



Institute of Biodiversity and Environmental Conservation

**Floral Biology, Reproductive Strategies, and Cluster Analyses of
Selected Calamoids of Sarawak (Arecaceae)**

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Floral Biology, Reproductive Strategies, and Cluster Analyses of Selected
Calamoids of Sarawak (Arecaceae)

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

The Calamoids are from the subfamily Calamoideae and account for one-fifth of Arecaceae, the palm family. The floral biology and breeding strategies in the tribe Calameae of Calamoideae is still relatively unknown despite its importance that supports a billion-dollar industry in rattan products. This study aims to investigate the floral biology and breeding strategies of three members of Calameae, *Calamus lobbianus*, *Calamus pygmaeus*, and *Salacca vermicularis*. The data on floral biology, floral visitors, and floral availability were collected and analysed. The Nectar availability was investigated by further measurement of nectar concentration and volume. Pollination studies were conducted to investigate the breeding mechanisms. Seed dispersal study was further conducted on *S. vermicularis* via camera trapping. Investigation on the population genomics of *C. pygmaeus* was conducted to elucidate on the asexual propagation as a viable breeding strategy by performing single nucleotide polymorphisms and insertion deletion analyses. For both species of *Calamus*, anthesis started in the early morning for pistillate and staminate plants, ended by late noon (staminate plants) but lasted till the next day in pistillate plants. Although both species appeared to be aseasional in flowering, *C. lobbianus* exhibited a male-biased population while *C. pygmaeus* did not exhibit any sex bias. Nectar was observed to be extruded from the base of the petals of the staminate flowers and sterile male flowers of the pistillate plants of *C. lobbianus*. The concentration and volume of the nectar of the staminate flower and sterile male flower of *C. lobbianus* peaked at c. 11% and 9 µl around 1100 (Day 1) and c. 13% and 8 µl around 0930 (Day 1) respectively but only appeared as a layer of glistening exudate in *C. pygmaeus*. Floral scent was not detected in any of the inflorescences of both species. Several insect visitors were observed to be the primary visitors of both *Calamus* species which include two species of *Tetragonula*, a species of *Liostenogaster* sp., and

Stenodyneriellus sp. Experiments on breeding mechanism of *C. lobbianus* and *C. pygmaeus* indicate that both species are most likely apomictic. Anthesis in staminate flowers of *S. vermicularis* began around noon and persisted until early morning on the second day whilst anthesis in pistillate flowers began around evening and took up to 40 hours to fully bloom. Floral scent was present throughout anthesis for staminate and pistillate plants. Apomixis is not present in this species. Several insect visitors were observed, namely *Heterotrigona* (Apidae) sp. as well as members of Curculionidae, Sciaridae, Formicidae, and Acaridae. Three species of visitors are likely the dispersal agents of *S. vermicularis*, namely *Hystrix* sp. (Hystricidae: Hystricinae), *Tragulus* sp. (Tragulidae), and *Lariscus insignis* (Sciuridae: Callosciurinae). A mongoose (Herspetidae) was observed foraging nearby. Phylogenetic analysis and DAPC analysis concluded that the *C. pygmaeus* found to consist of genetic clones forming genets with distinct genetic makeup among subpopulations.

Keywords: Pollinators, nectar, apomixis, cloning, population genetics

Biologi Bunga, Strategi Reproduksi, dan Genetik Populasi Kalamoid Terpilih di Sarawak (Arecaceae)

