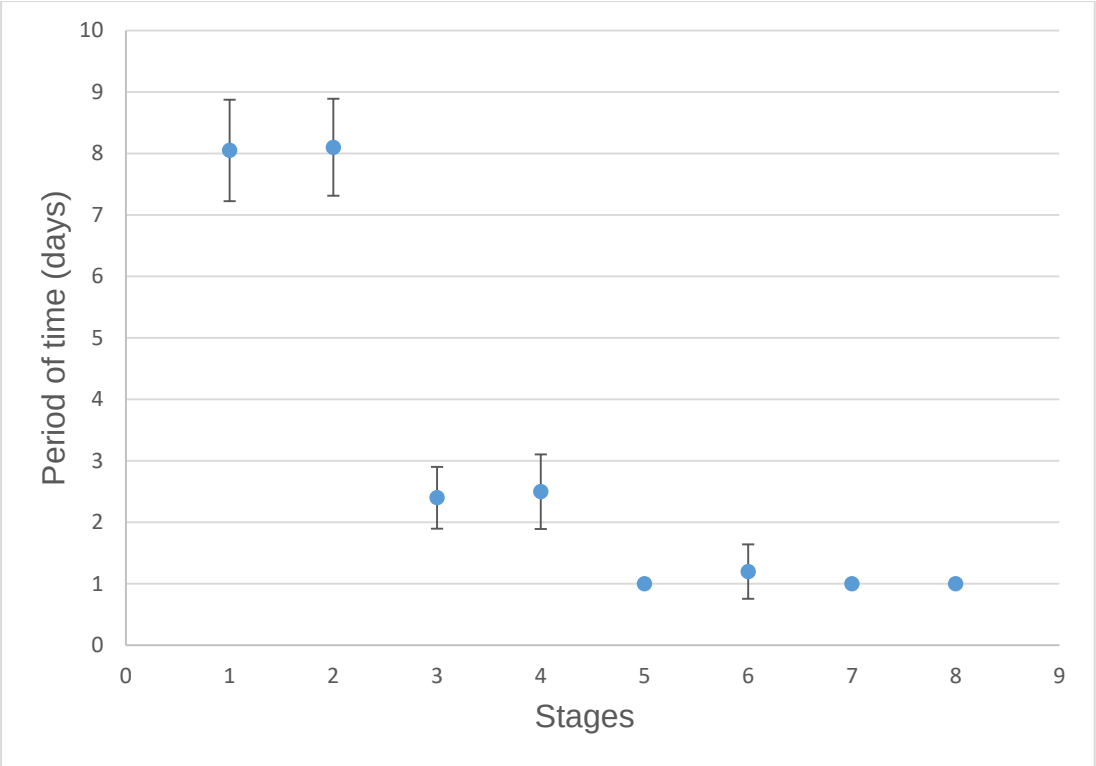

Figure 4.8 The average duration and standard deviation (SD) of flower development of *Ceropegia suddeei* from stages 1-9.

4.2.2 Blooming phenology

Twenty flowers of *C. thailandica* were observed at anthesis (the sixth stage) for five days (Figure 4.9). The result showed that the anthesis stage of these flowers took 1 day or 2 days. Twelve flowers took 1 day and eight flowers took 2 days. On each day, there were two to nine flowers at anthesis. Each plant had only one blooming flower per day. The flowers started to bloom in the morning, at about 6 am – 7 am. The corolla lobes split open, then the opening at their bases (widow-like structure) could be discerned. The flowers released strong scent that smelled like a rotten fruit. Most flowers withered in the evening, at about 7 pm (Figure 4.11A). However, some flowers remained open for 2 days. While a flower was withering, the pollinators detained inside were released.

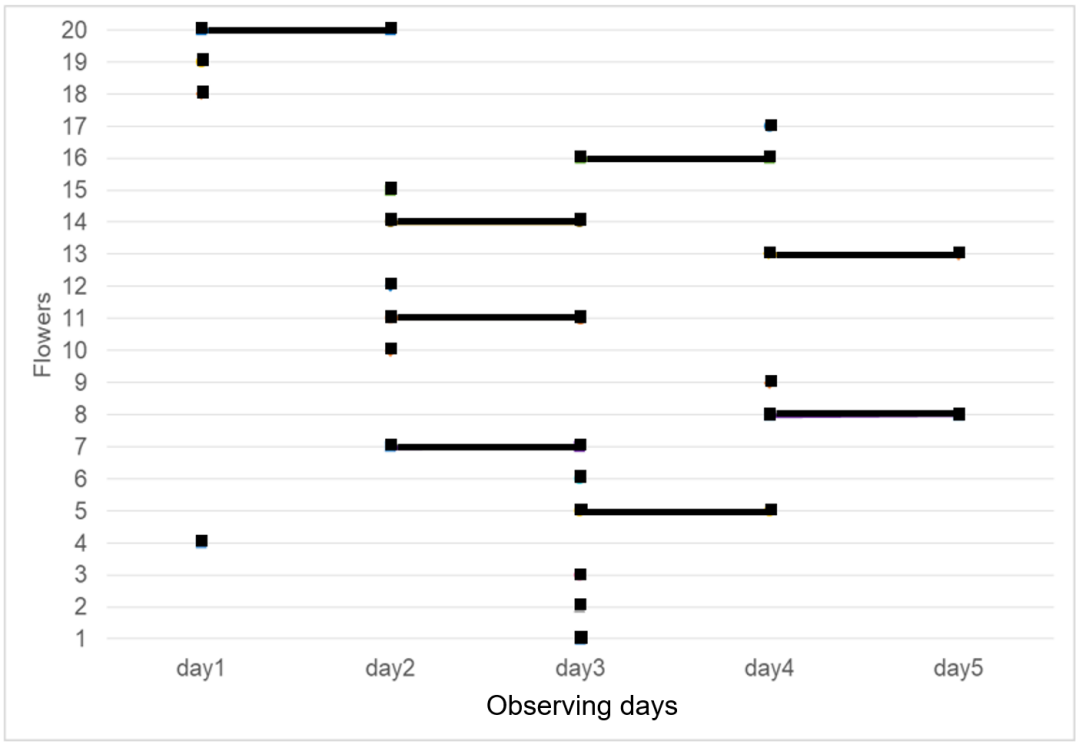

Figure 4.9 Anthesis period of flowers of *C. thailandica* (20 flowers), spot or line stand for period time of blooming. The symbols ■ stand for flowers blooming in each day.

As for *Ceropegia suddeei*, twenty flowers at anthesis were studied during six days (Figure 4.10). All flowers bloomed only 1 day. There were three to seven flowers newly opened in each day. Each plant had only one flower at anthesis per day. The corolla lobes split open and the window-like structure at their bases could be discerned. The flowers emitted a strong scent that smelled like a putrid fruit. After 6 pm, most flowers withered and their pollinators trapped inside were released due to reorientation of the flower from erect to horizontal (Figure 4.11B).

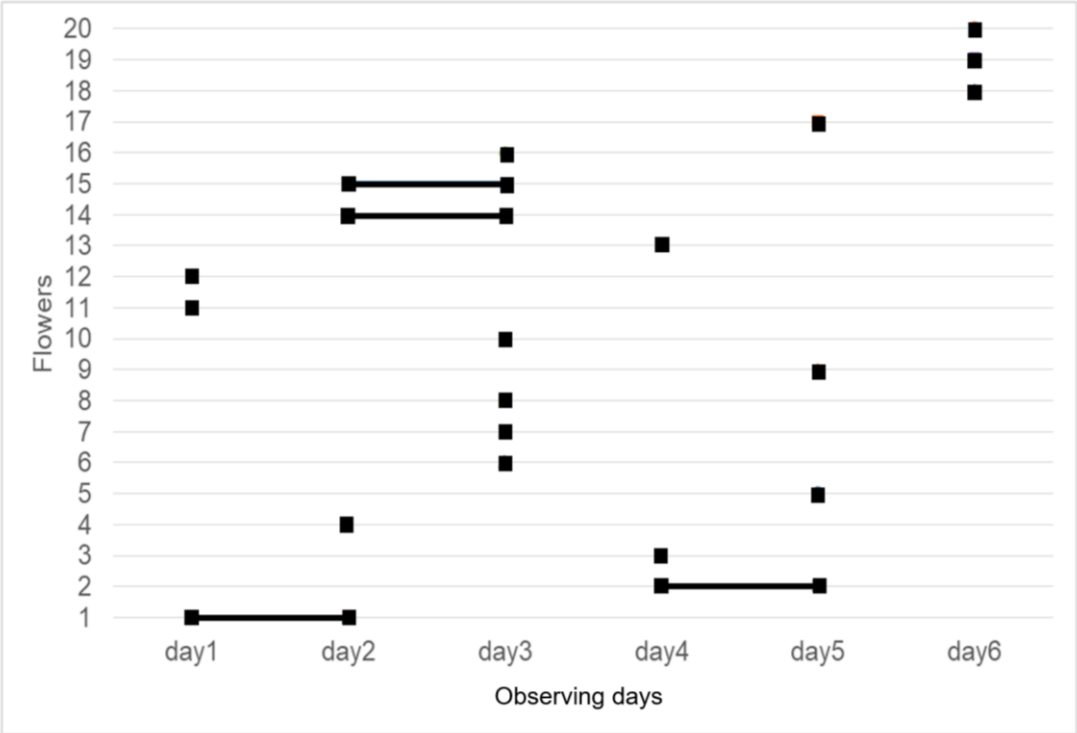

Figure 4.10 Anthesis period of flowers of *C. suddeei* (20 flowers), spot or line stand for period time of blooming. The symbols ■ stand for flowers blooming in each day.

Figure 4.11 Withered flower of *Ceropegia thailandica* compared to *C. suddeei*. A: *Ceropegia thailandica*. B: *C. suddeei.*

4.3. Interaction of flower and pollinator

4.3.1 Insect visitation

While the flowers of *Ceropegia thailandica* were in anthesis stage, the insects of four orders (Table 4.2) visited them, namely Diptera (fruit flies), Hymenoptera (ant), Orthoptera (grasshoper) and Lepidoptera (butterfly). There were only ants and fruit flies that could enter to flowers because their body size were smaller than the size of window at the base of corolla lobes and the smallest diameter of the corolla tube.

In *Ceropegia suddeei*, the flowers at anthesis were visited by insects of two orders (Table 4.3), namely Diptera (fruit flies) and Hymenoptera (ant). Both ant and dipterean fly could get in the flowers because their body size were smaller than size of window at the base of the corolla lobes and the smallest diameter of corolla tube.

Table 4.2 The insects visiting flowers of *Ceropegia thailandica.* Only the insect that could enter in the corolla tube and had pollinarium attarched to ther mouthparts were considered as pollinator based on camera recorded.

Date	Flowers	Pollinator of *C. thailandica*			Visitor of *C. thailandica*		
		Order Diptera					
		Entering flower	Leaving flower	Swarming flower	Order Hymenoptera	Order Orthoptera	Order Lepidoptera
24/5/2017	1	2^{P}	2^{P}	–	2	1	2
	2^{*}	$3+1^{P}$	–	4	4	–	–
	3	$3+1^{P}$	$2+1^{P}$	1	–	–	–
	4	$2+1^{P}$	1^{P}	–	1	–	–
27/5/2017	5	–	–	–	1	–	–
	6	–	–	1	–	1	–
	7^{*}	1^{P}	–	–	–	–	–
	8	–	–	1	–	–	–
	9	–	–	–	1	–	–
	10	–	–	2	–	–	–

* The flowers that remained in anthesis only 1 day, ^{P} insect with pollinarium attached to their mouthparts and – means cannot observe.

Table 4.3 The insects visiting flowers of *Ceropegia suddeei.* Only the insect that could enter in the corolla tube and had pollinarium attarched to ther mouthparts were considered as pollinator based on camera recorded.

Date	Flowers	Pollinator of *C. suddeei*			Visitor of *C. suddeei*		
		Order Diptera					
		Entering flower	Leaving flower	Swarming flower	Order Hymenoptera	Order Orthoptera	Order Lepidoptera
1/6/2017	1	2	2^{P}	–	2	–	–
	2	$3+3^{P}$	$1+2^{P}$	–	–	–	–
	3	$3+1^{P}$	$1+1^{P}$	–	–	–	–
	4	$3+6^{P}$	$2+2^{P}$	–	–	–	–
	5*	3	–	–	1	–	–
4/6/2017	6*	3	–	–	–	–	–
	7*	3	–	–	–	–	–
	8*	3	3^{P}	–	–	–	–
	9	–	–	2	–	–	–
	10	–	–	1	–	–	–

* The flowers that remained in anthesis only 1 day, ^{P} insect with pollinarium attached to their mouthparts and – means cannot observe.

4.3.2 Insect pollinators

The pollinators of *C. thailandica* belongs to order Drosophiliidae: family Millichiidae and Chloropidae (Table 4.4).

Milichiidae sp. 1 small, brown, long about 2.10 mm. Thorax; wide about 0.69 mm and a bit wider than abdomen. Wing line; 4 wing lines, 2 cross-veins.

Milichiidae sp. 2 small, dark brown at thorax, light brown at abdomen, long about 3.15 mm. Thorax; wide about 0.78 mm, as wide as abdomen. Wing line; 4 wing lines, 2 cross-viens.

Chloropidae sp. small, black, long about 3.23 mm. Thorax; wide about 1.05 mm, as wide as abdomen. Wing line; 4 wing line, 2 cross-veins.

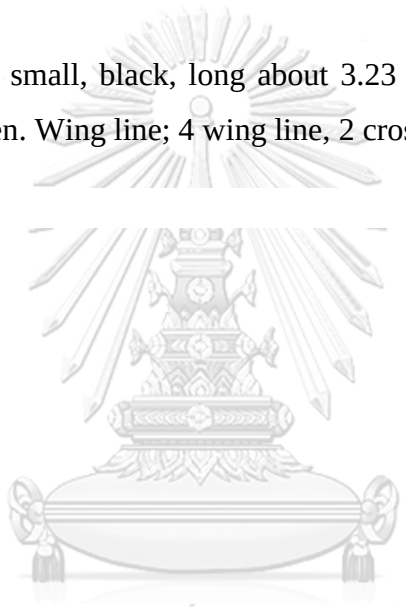

Table 4.4 Morphological characters insect pollinators of *C. thailandica*: family Millichiidae and Chloropidae.

	Milichiidae 1	Millichiidae 2	Chloropidae
Dorsal			
Ventral			
Wing line			

The pollinators of *C. thailandica* belongs to order Drosophiliidae: family Millichiidae and Chloropidae (Table 4.5).

Milichiidae sp. small, dark brown at thorax, light brown at abdomen, long about 3.06 mm. Thorax; wide about 1.08 mm, as wide as abdomen. Wing line; 4 wing lines, 2 cross-viens.

Chloropidae sp. small, black, long about 3.49 mm. Thorax; wide about 1.15 mm, as wide as abdomen. Wing line; 4 wing line, 2 cross-veins.

Table 4.5 Morphological characters insect pollinators of *C. suddeei*: family Millichiidae and Chloropidae.

	Milichiidae	Chloropidae
Dorsal		
Ventral		
Wing line		

4.4. Study of morphological characters of flowers associated with pollination

4.4.1 The floral structures that involve trapping in *Ceropegia thailandica* and *C. suddeei*

Ceropegia has remarkable flower. In general, its corolla can be divided into 3 parts namely (1) distal part: corolla lobes in form of a cage-like structure that twist at tip into a long tail. (2) middle part: a long narrow corolla tube and (3) basal part: an inflated portion of the corolla tube which function as a chamber, imprisoning their pollinator. However, the flower of *C. thailandica* constituted of only the distal and basal parts of corolla, i.e. the cage-like structure and twisted long tail, and the basal inflated tube, the middle long narrow tube is absent. Therefore, the flower is short and clearly different from other species (Figure 4.12A). At anthesis, the flower color become greenish-brown to reddish-brown and the corolla lobes split open forming the window-like structures at the base of the cage-like structure. These windows played an important role as the way for insect pollinators to get into the flower. After that, the insects fell into the corolla tube and detained in the flower. Escaping from there was restricted by the inflated tube and the hairs present inside the inflated tube. Nevertheless, the insect pollinators were released when the flowers wither.

In *Ceropegia suddeei*, the middle long narrow corolla tube is well developed (Figure 4.12B). At anthesis, color of the flower became reddish-brown or purple-brown and the corolla lobes split open forming the window-like structures at the base of the cage-like structure in the same way as *C. thailandica*. When the insect pollinators were attracted to the flower and got into the flower by entering the window-like structure, they were imprisoned until the flower withered, the pedicel of which bent down. Finally, the pollinator were released.

At the base of inflated tube of both species, there is a gynostegium. The detention of pollinator inside this part of the flower increased success in pollination. While the detained pollinator searched for the nectar around the gynostegium where the guide rails and the anther locules were located, the pollinium could be attached to its mouth parts (pollinium removal) or if the pollinator had entered with a pollinarium

already attached to its mouthparts, one or two pollinia could be inserted in the guide rails leading to pollination success (Figure 4.13).

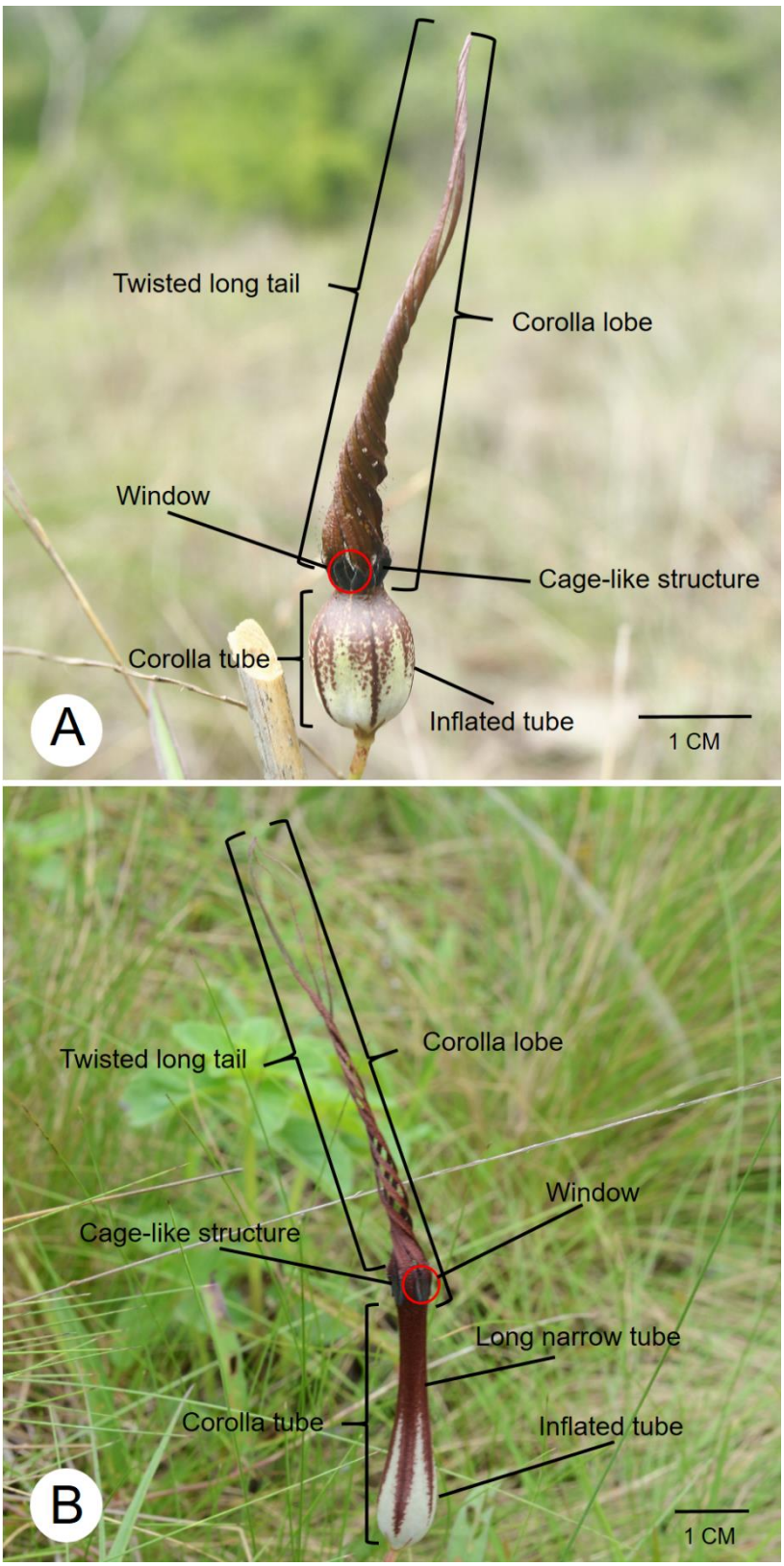

Figure 4.12 Flower morphology *Ceropegia thailandica* and *C. suddeei* A: Flower of *C. thailandica*. B: Flower of *C. suddeei*.

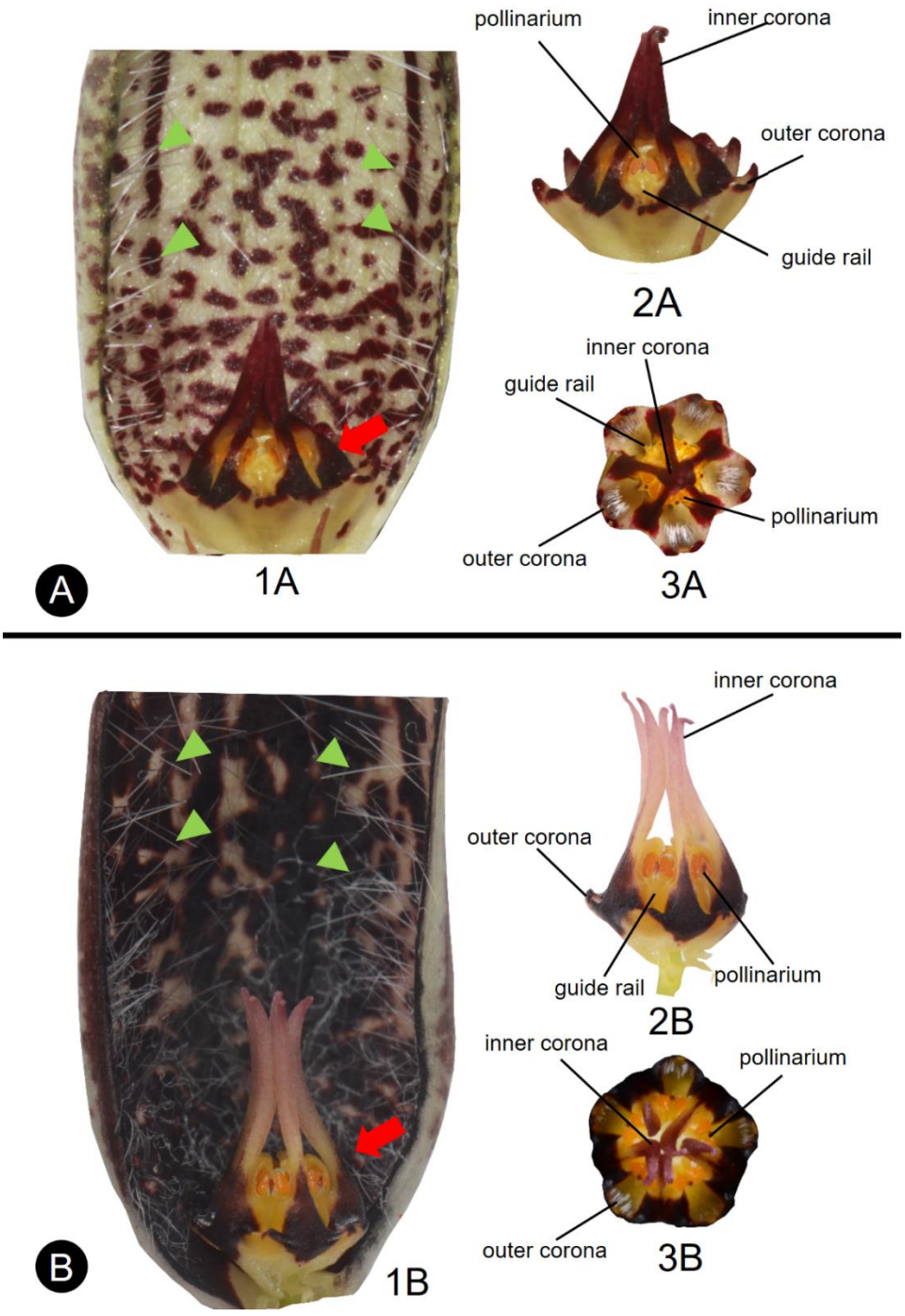

Figure 4.13 The internal structure of flower of *Ceropegia thailandica* and *C. suddeei*. A: *C. thailandica*, 1A: Long section of flower showing gynostegium (red arrow) and hairs (green arrow heads), 2A: Gynostegium (side view), 3A: Gynostegium (top view). B: *C. suddeei*, 1B: Long section of flower showing gynostegium (red arrow) and hairs (green arrow heads), 2B: Gynostegium (side view), 3B: Gynostegium (top view).

4.4.2 The quantitative characteristics of flower that involve in pollination mechanism

The quantitative characteristics of 10 flowers of each species were studied. Eight parts of flowers were measured (Table 4.6 and Figure 4.14).

Table 4.6 The size of the eight parts of flower of *Ceropegia thailandica* and *C. suddeei.*

No.	Part of flower	*C. thailandica* (mm)	*C. suddeei* (mm)
1	Total length of flower	58.8 ± 7.54	66.6 ± 9.45
2	Width of window	1.72 ± 0.13	1.72 ± 0.43
3	Length of window	3.01 ± 0.49	5.36 ± 0.42
4	Diameter of corolla tube at the narrowest point	3.99 ± 0.48	3.85 ± 3.53
5	Diameter of corolla tube at the widest point	9.25 ± 9.22	7.53 ± 0.48
6	Height of inflated tube	11.17 ± 1.28	13.35 ± 0.96
7	Height of gynostegium	4.95 ± 0.64	5.10 ± 0.50
8	Diameter of gynostegium	4.71 ± 0.27	3.72 ± 0.16

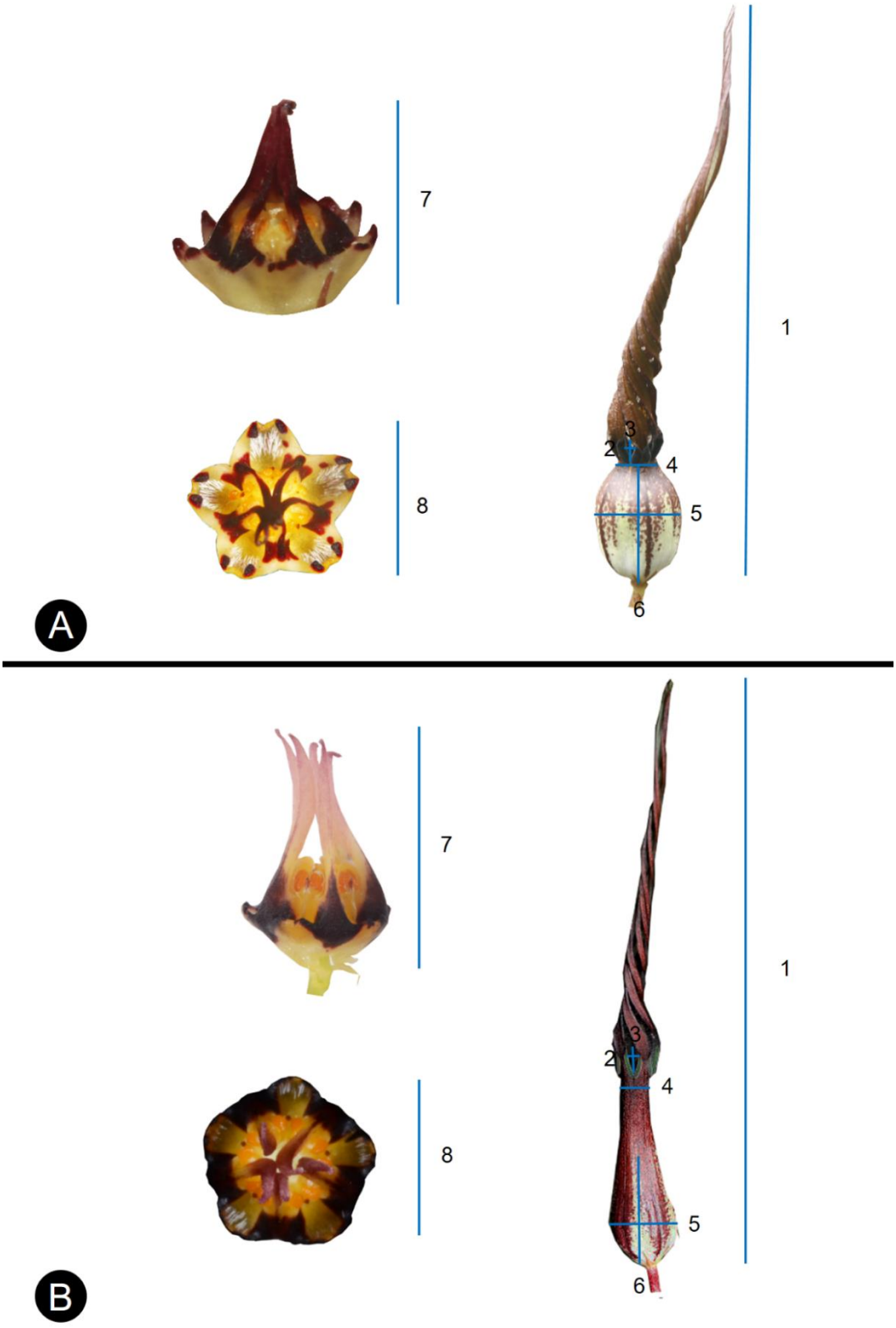

Figure 4.14 The eight parts of flower of which the size were measured. A: Flower of *Ceropegia thailandica*. B: Flower of *C. suddeei*.

4.4.3 The morphlogical characteristics and size of pollinarium of *Ceropegia thailandica* and *C. suddeei*

The pollinium of *Ceropegia thailandica* is oval. The width of pollinium is 318.54 ± 24.05 micrometer and the length of pollinium is 407.24 ± 20.36 micrometer. On the inner side of each pollinarium, there is a triangular shaped thin membrane called pellucid margin. The translator is a long flat structure which attached the pollinium and corpusculum. The corpusculum is long arrowhead shaped with a longitudinal groove and brown in color (Figure 4.15A).

The pollinium of *Ceropegia sudeei* is oval in shape like *C. thailandica*. The width of pollinium is 306.84 ± 30.66 micrometer and the length of pollinium is 409.16 ± 27.52 micrometer. There is a pellucid margin on the inner side of pollinium as found in *C. thailandica*. The translator is long flat and the corpusculum is long arrowhead with longitudinal groove (Figure 4.15B).