ABSTRAK

Kalamoids adalah daripada subfamily Calamoideae dan merangkumi satu per lima Arecaceae, keluarga pokok palma. Biologi bunga dan strategi pembiakan di tribus Calameae daripada Calamoideae adalah masih agak tidak ketahui walaupun kepentingannya yang menyumbang kepada industri berbilion dollar dalam produk rotan. Kajian ini bertujuan untuk menyiasat biologi bunga dan strategi pembiakan bagi tiga ahli Calameae, Calamus lobbianus, Calamus pygmaeus dan Salacca vermicularis. Data bagi biologi bunga, pengunjung bunga dan ketersediaan bunga telah dikumpulkan melalui pemerhatian langsung dan ketersediaan nektar telah dikaji melalui pengukuran kepekatan dan isipadu nektar. Kajian pendebungaan telah dikaji melalui kajian mekanisme pembiakaan. Kajian penyebaran biji telah dilakukan ke atas S. vermicularis melalui perangkap kamera untuk pemerhatian haiwan pengunjung. Kajian genomik populasi telah dilakukan ke atas C. pygmaeus bagi memahami perambatan vegetatif sebagai strategi pembiakaan yang berkesan dengan melakukan analisis SNP dan InDel ke atas genomnya. Untuk kedua-dua spesies Calamus, antesis bagi pokok betina dan jantan bermula daripada awal pagi dan berakhir lewat petang (pokok jantan) manakala antesis bagi pokok pistilat berlangsung sehingga hari seterusnya. Pemerhatian pembungaan bagi kedua-dua spesies adalah tidak bermusim tetapi pembungaan C. lobbianus mempamerkan berat sebelah ke populasi jantan manakala C. pygmaeus tidak mempamerkan berat sebelah terhadap kedua-dua jantina. Nektar hanya dihasilkan pada bahagian dasar kelopak bunga jantan dan bunga jantan steril diad di sebelah bunga betina C. lobbianus. Pengeluaran nektar bunga jantan C. lobbianus memuncak sekitar jam 11 (hari pertama) dengan kepekatan 11% dan isi padu sebanyak 9

μl . Manakala, pengeluaran bunga jantan steril memuncak sekitar jam 0930 (hari pertama) dengan kepekatan 13% dan isi padu sebanyak 8 μl manakala C. pygmaeus hanya menghasilkan lapisan cecair berkilat. Bau bunga kedua-dua spesies tidak dapat dikesan pada jambak bunga. Dua spesies Tetragonula dan satu spesies Liostenogaster sp. diperhatikan sebagai pelawat utama untuk kedua-dua spesis Calamus. Eksperimen terhadap mekanisma pembiakan menunjukan bagi kedua-dua spesies Calamus adalah berkemungkinan apomik. Antesis bagi bunga jantan S. vermicularis bermula sekitar tengah hari dan berlangsung sehingga awal pagi pada hari kedua manakala bunga betina bermula sekitar matang dan berkemungkinan mengambil sehingga 40 jam untuk berbunga sepenuhnya. Bau bunga dikesan sepanjang antesis bagi pokok jantan dan betina. Apomiksis adalah tidak dikesan dalam spesies ini. Beberapa serangga perhati, iaitu Heterotrigona (Apidae) sp. serta ahli family Curculionidae, Sciaridae, Formicidae, dan Acaridae. Tiga spesies pengunjung adalah berkemungkinan agen penyebar S. vermicularis, iaitu Hystrix sp. (Hystricidae: Hystricinae), Tragulus sp. (Tragulidae) dan Lariscus insignis (Sciuridae: Callosciurinae). Seekor bambun (Herspetidae) diperhati menua berhampiran. Analisis filogenetik dan analisis DAPC menyimpulkan bahawa subpopulasi sampel C. pygmaeus didapati terdiri daripada klon genetik yang membentuk genet.

Kata kunci: Pendebungaan, nektar, apomikis, pengklonan, genetik populasi

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LIST OF ABBREVIATIONS

ca.	<i>Circa</i>
cm	Centimetre
conc.	Concentration
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPC	Discriminant Analysis Of Principal Components
DNA	Deoxyribonucleic Acid
e.g.	<i>Exempli Gratia</i>
elev.	Elevation
et al.	<i>Et Alia</i>
G	Gigabases
GBS	Genotyping by Sequencing
GC	Guanine Cytosine
gDNA	Genomic DNA
i.e.	<i>Id Est</i>
InDel	Insertion And Deletion
inf	Inferred Groups
KNP	Kubah National Park
m	Meter
mg	Milligram
ML	Maximum Likelihood
mm	Millimetre

MP	Maximum Parsimony
ng	Nanogram
ng/μl	Nanogram Per Microliter
NJ	Neighbour Joining
ori	Original Groups
SNP	Single Nucleotide Polymorphism
μl	Microliter
%	Percentage
°C	Degrees Celsius

CHAPTER 1

INTRODUCTION

1.1 Study Background

The palm family (Palmae or Arecaceae) is a large and diverse family of iconic monocotyledonous flowering plants. It is distributed throughout tropical and subtropical areas of the world, but it is most diverse in highly threatened moist tropical forest habitats. The family comprises 181 genera and approximately 2,600 species (Yao et al., 2023). These are unevenly divided among the Americas (ca. 730 species), Africa (ca. 65 species), and the Asia-Pacific region (ca. 1,600 species). Palms are highly conspicuous in tropical ecosystems. Due to their diversity, abundance and interactions, many species play key ecological roles. They provide numerous ecosystem services and are of great economic importance, ranking third after grasses and legumes in usefulness (Palmweb, 2024).