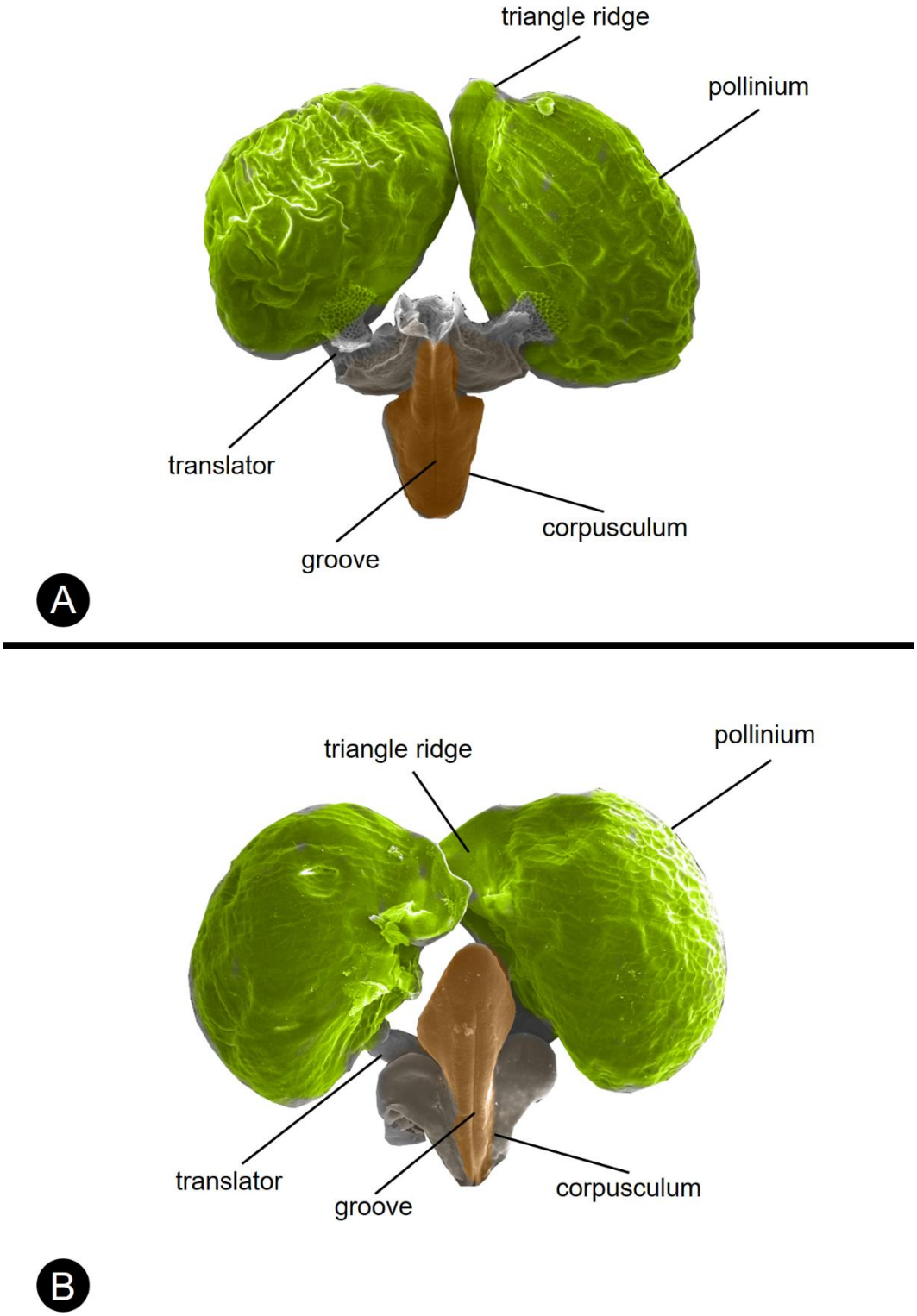

Figure 4.15 The pollinaria of *Ceropegia thailandica* and *C. suddeei*. A: The pollinarium of *C. thailandica*. B: The pollinarium of *C. suddeei*.

4.4.4 Attractants

4.4.1.1 Floral scent

The floral scents of *C. thailandica* and *C. suddeei* were examined by Gas chromatography/Mass spectrometry. The result showed that the floral scents of *C. thailandica* and *C. suddeei* comprised respectively 11 and 13 volatile organic compounds (Vocs). The main components of the floral scent emitted by *C. thailandica* were Isopentyl acetate, 1-Octen-3-yl-acetate and Benzyl acetate. While, the main components of floral scent of *C. suddeei* were Isopentyl acetate, 1-Octen-3-yl-acetate and (*E*)-2-Decenyl acetate (Table 4.7).

Table 4.7 Volatile organic compounds (VOCs) identified in floral scents collected from the flowers of *Ceropegia thailandica* and *C. suddeei*. Number of sampled flowers (each flower obtained from different plant individual), sampling times, total number of scent components, mean total amount of scent emitted per flower (ng/15 min ±SD), and mean (±SD) relative amounts of VOCs are provided. RI = retention index.

	RI	*C. thailandica*	*C. suddeei*
Number of sampled flowers:		6	5
Sampling time (h):		3	3
Total number of components:		11	13
Mean total amount emitted per flower:		8288.7 ± 9309.6	1107.4 ± 829.1
Fatty acid derivatives			
Isopentyl acetate	855.5	**5166.8 ± 2343.5**	**700.7 ± 556.4**
Hexyl acetate	990	134.1 ± 162.3	4.1
Cyclooctyl alcohol	1055	452.5	
Octyl formate	1054	194.1	
1-Octen-3-yl-acetate	1160	**3879. ± 4601.9**	**333.2 ± 175.1**
(*E*)-2-Decenyl acetate	1405	950.3 ± 1422.3	**99.1 ± 105.6**
2,5-Dimethyl-4-hydroxy-3-hexanone	820	79.6	
1-Methylpentyl hydroperoxide	1013	35	83.8 ± 9.7
3,3,6-Trimethyl-1,5-heptadien-4-one	914	670.3	
(*Z*)-2-Hexenyl acetate	990	754.3	4.1 ± 3.2
1-Ethylbutyl hydroperoxide	965		64.3
Eucalyptol	1059	7.4	
(*Z*)-2-Octen-1-ol	1039		3 ± 0.2
(*E*)-3-octenyl acetate	1100		7.2 ± 6.2
Linalyl acetate	1272		1.9
(*Z*)-5-Octen-1-ol	1048		3.1
(*Z*)-3-Decenyl acetate	1389		2.1
2-Ethylcyclohexanol	1068		21.5
Benzenoid compounds			
Benzyl acetate	1060	**1956.6 ± 908.9**	

4.4.4.2 Osmophore

The osmophore of *C. thailandica* was found located on the upper surface of the corolla lobe. It was composed of two cell layers, epidermal and subepidermal cell layers. The epidermal cells were broadly ovate shaped with acute to acuminate tip while the subepidermal cells were rounded with dense cytoplasm. Moreover, the subsequent cell layers were composed of large irregular shaped parenchyma cells with prominent intercellular spaces (Figure 4.16A). When the sections were stained with iodine solution, several starch grains were detected (Figure 4.16B). These starch grains lied at the middle and the base of the cells. Sudan IV staining detected the orange oval lipid droplets (Figure 4.16C) and neutral red revealed prominent red vacuole and red oval lipid droplets in broadly ovate epidermal cells (Figure 4.16D). When the sample was stained with Sudan black and Nadi reagent, the lipid (Figure 4.16E) and terpene (Figure 4.16F) were also detected in broadly ovate epidermal cells.

The osmophore of *C. suddeei* located on upper surface of corolla lobe. The osmophore was composed of two cell layers, epidermal and subepidermal cell layers. The epidermal cells were teardrop shaped with long acute tip, while the subepidermal cells were rounded with dense cytoplasm. The subsequent cell layers were composed of large irregular shaped parenchyma cells with prominent intercellular spaces (Figure 4.17A). When the sections were stained with iodine solution, several purple stained-starch grains were detected in the subepidermal cells (Figure 4.17B). These starch grains lied at the middle and the base of the cells .Sudan IV staining revealed the orange oval lipid droplets (Figure 4.17C) in this cell layer. When the samples were stained with neutral red, prominent red vacuoles could be observed in the teardrop shaped epidermal cells and subepidermal cells (Figure 4.17D). Moreover, lipids (Figure 4.17E) and terpenes (Figure 4.17F) were also detected in these cells by Sudan black and Nadi stains, respectively.

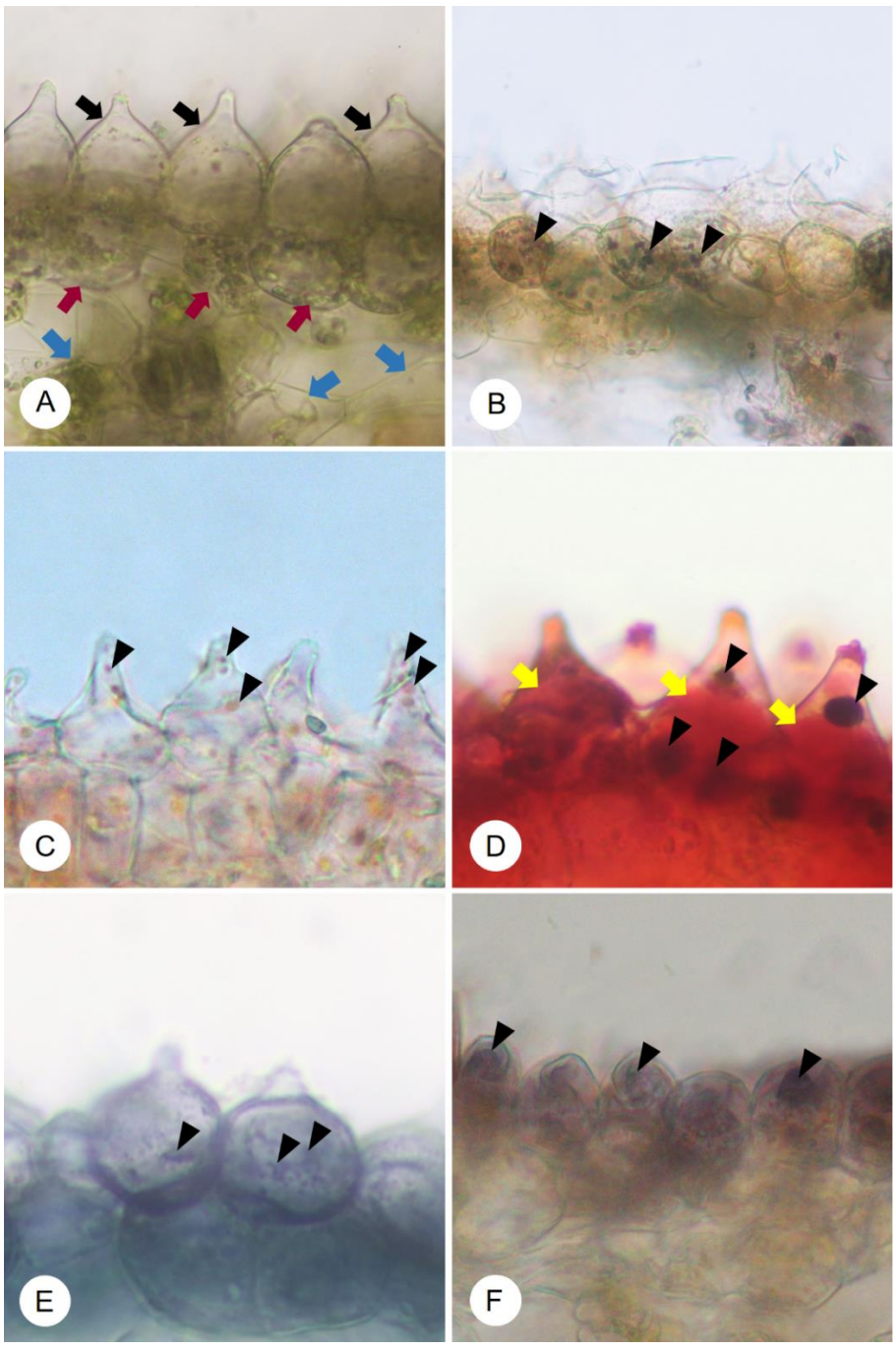

Figure 4.16 Transverse sections of the corolla lobe of *Ceropegia thailandica* stained with different dyes, showing the osmophore formed by the epidermal and subepidermal cells. A: Unstained section, the tear drop shaped epidermal cells (black arrows), the rounded subepidermal cells (brown arrows) and the large irregular shaped parenchyma cells (blue arrows). B : Starch grains (black arrow heads) detected with Iodine. C : Lipids (black arrow heads) detected with Sudan IV. D :Neutral red incorporated into the vacuoles (yellow arrows) of living cells and lipid droplets (black arrow heads). E :Lipids (black arrow heads) detected with Sudan black. F :Terpenes (black arrow heads) detected with Nadi reagent.

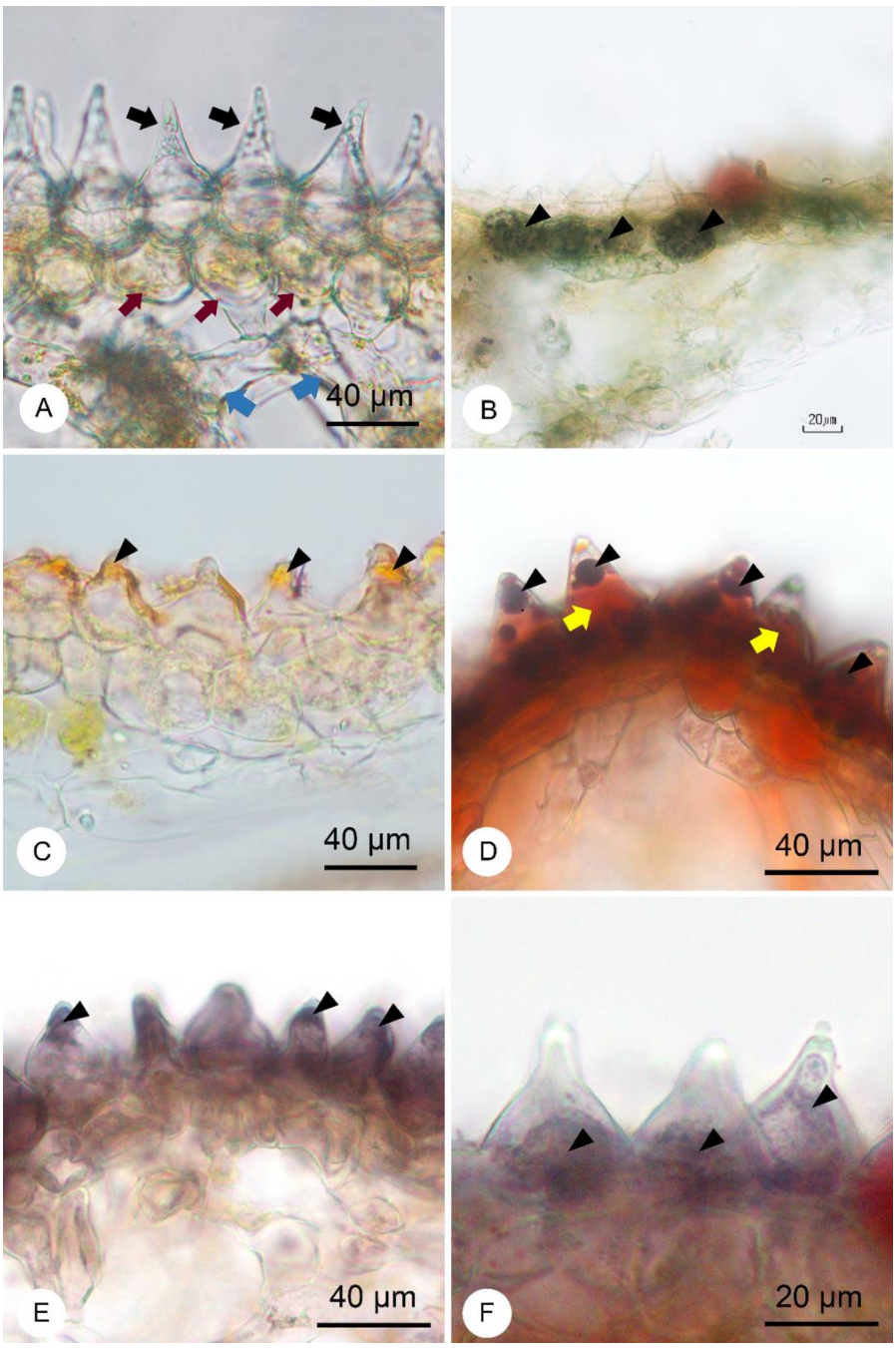

Figure 4.17 Transverse sections of the corolla lobe of *Ceropegia thailandica* stained with different dyes, showing the osmophore formed by the epidermal and subepidermal cells. A: Unstained section, the tear drop shaped epidermal cells (black arrows), the rounded subepidermal cells (brown arrows) and the large irregular shaped parenchyma cells (blue arrows). B : Starch grains (black arrow heads) detected with Iodine. C : Lipids (black arrow heads) detected with Sudan IV. D :Neutral red incorporated into the vacuoles (yellow arrows) of living cells and lipid droplets (black arrow heads). E :Lipids (black arrow heads) detected with Sudan black. F: Terpenes (black arrow heads) detected with Nadi reagent.

4.4.4.3 Nectar

The nectar samples of the two species extracted by distilled water were analyzed using High Performance Liquid Chromatography (HPLC) to detect glucose, fructose and sucrose, the main composition of nectar (Nicolson and Thornburg, 2007). It was found that the nectar of *C. thailandica* contained 43% and 57% of glucose and fructose, respectively. While, the nectar of *C. suddeei* constituted of 46%, 51% and 3% of glucose, fructose and sucrose, respectively (Figure 4.18).

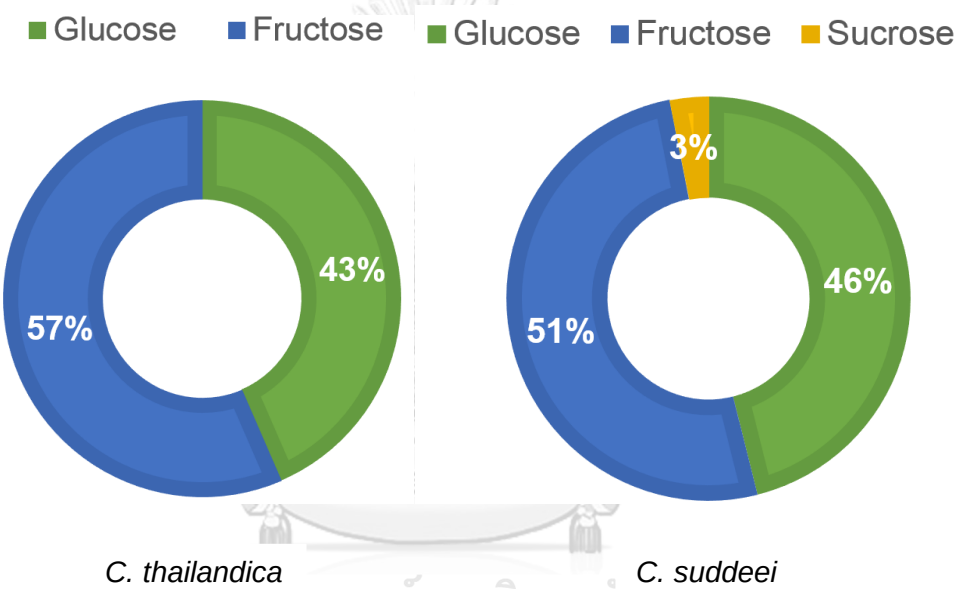

Figure 4.18 The major sugar composition of nectar of *Ceropegia thailandica* and *C. suddeei*.

4.4.4.4 Nectary

Cross sections of the lower part of gynostegium observed under light microscope showed that the nectariferous tissue of the two species was located in the guide rails. In each species, five nectaries behind the guide rails were stained in purple. The nectariferous tissue of *Ceropegia thailandica* was composed of an epidermal layer of anticlinal elongate papillose cells and one to two subepidermal layers of loosely arranged polygonal parenchyma (Figure 4.19) cells. The nectariferous tissue of *C. suddeei* was composed of an epidermal layer of anticlinally arranged elongate papillose cells and one to two subepidermal layers of loosely arranged polygonal or irregular shaped parenchyma cells (Figure 4.20).

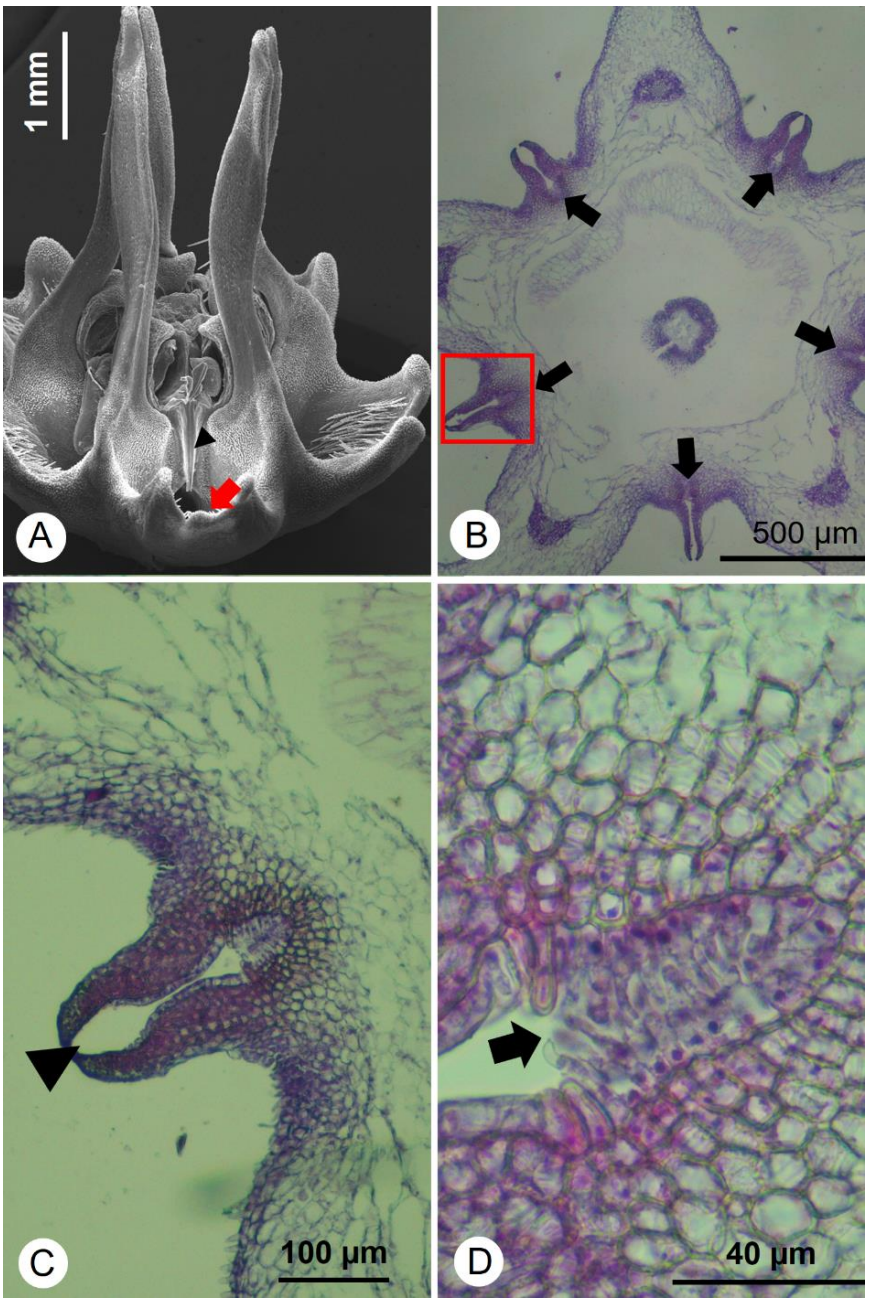

Figure 4.19 SEM (A) and LM photographs (B-C) showing guide rails, nectaries and nectariferous tissue of *Ceropegia thailandica*. A: Gynostegium on which the guide rails (black arrow heads) and nectar cup (red arrow) were found. B: Transverse sections of the lower part of gynostegium double stained with safranin O and fast-green, showing five nectaries hidden behind the guide rails (black arrows). C: High magnification of the red framed zone in B, nectary located behind the guide rails (black arrow head). D: Nectariferous tissue composed of an epidermal and one to two subepidermal layers.

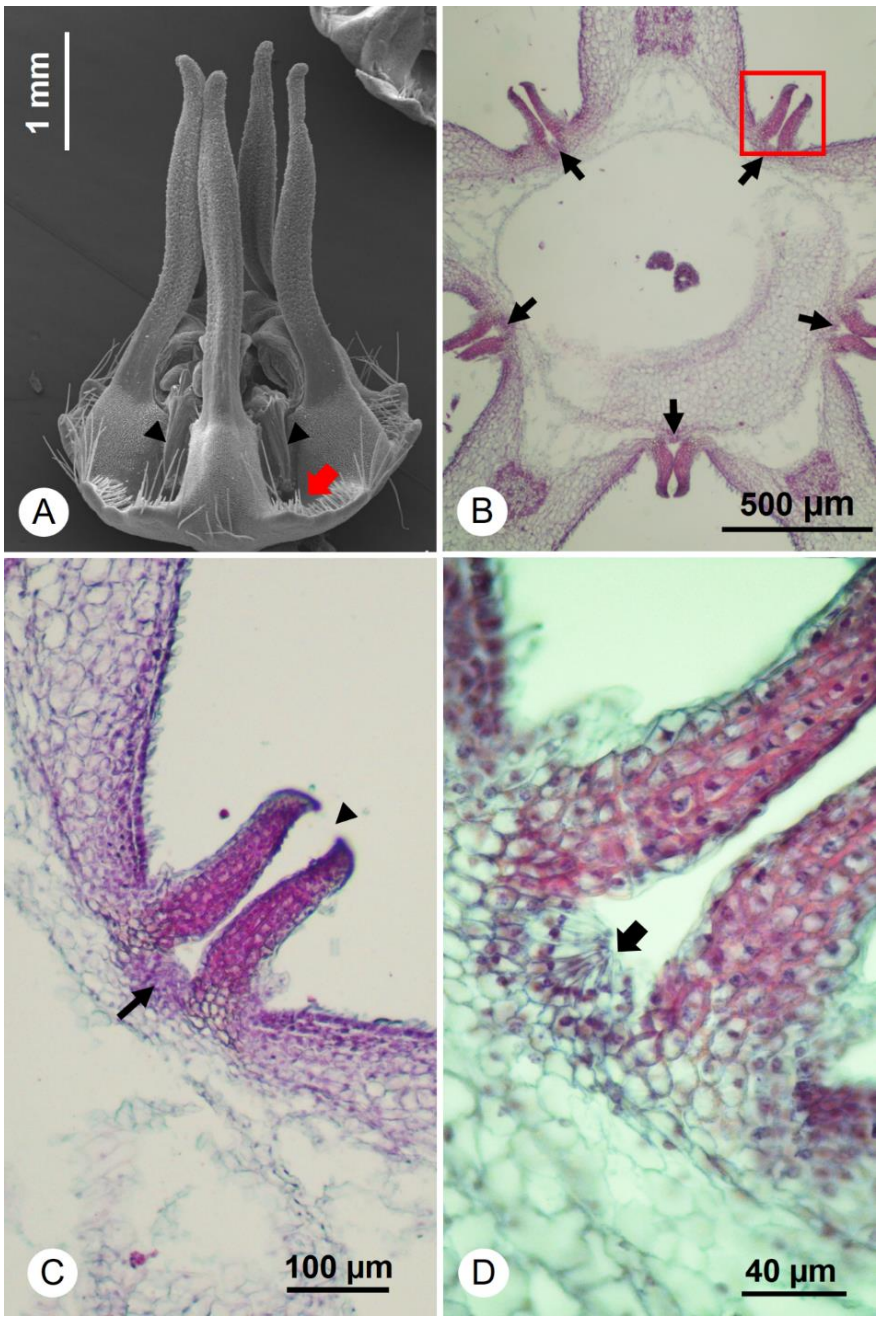

Figure 4.20 SEM (A) and LM photographs (B-C) showing guide rails, nectaries and nectariferous tissue of *C. suddeei*. A: Gynostegium on which the guide rails (black arrow heads) and nectar cup (red arrow) were found. B: Transverse sections of the lower part of gynostegium double stained with safranin O and fast-green, showing five nectaries hidden behind the guide rails (black arrows). C: High magnification of the red framed zone in B, nectary (black arrow) located behind the guide rails (black arrow head). D: Nectariferous tissue constituted of an epidermal and one to two subepidermal layers.

4.5. Visiting behavior of insect pollinators of *Ceropegia thailandica* and *C. suddeei*

The flowers of *Ceropegia thailandica* and *C. suddeei* attracted the pollinators from long distance using floral scent produced from osmophore located at the corolla lobe. After that, the pollinators landed on the middle part of corolla lobes. When they crawled around the windows at the base of the corolla lobes, they often felt into the inflated tube of the flower. Inside the inflated tube, there were many long hairs which acted as barrier to prevent pollinators from escaping (Figure 4.21C). While the pollinators were detained in the flower, they tried to find exit and nectar accumulated in the nectar cup and behind the guide rail. The anther locules were located above the guide rails. When the pollinators searched for some reward nearby (Figure 4.21A; 4.21B), the pollinarium could possibly attach to their mouthparts (Figure 4.22A; 4.22B; 4.22D; 4.22E; 4.22F) on which there were long hairs that could prick trough the groove of the corpusculum (Figure 4.23C). The pollinarium removal could then be occurred. The pollinarium attached the mouth might annoy the insects, as it was observed in the video recording that they try to remove it, but in vain.

When the pollinators visited and trapped in another flower, with the pollinarium (or sometimes pollinaria) attached to their mouthparts, the pellucid margins on the inner side of the pollinia could be inserted in the guide rails, leading to pollinia deposition and pollination.

The last step of pollination processes was “untrapping”. *Ceropegia thailandica* and *C. suddeei* could detain the pollinators inside their flowers for about 24 hours. At post anthesis stage, the flower withered and the hairs inside the inflated tube were dehydrated and shriveled, allowing the pollinators to escape. Interestingly, the untrapping process of the flower of *C. suddeei* was not only owing to flower withering, but there was also the curling of pedicel, leading to reorientation of the flower from vertical to horizontal positions and the pollinating insects could thereby release.