The subfamily Calamoideae is a major lineage of palms, accounting for one quarter of all palm species. Ninety percent of these are rattans, i.e. spine-bearing climbing palms. Currently, 604 rattan species are known (Vorontsova et al., 2016; Kuhnhäuser et al., 2021). A rattan (*Calamus manan* Miq.) holds the record for the longest unbranched stem of any plant (Burkill, 1936). With their climbing habit, rattans form an integral part of Southeast Asian rainforests. After bamboo, they are the second-most important non-timber product of the tropical rainforests of Southeast Asia and the basis of a multi-billion dollar international trade in rattan products (Henderson, 2009). The versatility of the different species provides a wealth of uses to local communities, ranging from edible fruits to furniture, basketry and traditional medicine (Dransfield et al., 2008).

For a long time, it was assumed that palms are wind- pollinated (Delpino, 1870), but a growing number of detailed studies show that wind pollination is an exception, and that the overwhelming majority of palms studied are insect pollinated. There is also a single well-documented instance of bat pollination in *Calyptranthes ghiesbreghtiana* (Linden & H.Wendl.) H.Wendl. (Beach, 1986; Cunningham, 1995; Tschapka, 2003), and a remarkable example of pollination by primates in *Eugeissona*. It seems reasonable to predict that most palms will be shown to be insect pollinated. Large quantities of pollen, a feature of the wind-pollination strategy, seem, at least in some palms, to be an adaptation to predation by insects. True wind-pollination does indeed occur, for example, in *Thrinax* (Read, 1975) and *Howea* (Savolainen et al., 2006), but even the date palm, *Phoenix dactylifera* L., may in fact be pollinated only secondarily by wind (Henderson, 2009).

In tropical forests, the abundance of palms in the community and their simple architectural characteristics have made them a focus of quantitative studies on plant growth and survival strategies (Bullock, 1980; Ash, 1988; Martínez-Ramos et al., 1988; De Steven, 1989; Oyama, 1990; Chazdon, 1992; Bernal, 1998; Mendoza & Franco, 1998; Barot & Gignoux, 1999; Svenning, 2000). Although a full understanding of the role of clonal growth in the maintenance of palm populations in tropical forests is far from complete, a number of generalizations on the size, growth, and reproduction of tropical palms (clonal and nonclonal) have been advanced. These generalizations provide a foundation for further research into the ecological and evolutionary significance of clonal growth in these ecosystems (Mendoza & Franco, 1998; Barot & Gignoux, 1999; Svenning, 2000).

1.2 Research Gap

Majority of studies conducted on Arecaceae are primarily focused on the species with commercial value while lesser-known species occurring in Borneo are less studied. Thus, this study aims to contribute to the pollination studies and population genetics of Arecaceae in Borneo focusing on the tribe Calameae namely the genera *Calamus* L. and *Salacca* Reinw.

1.3 General Objectives

- To investigate the floral biology and breeding strategies of *Calamus lobbianus* and *Calamus pygmaeus*
- To investigate the floral biology and breeding strategies of *Salacca vermicularis*
- To understand the population structure of *Calamus pygmaeus*

CHAPTER 2

LITERATURE REVIEW

2.1 Tribe Calameae

Since its first description in Dictionnaire Raisonne des Termes de Botanique et des Familles Naturelles by Karl Sigismund in 1831, Tribe Calameae has undergone many iterations. In its most recent delimitation, Calameae now contains 498 species comprising of six subtribes (Korthalsiinae Becc., Salaccinae Becc., Metroxylinae Blume, Pigafettinae J.Dransf. & N.W.Uhl, Plectocomiinae J.Dransf. & N.W.Uhl, and Calaminae Meisn.) and within the six tribes, nests nine genera (*Korthalsia* Blume, *Eleiodoxa* (Becc.) Burret, *Salacca* Reinw., *Metroxylon* Rotb., *Pigafetta* (Blume) Becc., *Plectocomia* Mart. & Blume, *Myrialepsis* Becc., *Plectocomiopsis* Becc., and *Calamus* L.) distributed mostly from Mainland Southeast Asia to New Guinea with its largest members from genus *Calamus* standing at 415 members which can be found from India to Fiji (Baker & Dransfield, 2016; Henderson, 2020; Kuhnhäuser et al., 2021). Being a strongly supported sister to all other palms, many attempts have been made to resolve its phylogeny. The most recent attempt was by Kuhnhäuser et al. (2021) where they resolved the phylogenomics by sequencing almost 1000 nuclear genomics regions for 75 systematically selected Calamoideae to represent the whole subfamily revealing complex deep evolutionary histories in tribes Eugiessoneae, Lepidocaryae and Calameae.