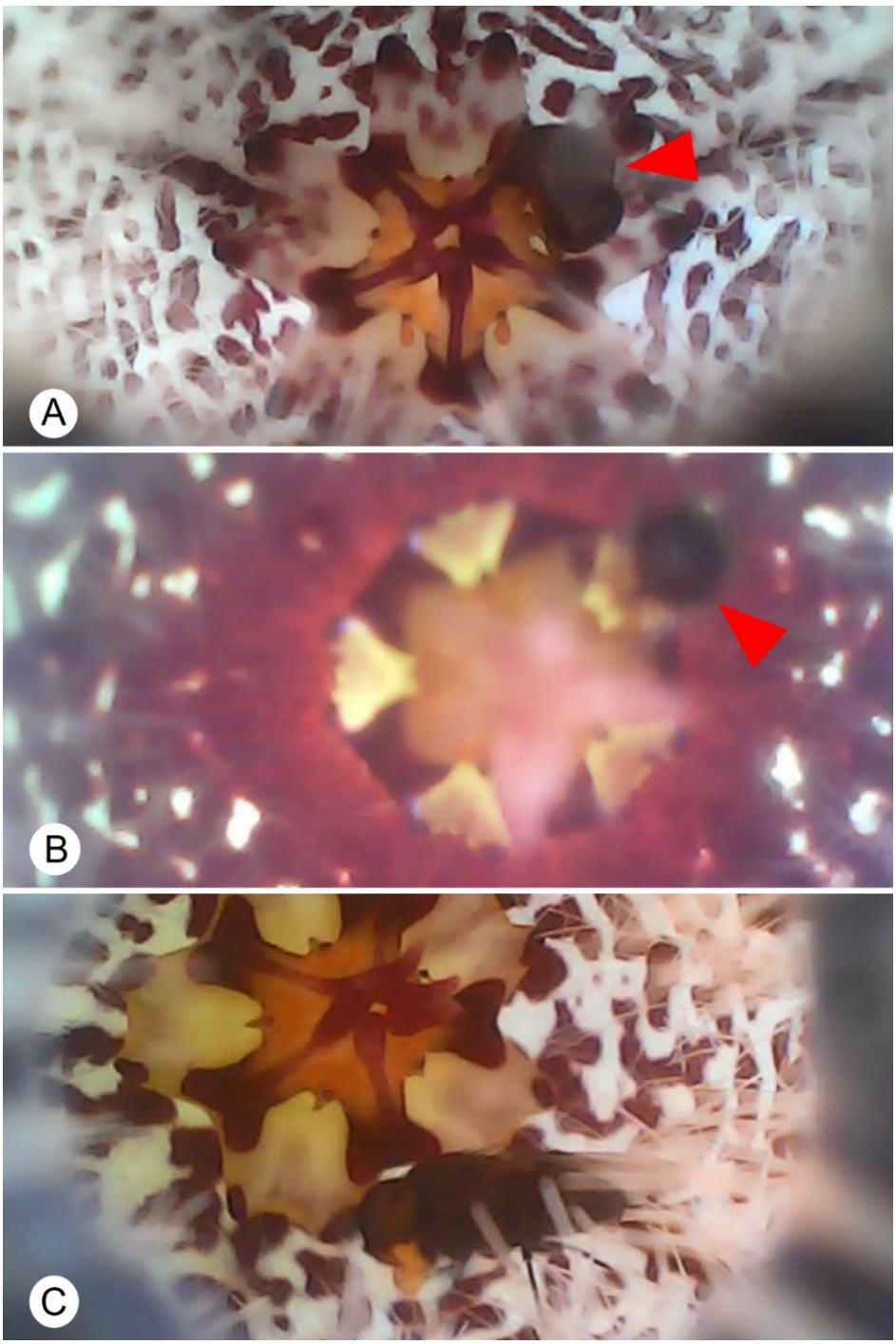

Figure 4.21 Visiting behaviors of insect pollinators while remained inside the flower of *Ceropegia thailandica* and *C. suddeei*. A: The pollinators were searching for some reward or exit around the gynostegium inside the flower of *C. thailandica*. B: The pollinators were searching for some reward or exit around the gynostegium inside flower of *C. suddeei*. C: The long hairs present inside the inflated tube of *C. thailandica* prevented insect from escaping.

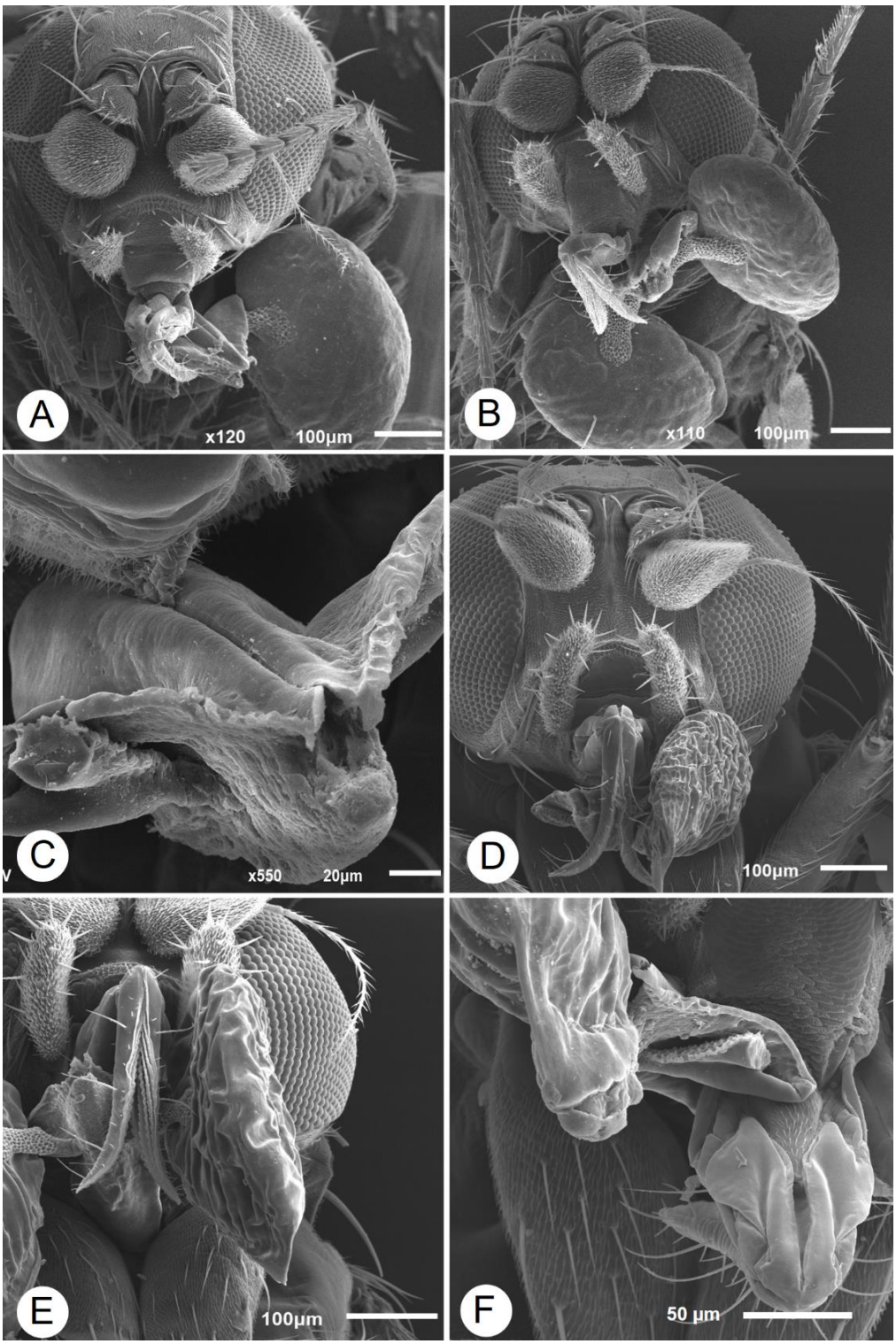

Figure 4.22 The attaching pollinia on the mouthpart of pollinators. A and B: Attaching pollinium of pollinator of *C. thailandica*. C: The pricking of long hair trough the groove of the corpusculum. D, E and F: Attaching pollinium of pollinator of *C. suddeei*.

4.6. Environmental factors in relation to blooming of flowers and behavior of insect pollinators

The average hourly temperature, relative humidity and light intensity obtained from data logger in Phu Wau Wildlife Sanctuary showed that the lowest temperature is 25.62 degree Celsius at 11:00 PM, 12:00 PM and 2:00 AM. While, the highest temperature is 33.24 degree Celcius at 13:00 AM. The lowest and highest relative humidity are 62.26% and 90.39% at 3:00 PM and 5:00 AM respectively (Figure 4.23). The lowest light intensity is 43.29 lux at 6:00 AM and the highest is 2021.23 lux at 1:00 PM (Figure 4.24).

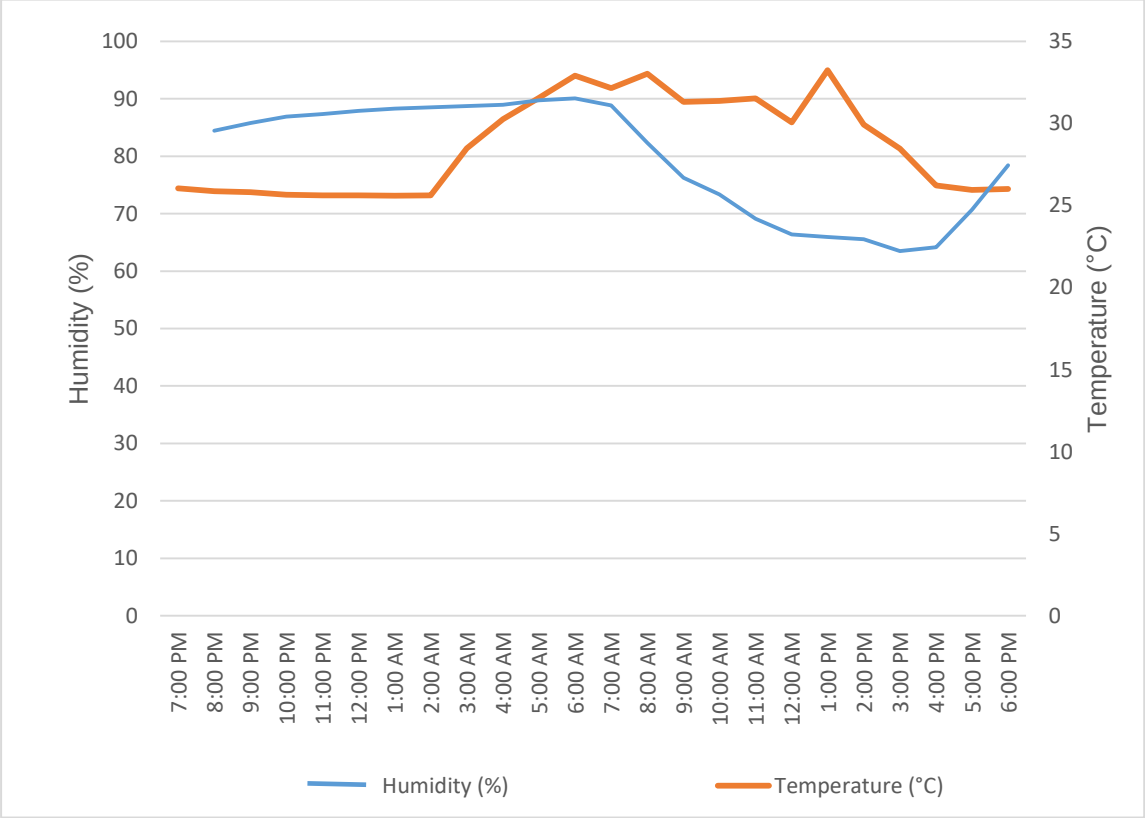

Figure 4.23 Average hourly relative humidity and temperature at Phu Wau Wildlife Sanctuary (May to October 2017).

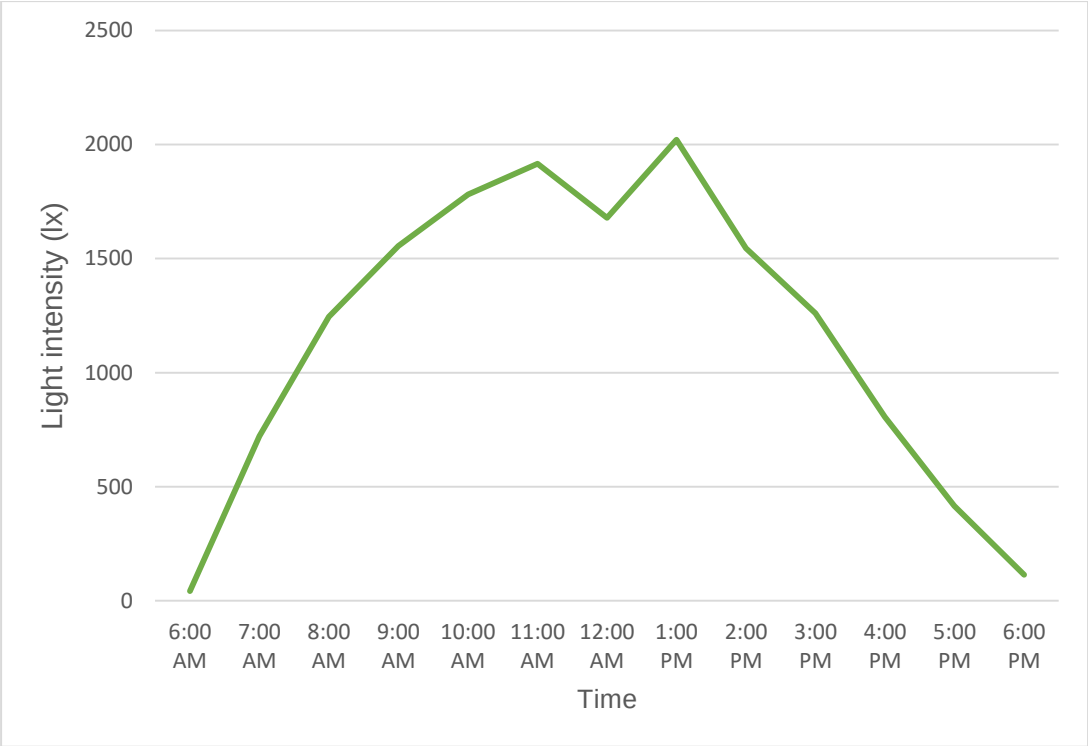

Figure 4.24 Average hourly light intensity of Phu Wau Wildlife Sanctuary (May to October 2017).

The number of insect pollinator entering flower of *Ceropegia thailandica* on 24th May and 27th May 2017 revealed the visitation of insects was most abundant during 8:00-10:00 AM, with the visitation peak at 9:00 AM where the temperature and light intensity was increasing and the relative humidity was high (Figure 4.25).

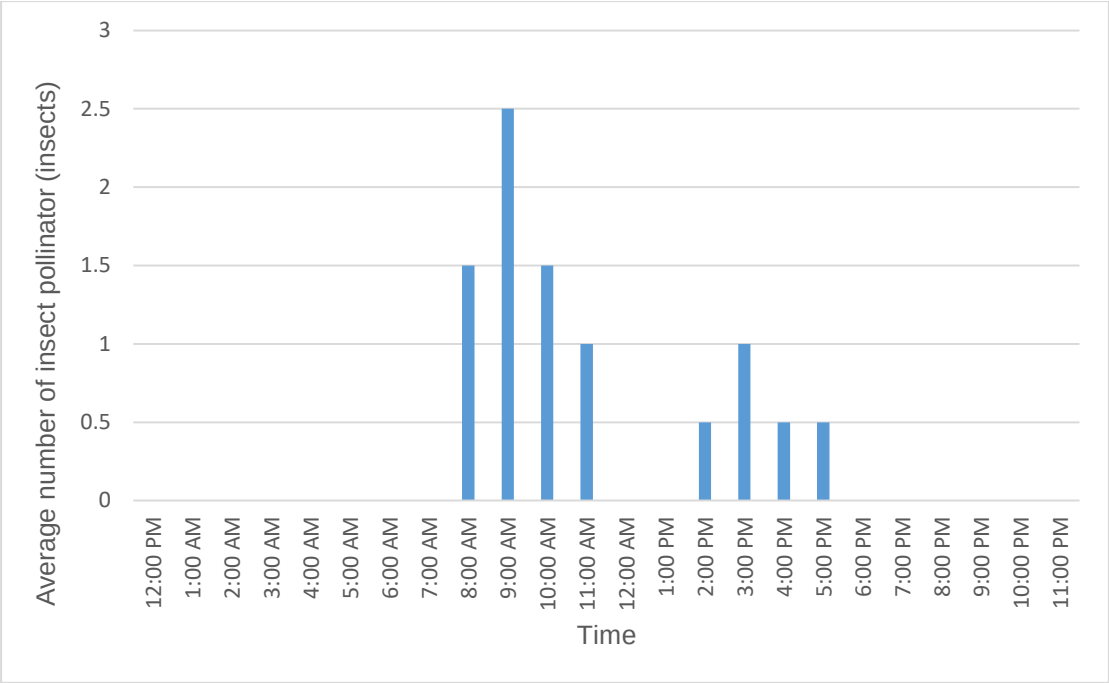

Figure 4.25 The average number of insect pollinators entering flower of *Ceropegia thailandica* for each hour (May to July 2017).

Phu Phan National Park, the study site of *Ceropegia suddeei*, the average hourly temperature, relative humidity and light intensity obtained from data logger showed that the lowest temperature and the highest temperature are 18.93 degree Celsius at 11:00 PM and 28.29 degree Celsius at 1:00 PM. The lowest and highest relative humidity are 70.96% at 3:00 PM and 99.28% at 5 AM (Figure 4.26). While, the lowest light intensity is 77.41 lux at 6:00 AM and the highest is 4339.06 lux at 12:00 AM (Figure 4.27).

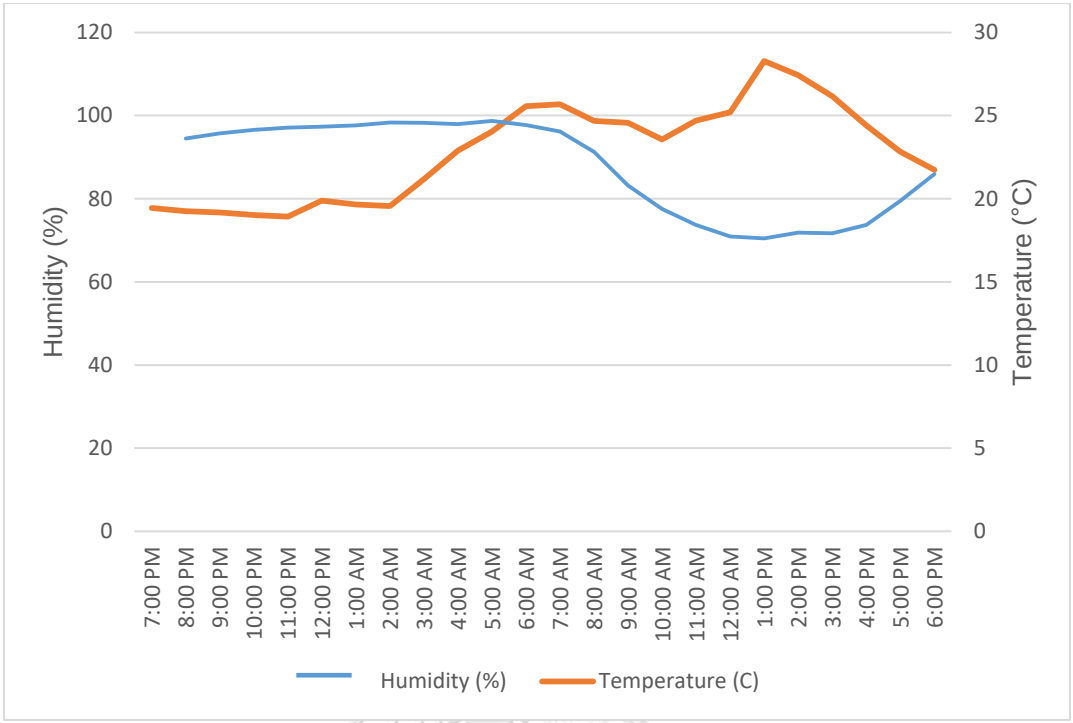

Figure 4.26 Average hourly relative humidity and temperature at Phu Phan National Park (May to October 2017).

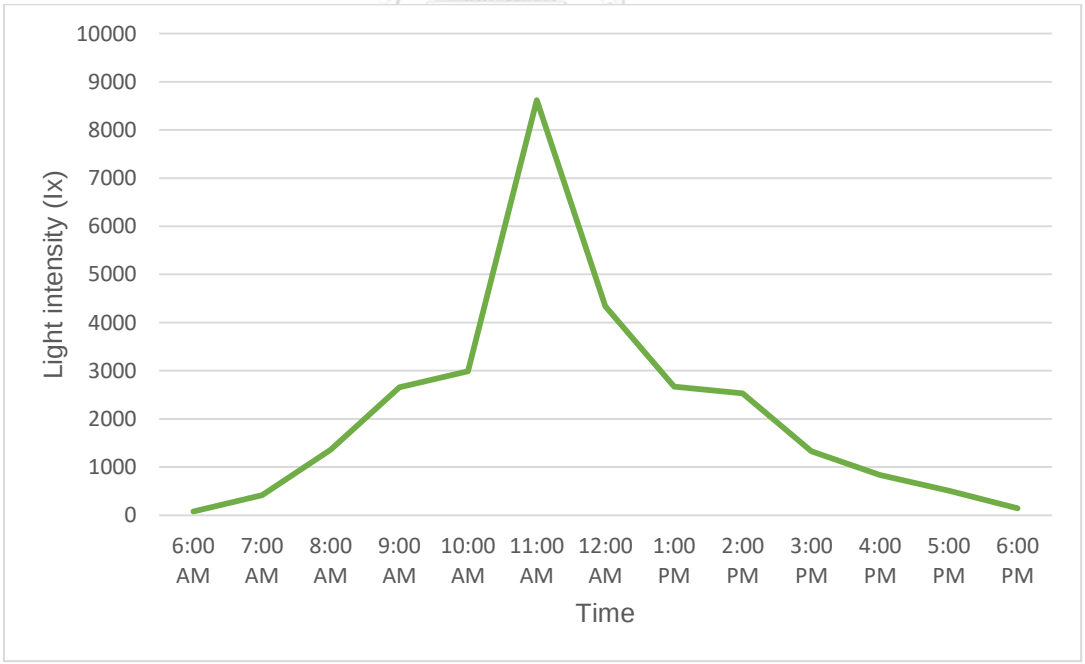

Figure 4.27 Average hourly light intensity at Phu Phan National Park (May to October 2017).

The number of insect pollinators entering flower of *Ceropegia suddeei* on 1st June and 4th June 2017 were abundant between 4:00-6:00 PM with the visitation peak at 4:00 PM where the relative humidity and light intensity were low, and the temperature was moderate (Figure 4.28).

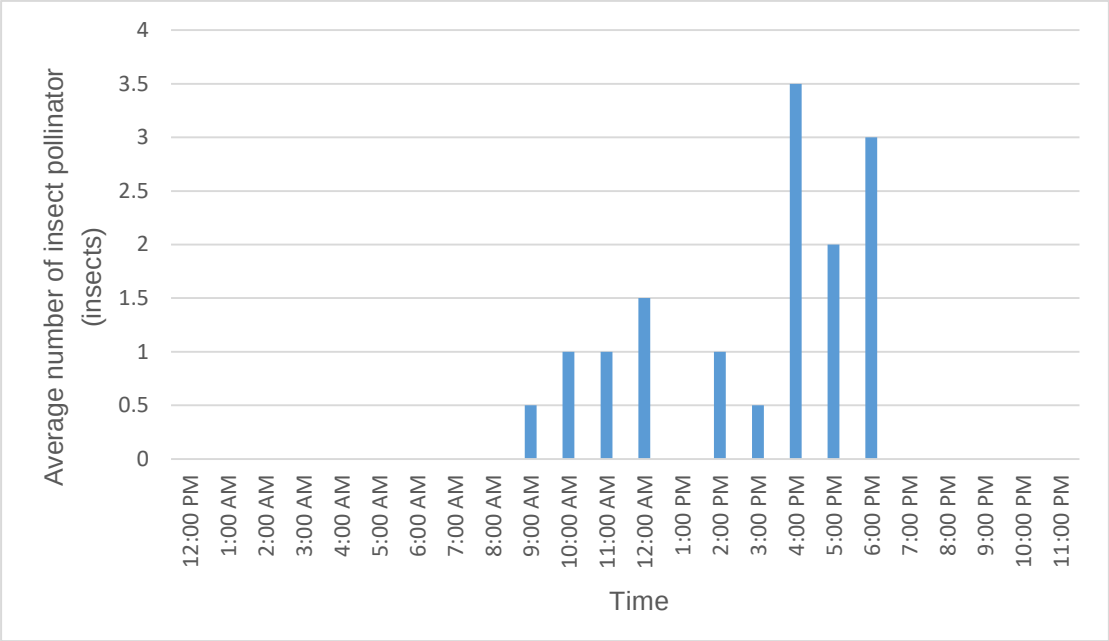

Figure 4.28 The average number of insect pollinators entering flower of *Ceropegia suddeei* (May to July 2017).

4.7. Pollen Transfer Efficiency (PTE)

The pollination of *Ceropegia thailandica* and *C. suddeei* could take place by the activity of when the pellucid margin of a pollinium was inserted between the guide rails from where the pollen tubes could grow downward to the ovaries (Figure 4.29). Fifteen flowers from the population of each species were examined for the number of pollinaria removed from anther and the number of pollinia inserted between the guiderails. The result is shown in the Table 4.8. The obtained pollen-transfer efficiency (PTE) of *C. thailandica* was 0.16.

Table 4.8 Blooming period, number of pollinaria that were removed from anthers and number of pollinia that were inserted between the guiderails in the flowers of *Ceropegia thailandica.*

Number of flower	Blooming period (day)	Number of pollinaria removed	Number of pollinia inserted
1	1	1	0
2	1	1	0
3	1	0	0
4	1	0	0
5	1	0	0
6	2	0	5
7	1	3	2
8	1	0	0
9	2	0	5
10	1	0	0
11	2	1	1
12	1	0	0
13	1	0	0
14	2	0	5
15	1	0	1

The result obtained for *Ceropegia suddeei* is shown in the Table 4.9. The pollen-transfer efficiency (PTE) calculated for this species was 0.21.

Table 4.9 Blooming period, number of pollinaria that were removed from anthers and number of pollinia that were inserted between the guiderails in the flowers of *Ceropegia suddeei.*

Number of flower	Blooming period (day)	Number of pollinaria removed	Number of pollinia inserted
1	2	4	4
2	1	5	0
3	1	0	0
4	1	5	0
5	1	4	2
6	1	0	0
7	2	2	4
8	1	5	2
9	2	4	0
10	1	1	1
11	2	4	2
12	1	5	1
13	1	3	3
14	1	3	0
15	2	4	2

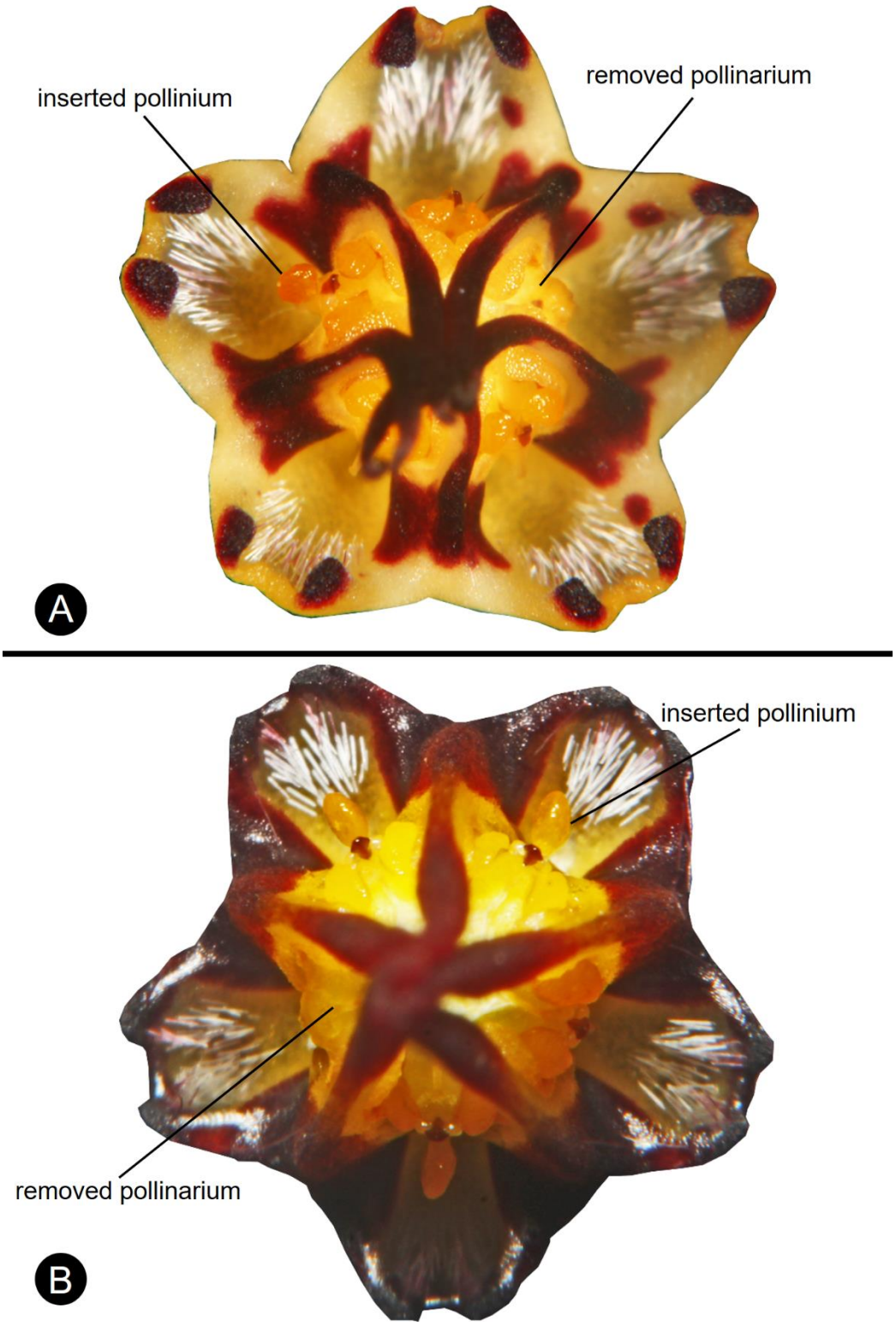

Figure 4.29 The sections of gynostegium where the pollinarium was removed and a pollinium was inserted between the guide rails(arrows). A: *Ceropegia thailandica*. B: *C. suddeei*.

CHAPTER V
DISCUSSION

5.1. Natural habitats of *Ceropegia thailandica* and *C. suddeei*

Ceropegia thailandica and *C. suddeei* grow on sandy soil in open area of dry dipterocarp forest in Phu Wau Wildife Sanctuary and Phu Phan National Park respectively. The average temperature throughout the year of Phu Wau Wildlife Sanctuary and Phu Phan National park were very similar, but the average humidity throughout the year of the two localities were slightly different. Considering the habitats, both species occur in the habitats that are extremely threaten by human activities and wildfire. Moreover, there are low number of individuals in the population of the two species and they are classified as critically endangered (Forest and Plant Conservation Research Office, 2015).

5.2. Flowering phenology of *Ceropegia thailandica* and *C. suddeei*

Ceropegia thailandica and *C. suddeei* grew very rapidly in early rainy season (May) and started to bloom in late May until early October. This developmental period is similar to those of the other species of genus *Ceropegia* found in Thailand (Kidyoo, 2014a; Kidyoo, 2014b; Kidyoo, 2015). There is no great different of the total period of time took for flower development from young flower (stage 1) to fallen flower (stage 8) between the two species, i.e. 26.20 days and 25.05 days for *C. thailandica* and *C. suddeei*, respectively. Their flowers took the longest time to develop in the first and second stages. This might be related to resource allocation for development of male and female organs, as well as other structures involving in pollination (Bazzaz, et al., 2000; Ichie, et al., 2005).

The anthesis stage of *Ceropegia thailandica* and *C. suddeei* took an average of 1 and 2 days, respectively. The flowers started blooming in the morning, about 6:00-7:00 A.M., where the temperature was getting higher, while the humidity was getting lower. Moreover, the flowers of the two species started opening when there was the first light of the day like most diurnal flowers (Doorn and Meeteren, 2003; Kidyoo

and McKey, 2012). Temperature and light intensity clearly affected the opening of flowers in several plant species, such as *Gentiana rhaetica*, *Portulaca* ssp., *Oxalis martiana*, *Nymphaea alba* and several species in the Asteraceae. (Doorn and Meeteren, 2003). However, floral opening in most species tested did not response to change in relative humidity.