A major taxonomic revision of the largest genus in Calameae, *Calamus* was performed by Henderson (2020) where 411 species was ultimately recognised. Of the recognised species, 38 was recognized as new species, 11 species was divided into 36 subspecies and eight was considered to be oculospecies. The delimitation by Henderson

(2020) recognises the transfer of *Ceratolobus* Blume ex Schult. & Schult.f., *Daemonorops* Blume, and *Pogonotium* J.Dransf. into *Calamus* (Baker, 2015), subsuming *Calospatha* Becc. (Baker & Dransfield, 2008) and *Retispatha* J.Dransf. into *Calamus* (Henderson & Floda, 2014).

2.1.1 Taxonomy of *Calamus*

Calamus currently comprises ca. 520 species which now also include *Ceratolobus* Blume, *Daemonorops* Blume ex Schult. f., *Pogonotium* J.Dransf., and *Retispatha* J.Dransf. sensu Baker (2015) while Henderson (2020) recognizes 411 phylogenetic species out of 516 sampled species. *Calamus* is distributed across six main regions (Afro-India, Indo-Burma, Philippines, Sundaland, Wallacea, and Sahul with Borneo alone having 111 species) and divided into distinct groups where only a few species are widespread over three or more regions (Henderson, 2020). Several *Calamus* species stand out with certain unique features such as the following three species where the inflorescence apices can develop into new plants, e.g., *Calamus pygmaeus* Becc., *Calamus castaneus* Griff. (Ruppert et al., 2012), and *Calamus kampucheaensis* A.J.Hend. & Hourt (Henderson, 2020). Even the flagellate apices can develop into new plants, as in *Calamus gamblei* Becc. which appears to be common within the species (Renuka & Nambiar, 1985). *Calamus pygmaeus* is placed in a group of six other flagellate species that occur within Sundaland (*Calamus comptus* J.Dransf., *Calamus gaharuensis* A.J.Hend, *Calamus lengguanii* A.J.Hend, *Calamus nematospadix* Becc., *Calamus nielsenii* J.Dransf., and *Calamus psilocladus* J.Dransf.) (Henderson, 2020). Several characters that members of this group shared are the reddish-brown indumentum along rachis and pinnae base and small whitish globose fruits with explanate fruiting perianths and scarcely developed knees (absent in *C. pygmaeus*) (Henderson, 2020).

Henderson (2020) proposed several points on the complexity of the calamoid group. Firstly, at least 14 out of 20 species recognized in his revision are represented by one or a few specimens, with sparse data. Secondly, several characters are difficult to score as in some species, it is difficult to determine if pinnae are linear or elliptic, and most species have a tendency for pinnae to be divaricate although only two appear to be obviously divaricate (and the apical pair of pinnae in all species are only briefly joined at their bases). Several species have red-brown, long, papery spines on the margins of the leaf sheaths and apices of the rachis bracts, although this is not consistent within species. Moreover, there is a high possibility of species hybridisation (Dransfield, 1992), which adds further complications. Lastly, most species have a small region of distribution.

At least twelve species are known from single localities where most are from relatively well-collected areas and thus unlikely to be artifacts of insufficient collecting. Of the remaining species, few can be considered widespread, and even such species as *Calamus laxissimus* Ridl. and *Calamus perakensis* Becc. seem to consist of several, more narrowly distributed populations (Henderson, 2020). Although this narrowness of distributions does not necessarily lead to difficulties in species delimitation, it does seem different from distribution patterns in other groups of species and, together with the difficulty of scoring characters, gives the impression that there may be fewer species in the group and these may comprise numerous, local populations rather than distinct species (Henderson, 2020).