Immediately after the flowers opened, they had not yet visited by insects. In *Ceropegia thailandica*, the first insect pollinator entered the flower at 8:00 A.M., while the first insect pollinator of *C. suddeei* entered the flower at 9:00 A.M. These results conform to those found in the previous studies. The insect pollinators of diurnal flowers visited the flowers from morning to mid-day. Their visiting behavior was likely to be affected by light intensity and temperature (Howe *et al.*, 1990; Yokota and Yahara, 2012; Auttama *et al.*, 2018). However, the insect pollinator of *C. thailandica and C. suddeei* also got into the flowers until 5:00 P.M. and 6:00 P.M., respectively. This might be due to the locations of the plant individuals studied that were situated in the expose area. The flowers at post-anthesis stage of both species were quite different. The flower of *C. thailandica* simply wilted from 5:00 P.M. to the night or the next morning, while the flower of *C. suddeei* wilted and the pedicel curled down from 6:00 P.M. to the night or next morning.

5.3. Floral scent of *C. thailandica* and *C. suddeei*

The floral fragrance of *Ceropegia thailandica* comprised as main components Isopentyl acetate, 1-Octen-3-yl-acetate and Benzyl acetate. While, the main components of the floral scent of *C. suddeei* were Isopentyl acetate, 1-Octen-3-yl-acetate and (*E*)-2-Decenyl acetate. The result from the anatomical study showed that the osmophore of both species was located on the upper surface of the corolla lobe and it was composed of epidermal and subepidermal cell layers. This structure of osmophore was similar to that found in the other *Ceropegia* species previously studied by Vogel in 1990. The epidermal cells were broadly ovate shaped or teardrop shaped with acute to acuminate tip. While, the subepidermal cell layer were rounded with dense cytoplasm, which is quite typical in secretory cells that might be related to

accumulation of the substrate for synthesizing the floral scent (Vogel and Renner, 1990).

The pollination mechanism of *Ceropegia thailandica* and *C. suddeei* began by odor emission attracting the insect pollinators from a long distance. This genus has outstanding process of pollination mechanism that had long been known over fifty years ago (Vogel, 1961). The previous studies found that the flower of *Ceropegia* species emitted the scents that were highly variable in compositions among species (Heiduk *et al.*, 2015; Heiduk *et al.*, 2016; Auttama *et al.*, 2018).

The main components 1-Octen-3-yl-acetate and (*E*)-2-Decenyl acetate of the floral scent of *C. suddeei* were similar to those of *C. tenuicaulis* from Pha Taem National Park, Ubonratchathani province (Wattana, 2017). While, Isopentyl acetate found in *C. thilandica* and *C. suddeei*, and Benzyl acetate found in *C. thailandica* had not been previously reported in floral components of other *Ceropegia* species. However, a previous studie found the Isopentyl acetate was known as the most important impact compound of banana aroma (Berger, 2007). Lastly, the flowers of *C. thailandica* and *C. suddeei* at second day of anthesis had not emitted any volatile compounds.

5.4. Trapping pollination mechanism of *Ceropegia thailandica* and *C. suddeei*

The overall results of this study allowed to conclude that the pollination mechanisms of *C. thailandica* and *C. suddeei* occurred in 5 steps, including:

1. Advertising

2. Trapping

3. Rewarding

4. Pollinating

5. Untrapping

Firstly, the flowers of the two species emitted floral scent as advertisement from osmophore (advertising). After that, the insect pollinators were attracted to the flower from a long distance. They always landed on the middle part of corolla lobe, they then crawled around the corolla lobe and finally falled or crawled into the inflated tube of the flower (trapping) where the gynostegium, consisting of male and female reproductive structures, was located. Escaping from there was restricted by presence of long hairs inside the inflated tube. While the pollinators were detained in the flower, they tried to find nectar that was produced by nectariferous tissue located behind the guide rails and flowed to accumulate in nectar-holding cup formed by the corona located at the base of the guide rails (rewarding). After the pollinator fed on nectar, the also searched for more nectar around the guide rails as well as on the other sectors of gynostegium. They crawled around the anther locule, thereby increasing the chance for pollinarium load by attachment of the insects' hairs on the mouthparts to the corpusculum. In contrast, if the insects entered the flower with a pollinarium already attached to their mouthparts, while they tried to find nectar around the guide rails, the pollinia could possibly be inserted between the guide rails, resulting in pollination success (pollinating). Lastly, when the flowers of the two *Ceropegia* species developed into post-anthesis stage, the hairs inside the inflated portion of the corolla tubes and the other parts of flowers wilted, the pollinator could then be released from the flowers (Figure 5.1). All processes of trapping pollination mechanism of *C. thailandica* and *C. suddeei* conform to those found in the previous studies of *Ceropegia*'s pollination in Thailand (Auttama *et al.*, 2018; Wattana, 2018).

The marked difference of pollination mechanism between *Ceropegia thailandica* and *C. suddeei* was the untrapping process that released the pollinators inside the flowers. In *C. thailandica*, its floral parts wilted after anthesis without the curling of pedicel. Conversely, the floral parts of *C. suddeei* wilted and the flower reoriented from erect to horizonal positions, owing to bending of the pedicel. This reorientation of flower could also be found in other species with long corolla tube such as *C. thaithongiae, C. tenuicaulis* and *C. dolichophylla* (Heiduk *et al.*, 2010; Auttama *et al.*, 2018; Wattana, 2018)

Figure 5.1 Diagram showing pollination mechanism of *Ceropegia thailandica* and *C. suddeei*.

5.5. Effective pollinator of *C. thailandica* and *C. suddeei*

In the blooming period, *C. thailandica* has the 4 orders namely order Diptera, Hymenoptera, Orthoptera and Lepidoptera. However, there are only dipterean fly (order Diptera) i.e. family Milichiidae and Chloropidae that can enter the flower and ensure pollination successful by carrying the pollinarium on their mouth part. In contrast, if the insects entered the flower with a pollinarium already attached to their mouthparts. The pollinium will be pollinated into stigma because they try to find nectar that was produced by nectariferous tissue located behind the guide rails. As well as *C. suddeei*, the effective pollinators are family family Milichiidae and Chloropidae (order Diptera). From previous study, the Milichiidae and Chloropidae were the effective pollinators of *C. Arabica* and *C. robynsiana* found in Kenya (Masinde, 2004b).

5.6. Breeding system of *Ceropegia thailandica* and *C. suddeei*

This study did not investigate breeding systems by hand-pollination. However, there were few characteristics indicating mechanism for protecting self-pollination which described as follow:

1. Flowering phenology prevented geitonogamy (self-pollination between two flowers of the same plant). Each individual plant of *C. thailandica* and *C. suddeei*, develop flowers in different stages, but each of these flowers bloomed one after another. Therefore, there was only a single flower at anthesis per plant per day. This kind of flowering phenology was also revealed in *C. thaithongiae* and *C. tenuicaulis* (Auttama, McKEY and Kidyoo, 2018; Wattana, 2018). This results conform to a previous study by Karuppusamy and Pullaiah (2009), they found the most *Ceropegia* species such as *C. cumingiana*, *C. nilotica* and *C. elegans* did not permit geitonogamy be self-incompatability.

2. Spatial separation of male and female reproductive structures on the gynostegium (herkogamy) prevented autogamy (self-pollination within a flower). Self-pollination was almost impossible without insect activities inside a flower.

3. Pollinarium reconfiguration promoted xenogamy (cross-pollination between two flowers of different plants). When the pollinatrium had just removed from an anther locule, its alignment was parallel to the face and perpendicular to the mouthparts of the insect (Figure 5.2). This arrangement prevent insertion of a pollinium into the guiderails. After the insect was released to from flower to the outside, the pollinarium got dehydrated and the alignment plane was twisted. In this way, the pellucid margin of a pollinium became reoriented in the same plane of the guide rails (Figure 5.2), this pollinium could then be inserted inside leading to pollination. This occurrence is called pollinarium reconfiguration. Protection of self-pollination by pollinarium configuration were previously reported many orchid species (Shukla *et al.*, 1998; Singer, 2002; Peter and Johnson, 2006) and some species of the genus *Asclepias* (Asclepiadoideae) (Bookman, 1981).

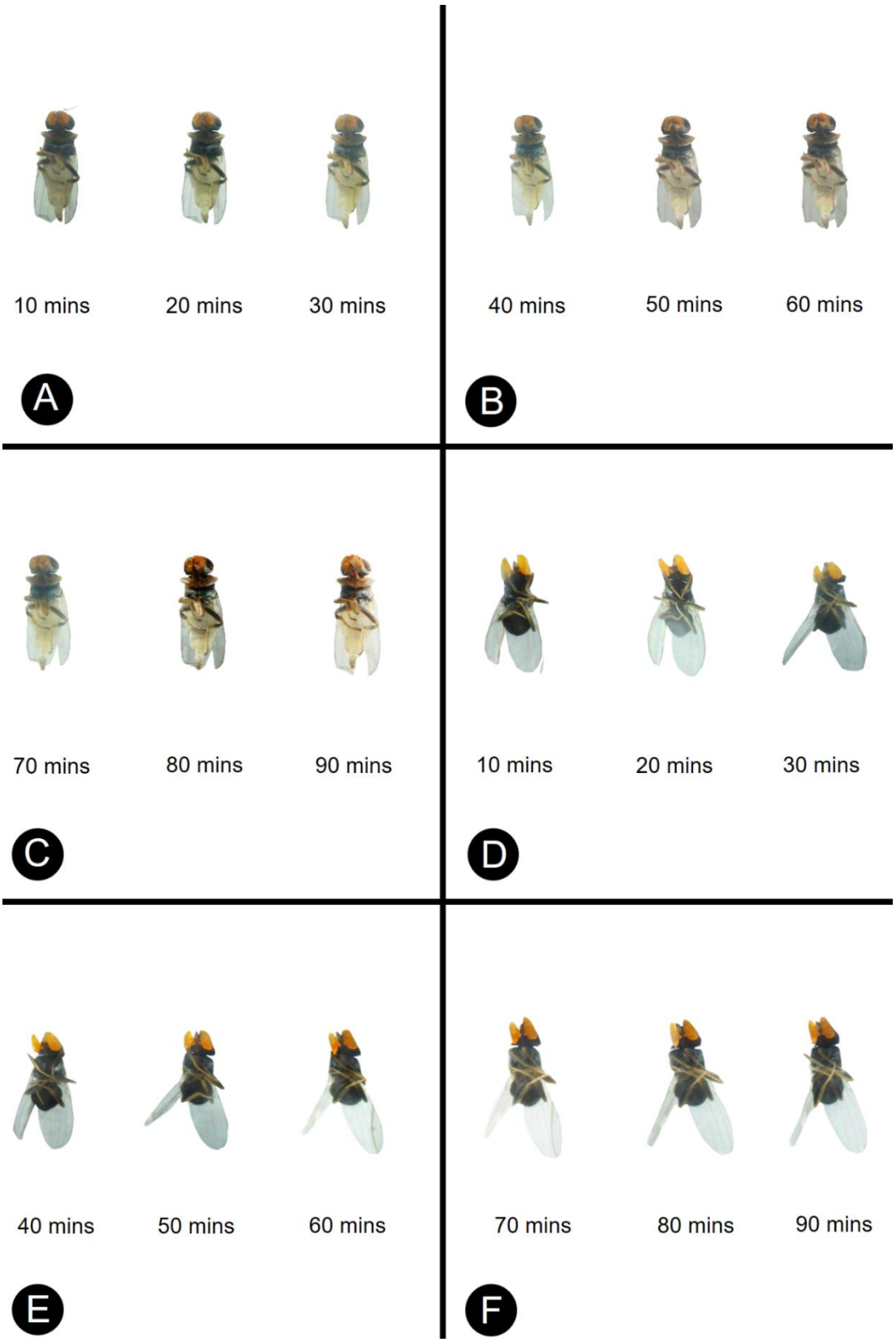

Figure 5.2 Pollinarium configuration of *Ceropegia thailandica* (A, B and C) and *C. suddeei* (D, E and F).

5.7. Pollen Transfer Efficiency (PTE)

Pollen Transfer Efficiency (PTE) of *Ceropegia thailandica* and *C. suddeei* are 0.16 and 0.21 respectively. These values were fairly different comparing to those reported the other Thai species, i.e. *C. thaithongiae* (PTE = 0.067) and *C. tenuicaulis* (PTE = 13.64) (Auttama *et al.*, 2018).

Considering the values revealed in the other *Ceropegia* species from the other countries, it was found that the PTE values were comparable those of two *C. thailandica* and *C. suddeei* (Coombs *et al.*, 2011; Heiduk *et al.*, 2015; Heiduk *et al.*, 2017). Lastly, when the PTE of *C. thailandica* and *C. suddeei* were compared to the other genera of Asclepiadoideae, It was found that their PTE values were lower than the other genera, such as *Asclepias* and *Araujia*.

The low PTE of *C. thailandica* and *C. suddeei* may contribute to pollen limitation and thus limit seed production at the population level that can lead to the extinction of these species (Harder and Aizen, 2010). However, the species with pollinia especially Asclepiadoideae has the pollen transfer efficiencies more than the species with monads (Harder and Johnson, 2008). Moreover, the pollinium deposition by inserting in the guide rails was hypothesized to increase the precision of pollen transfer and may also increase PTE (Livshultz *et al.*, 2018).

CHAPTER VI
CONCLUSION

Ceropegia thailandica and *C. suddeei* are endemic species of Thailand occurred in open area of dry dipterocarp forest in Phu Wau Wildife Sanctuary and Phu Phan National Park respectively. The blooming period of flower of *C. thailandica* and *C. suddeei* occur in late May to early October yearly. The average period for flower development of *C. thailandica* was 26.2 days. While, the average period of flower development of *C. suddeei* was 25.05 days. There is no great different of the total period of time took for flower development flower of the flower between the two species. The anthesis stage of *Ceropegia thailandica* and *C. suddeei* took an average of 1 and 2 days, respectively. The flowers started blooming in the morning, about 6:00-7:00 A.M. At that time, the temperature was getting higher, while the humidity was getting lower. Moreover, each individual plant of *C. thailandica* and *C. suddeei*, develop flowers in different stages, but each of these flowers bloomed one after another. Therefore, there was only a single flower at anthesis per plant per day.

The flowers of the two species emitted floral scent as advertisement from osmophore (advertising). The floral fragrance of *Ceropegia thailandica* comprised as main components Isopentyl acetate, 1-Octen-3-yl-acetate and Benzyl acetate. While, the main components of the floral scent of *C. suddeei* were Isopentyl acetate, 1-Octen-3-yl-acetate and (*E*)-2-Decenyl acetate released from osmophore. The pollination mechanism of *Ceropegia thailandica* and *C. suddeei* began by odor emission attracting the insect pollinators from a long distance. After that, the insect pollinators were attracted to the flower from a long distance. They always landed on the middle part of corolla lobe, they then crawled around the corolla lobe and finally falled into the inflated tube of the flower (trapping). While the pollinators were detained in the flower, they tried to find nectar that was produced by nectariferous tissue located behind the guide rails and flowed to accumulate in nectar-holding cup (rewarding). They crawled around the anther locule, thereby increasing the chance for pollinarium load by attachment of the insects' hairs on the mouthparts to the corpusculum. In contrast, if the insects entered the flower with a pollinarium already

attached to their mouthparts, while they tried to find nectar around the guide rails, the pollinia could possibly be inserted between the guide rails, resulting in pollination success (pollinating). In the post-anthesis stage, the hairs inside the inflated portion of the corolla tubes and the other parts of flowers wilted, the pollinator could then be released from the flowers. However, the great difference of pollination mechanism between *Ceropegia thailandica* and *C. suddeei* was the untrapping process that released the pollinators inside the flowers. In *C. thailandica*, its floral parts wilted after anthesis without the curling of pedicel. Conversely, the floral parts of *C. suddeei* wilted and the flower reoriented from erect to horizonal positions, owing to bending of the pedicel.

The effective pollinators of *C. thailandica* and *C. suddeei* were dipterean flies (order Diptera) in family Milichiidae and Chloropidae. They can enter the flower and ensure pollination successful by carrying the pollinarium on their mouth part. In contrast, if the insects entered the flower with a pollinarium already attached to their mouthparts. The pollinium will be pollinated into stigma because they try to find nectar that was produced by nectariferous tissue located behind the guide rails.

Interestingly, the breeding system of *Ceropegia thailandica* and *C. suddeei*, there is the pollinarium reconfiguration promoted xenogamy (cross-pollination). When the pollinatrium had just removed from an anther locule, its alignment was parallel to the face and perpendicular to the mouthparts of the insect. This arrangement prevent insertion of a pollinium into the guide rails. After the insect was released from flower to the outside, the pollinarium got dehydrated and the alignment plane was twisted. In this way, the pellucid margin of a pollinium became reoriented in the same plane of the guide rails, this pollinium could then be inserted inside leading to pollination.

Lastly, pollen transfer efficiency (PTE) of *Ceropegia thailandica* and *C. suddeei* are 0.16 and 0.21 respectively. These values were fairly different comparing to those reported the other Thai species, i.e. *C. thaithongiae* (PTE = 0.067) and *C. tenuicaulis* (PTE = 13.64).

REFERENCES

Abrol, D. P. 2011. *Pollination Biology: Biodiversity conservation and agricultural production*. New York: Springer.

Ali, T. 1994. *Pollination ecology of some asclepiads (Asclepiadaceae) from Pakistan.* Ph. D. Thesis, Department of Botany, University of Karachi, Karachi, Pakistan.

Auttama, P., McKey, D. and Kidyoo, A. 2018. Flowering phenology and trap pollination of the rare endemic plant *Ceropegia thaithongiae* in montane forest of northern Thailand. *Botany* 96(9): 601-620.

Bookman, S. S. 1981. The floral morphology of *Asclepias speciosa* (Asclepiadaceae) in relation to pollination and a clarification in terminology for the genus. *American Journal of Botany* 68(5): 675-679.

Brown, R. 1833. Observations on the organs and mode of fecundation in Orchideae and Asclepiadeae. *Transactions of the Linnean Society of London (Botany)* 16: 685-745.

Coombs, G., Dold, A. P. and Peter, C. I. 2011. Generalized fly-pollination in *Ceropegia ampliata* (Apocynaceae–Asclepiadoideae): the role of trapping hairs in pollen export and receipt. *Plant systematics and evolution* 296(1-2): 137-148.

Corry, T. 1884. On the Structure and Development of the Gynostegium, and the Mode of Fertilization in Asclepias Cornuti, Decaisne (*A. syriaca* L.). *Transactions of the Linnean Society of London (Botany)* 2(8): 173-207.

Demarco, D. 2017. Floral glands in asclepiads: structure, diversity and evolution. *Acta Botanica Brasilica* 31(3): 477-502.

Doorn, W. G. and Meeteren, U. 2003. Flower opening and closure: A review. *Journal of Experimental Botany* 54(389): 1801-1812. doi:10.1093/jxb/erg213

Dormer, K. J. 1960. The truth about pollination in Arum. *New Phytologist* 59(3): 298-301.

Endress, P. K. 1994. Floral structure and evolution of primitive angiosperms: recent advances. *Plant Systematics* 192(1-2): 79-97.

Francisco, A. and Ascensao, L. 2013. Structure of the osmophore and labellum micromorphology in the sexually deceptive orchids *Ophrys bombyliflora* and *Ophrys tenthredinifera* (Orchidaceae). *International Journal of Plant Sciences*

174(4): 619-636.

Harder, L. D. and Aizen, M. A. 2010. Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology* 88(2): 271-281.

Harder, L. D. and Johnson, S. D. 2008. Function and evolution of aggregated pollen in Angiosperms. *International Journal of Plant Sciences* 169(1): 59-78.

Heiduk, A., Brake, I., Tolasch, T., Frank, J., Jürgens, A., Meve, U. and Dötterl, S. 2010. Scent chemistry and pollinator attraction in the deceptive trap flowers of *Ceropegia dolichophylla*. *South African Journal of Botany* 76(4): 762-769.

Heiduk, A., Brake, I., von Tschirnhaus, M., Göhl, M., Jürgens, A., Johnson, S. D., Meve, U. and Dötterl, S. 2016. *Ceropegia sandersonii* mimics attacked honeybees to attract kleptoparasitic flies for pollination. *Current Biology* 26(20): 2787-2793.

Heiduk, A., Brake, I., von Tschirnhaus, M., Haenni, J.-P., Miller, R., Hash, J., Prieto-Benítez, S., Jürgens, A., Johnson, S. D. and Schulz, S. J. F. 2017. Floral scent and pollinators of *Ceropegia* trap flowers. 232: 169-182.

Heiduk, A., Kong, H., Brake, I., von Tschirnhaus, M., Tolasch, T., Tröger, A., Wittenberg, E., Francke, W., Meve, U. and Dötterl, S. 2015. Deceptive *Ceropegia dolichophylla* fools its kleptoparasitic fly pollinators with exceptional floral scent. *Frontiers in Ecology Evolution* 3: 66.

Howe, H. F., Bawa, K. S. and Hadley, M. 1990. *Reproductive ecology of tropical forest plants*. Paris: Unesco and The Parthenon Publishing Group.

Ibanez, S., Dötterl, S., Anstett, M. C., Baudino, S., Caissard, J. C., Gallet, C. and Després, L. 2010. The role of volatile organic compounds, morphology and pigments of globeflowers in the attraction of their specific pollinating flies. *New Phytologist* 188(2): 451-463.

Irwin, R. E., Adler, L. S. and Brody, A. K. 2004. The dual role of floral: pollinator attraction and plant defense. *Ecology* 85(6): 1503-1511. doi:10.1890/03-0390

Johansen, D. A. 1940. *Plant microtechique*. London: McGraw-Hill Book Company, Inc.

Johnson, S. D. and Steiner, K. E. 2000. Generalization versus specialization in plant pollination systems. *Tree* 15(4): 140-143.

Karuppusamy, S. and Pullaiah, T. 2009. Pollination system and ex situ fruit set in

Ceropegia juncea Wight (Apocynaceae)—An endemic species of India. *Academic Journal of Plant Sciences* 2: 242-245.

Kidyoo, A. M. and McKey, D. 2012. Flowering phenology and mimicry of the rattan *Calamus castaneus* (Arecaceae) in southern Thailand. *Botany* 90(9): 856-865.

Kidyoo, M. 2014a. *Ceropegia suddeei* sp. nov. (Apocynaceae, Asclepiadoideae) from northeastern Thailand. *Nordic Journal of Botany* 32: 569-574. doi:10.1111/njb.00418

Kidyoo, M. 2015. *Ceropegia tribounii* (Apocynaceae, Asclepiadoideae), a new species from western Thailand. *Phytotaxa* 205(1): 59-64.

Kidyoo, M. 2018. A new species of *Ceropegia* (Asclepiadoideae, Apocynaceae) from eastern Thailand. 385(1): 31-36.

Kullayiswamy, K. R., Sandhyarani, S. and Karuppusamy, S. 2013. *Ceropegia pullaiahii* sp. nov. (Apocynaceae, Asclepiadoideae) from India. *Nordic Journal of Botany* 31(2): 166-169. doi:10.1111/j.1756-1051.2012.01557.x

Kunze, H. 1991. Structure and function in asclepiad pollination. *Plant Systematics Evolution* 176(3-4): 227-253.

Liede, S. and Kunze, H. 1993. A descriptive system for corona analysis in Asclepiadaceae and Periplocaceae. *Plant Systematics Evolution* 185(3-4): 275-284.

Lipow, S. and Wyatt, R. 2000. Towards an understanding of the mixed breeding system of swamp milkweed (*Asclepias incarnata*). *The Journal of the Torrey Botanical Society* 127(3): 193. doi:10.2307/3088756

Livshultz, T., Hochleitner, S. and Lakata, E. 2018. Pollen transfer efficiency of *Apocynum cannabinum* (Apocynaceae): a comparative perspective. *Journal of Pollination Ecology* 22(4): 35-48.

Masinde, P. 2004a. Trap-flower fly pollination in East African *Ceropegia* L.(Apocynaceae). *International Journal of Tropical Insect Science* 24(1): 55-72.

Masinde, P. S. 2004b. Trap-flower fly pollination in East African *Ceropegia* L. (Apocynaceae). *International Journal of Tropical Insect Science* 24(1): 55-72.

Medrano, M., Herrera, C. M. and Barrett, S. C. H. 2005. Herkogamy and mating patterns in the self-compatible daffodil *Narcissus longispathus*. *Annals of Botany*

95(7): 1105-1111. doi:10.1093/aob/mci129

Meekijjaroenroj, A., Bessière, J.-M. and Anstett, M.-C. 2007. Chemistry of floral scents in four *Licuala* species (Arecaceae). *Flavour and Fragrance Journal* 22(4): 300-310. doi:10.1002/ffj.1797

Meve, U. 2009. *Ceropegia thailandica* (Asclepiadoideae-Ceropegieae), a spectacular new Thai species. *Bradleya* 27: 161-165.

Meve, U. and Liede, S. 1994. Floral biology and pollination in stapeliads—new results and a literature review. *Plant Systematics Evolution* 192(1-2): 99-116.

Murthy, K. S. R., Kondamudi, R., Karuppusamy, S. and Pullaiah, T. 2012. Check-list and conservation strategies of the genus *Ceropegia* in India. *International Journal of Biodiversity Conservation* 4(8): 304-315.

Ollerton, J. and Liede, S. 1997. Pollination systems in the Asclepiadaceae: a survey and preliminary analysis. *Biological Journal of the Linnean Society* 62(4): 593-610.

Pellmyr, O. 1992. The phylogeny of a mutualism: evolution and coadaptation between *Trollius* and its seed-parasitic pollinators. *Biological Journal of the Linnean Society* 47(4): 337-365. doi:10.1111/j.1095-8312.1992.tb00674.x

Peter, C. I. and Johnson, S. D. 2006. Doing the twist: A test of Darwin's cross-pollination hypothesis for pollinarium reconfiguration. *Biology Letters* 2: 65-68.

Rahman, M. and Wilcock, C. 1992. Diversity of Life-Form and Distribution of the Asclepiadaceae in South-West Asia and the Indian Subcontinent. *ournal of Biogeography* 19: 51-58. doi:10.2307/2845619

Ruzin, S. E. 1999. *Plant microtechnique and microscopy*, Vol. 198. New York: Oxford University Press

Shukla, A. K., Vijayaraghavan, M. R. and Chaudhry, B. 1998. *Biology of pollen*. New Delhi: APH Publishing.

Singer, R. B. 2002. The Pollination Mechanism in *Trigonidium obtusum* Lindl. (Orchidaceae:Maxillariinae): Sexual Mimicry and Trap-fowers. *Annals of Botany* 89: 157-163.

Vogel, S. and Renner, S. S. 1990. *The role of scent glands in pollination: on the structure and function of osmophores*. Washington, D.C.: Amerind Publishing.

Wattana, P. 2018. *Flowering phenology and pollination mechanism of Ceropegia*

tenuicaulis Kidyoo. Bachelor's Thesis, Department of Botany, Faculty of Science, Chulalongkorn University,

Wiemer, A. P., Sersic, A. N., Marino, S., Simoes, A. O. and Cocucci, A. A. 2012. Functional morphology and wasp pollination of two South American asclepiads (Asclepiadoideae-Apocynaceae). *Annals of Botany* 109(1): 77-93. doi:10.1093/aob/mcr268

Yokota, S. and Yahara, T. 2012. Pollination biology of *Lilium japonicum* var. *abeanum* and var. *japonicum:* evidence of adaptation to the different availability of diurnal and nocturnal pollinators. *Plant Species Biology* 27(1): 96-105. doi:10.1111/j.1442-1984.2011.00336.x

APPENDIX

1. Nadi reagent

The solution was prepared at the time of use by mixing 0.5 ml of 1% dimethyl-p-phenylenediamine chloride in water with 0.5 ml of 1% dimethyl-p-phenylenediamine chloride in water and with 49 ml of phosphate buffer 0.05 M (pH 7.2).

2. Kovat retention index

In gas chromatography, Kovats retention index or retention index are used to convert retention times into system-independent constants. The Kovats retention index of a certain chemical compound is its retention time normalised to the retention times of adjacently eluting n-alkanes, the Kovats index is given by the equation.

$$I_i = 100 \left(\frac{Tx - Tn}{(Tn+1) - Tn} \right)$$

I_i the Kováts retention index of peak i,

Tx retention time of compound i

Tn retention time of carbon number of n-alkane peak before peak i

Tn+1 retention time of carbon number of n-alkane peak after peak i

3. The quantitative characteristics comparison of *Ceropegia thailandica* and *Ceropegia suddeei.*

3.1 Total length of flower

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. thailandica	10	587.7815	58.77815	56.851677
C. suddeei	10	666.4639	66.64639	89.302554

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	309.5459	1	309.5459	85.21881	3.01×10^{-8}	4.413873
Within Groups	65.38259	18	3.632366			
Total	374.9285	19				

3.2 Width of window

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. suddeei	10	17.16426	1.716426	0.184931
C. thailandica	10	17.21766	1.721766	0.016995

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000143	1	0.000143	0.001436	0.970184	4.413873
Within Groups	1.786987	18	0.099277			

3.3 Length of window

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. thailandica	10	30.11531	3.011531	0.240123
C. suddeei	10	53.59139	5.359139	0.176410

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27.5563	1	27.55631	232.9211	9.64×10^{-12}	4.413873
Within Groups	2.12953	18	0.118307			
Total	29.6858	19				

3.4 Diameter of corolla tube at narrowest

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. suddeei	10	3.849654	0.384965	12.46102
C. thailandica	10	3.990386	0.399039	0.230430

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00099	1	0.00099	0.390455	0.539901	4.413873
Within Groups	0.045652	18	0.002536			
Total	0.046642	19				

3.5 Diameter of corolla tube at the widest point

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. thailandica	10	7.533234	0.753323	85.008455
C. suddeei	10	9.253837	0.925384	0.235121

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.148024	1	0.148024	27.3539	0.00005657	4.413873
Within Groups	0.097406	18	0.005411			
Total	0.245429	19				

3.6 Height of inflated tube

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. thailandica	10	100.5658	11.17398	1.638541
C. suddeei	10	120.1177	13.34641	0.921742

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.2375	1	21.23753	52.16661	0.00000203	4.493998
Within Groups	6.51375	18	0.40711			
Total	27.7512	19				

3.7 Hieght of gynostegium

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. suddeei	10	50.99918	5.099918	0.409721
C. thailandica	10	49.54808	4.954808	0.25001

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.10528	1	0.10528	0.31558	0.581199487	4.413873
Within Groups	6.00508	18	0.33361			
Total	6.11036	19				

3.8 Diameter o gynostegium

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
C. suddeei	10	37.24642	3.724642	0.072971
C. thailandica	10	47.06132	4.706132	0.025631

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.816614	1	4.816614	98.54064	1.00×10^{-9}	4.413873
Within Groups	0.87983	18	0.048879			
Total	5.696444	19				

VITA

NAME	Chayanin Kraithep
DATE OF BIRTH	07/02/1993
PLACE OF BIRTH	Nakhon Si Thammarat
INSTITUTIONS ATTENDED	Bechelor degree from Prince of Songkla University, Hat-Yai campus
HOME ADDRESS	9/578 Plum condo soi Ladpraw 101 Ladpraw road Klong-Jun subdistrict Bang Kapi district Bankok 10240