2.1.2 Taxonomy of *Salacca*

Salacca is one of two genera in subtribe Salaccinae Becc, the other being *Eleiodoxa*, distributed from the eastern Himalaya to South Central China and West Malesia with its

centre of diversity in Borneo (Zumaidar & Miftahuddin, 2018; POWO, 2023). *Salacca* comprises 23 species to date (Zumaidar & Miftahuddin, 2018; Kuhnhäuser et al., 2021; POWO, 2023). *Salacca*, characterized as dioecious, exhibits staminate and pistillate flowers on separate trees. Staminate trees only bear staminate flowers, while pistillate trees bear dyads consisting of one pistillate flower and a sterile staminate flower (Mogea, 1978). *Salacca* is divided into two sections: section *Salacca* with spine-tipped scales and pistillate rachillae bearing dyads of a fertile pistillate and a sterile staminate flower and section *Leiosalacca* with smooth scales and pistillate rachillae bearing solitary pistillate flowers (Furtado, 1949; Dransfield et al., 2008).

2.2 Calameae of Borneo

Within the nine genera in Calameae, six can be found in Borneo namely, *Korthalsia*, *Eleidoxa*, *Salacca*, *Plectocomia*, *Plectocomiopsis*, and *Calamus*. A noteworthy member would be *Metroxylon sagu* Rottb. that is native from New Guinea to the Moluccas but has been naturalised and is found widespread in the Southeast Asian region (Dransfield et al., 2008). Within the six genera present in Borneo, 15 members of *Korthalsia* can be found in Korthalsiinae (Shahimi et al., 2023), one member of *Eleidoxa* (Furtado, 1949; Dransfield et al., 2008) and 11 members of *Salacca* (Furtado, 1949; Zumaidar & Miftahuddin, 2018) in Salaccinae, three members of *Plectocomia* and three members of *Plectocomiopsis* in Plectocomiinae (Dransfield, 1982; Govaerts et al., 2024), and 111 members of *Calamus* in Calaminae (Henderson, 2020).

2.2.1 *Calamus* of Borneo

There are 111 species of *Calamus* occurring in Borneo, at least 69 of them endemic to the island with ca. 20 species considered widespread in Borneo with uneven distribution and several areas having higher concentration of species (Henderson, 2020). This uneven distribution is most prevalent in western Sarawak and West Kalimantan. There are 16 endemic species, many of which can be found on Mount Matang, Kuching (Pearce, 1992; Henderson, 2020). There is also a noticeable concentration of species in northeastern Borneo, mostly in Sabah, where six species are endemic. Additionally, there is a third area of endemism in northcentral Borneo, including Brunei and eastern Sarawak, where about nine species are endemic. Three species occur only in eastern Borneo, *Calamus disjunctus* A.J.Hend, *Calamus praetermissus* J.Dransf., and *Calamus longipes* Griff. (Henderson, 2020). In Gunung Mulu National Park, 34 *Calamus* (sensu Henderson) which includes three *Ceratolobus*, four *Daemonorops*, one *Pogonotium*, and one *Retispatha* was recorded (Dransfield, 1984) while Pearce (1992) reported 45 *Calamus* (sensu Henderson) which includes two *Ceratolobus*, thirteen *Daemonorops*, one *Pogonotium*, and one *Retispatha* in Kubah National National Park. More recently, Petoe et al. (2020) recorded 18 species belonging to the genera *Calamus* (sensu Henderson) in Lanjak Entimau Wildlife Sanctuary.

2.2.2 *Salacca* of Borneo

In its latest delimitation, 11 species of *Salacca* can be found in Borneo: namely *Salacca affinis* Griff., *Salacca bakeriana* J.Dransf., *Salacca clemensiana* Becc., *Salacca dolicholepis* Burret, *Salacca dransfieldiana* Moge, *Salacca lophospatha* J.Dransf. & Moge, *Salacca magnifica* Moge, *Salacca ramosiana* Moge, *Salacca rupicola* J.Dransf.,

Salacca sarawakensis Moge, and *Salacca vermicularis* Becc. (Zumaidar & Miftahuddin, 2018). Zumaidar and Miftahuddin (2020) concluded that there is a high level of probability of discovering new species in *Salacca* in Borneo which also indicates that the island is not only a centre of diversity for *Salacca*, but it could also be considered as a centre of origin for *Salacca*. In Mulu National Park, four species was previously recorded; namely they are *S. affinis*, *S. magnifica*, *S. rupicola*, and *S. vermicularis* (Dransfield, 1984) while Pearce (1992) confirmed two species namely, *S. affinis* and *S. vermicularis*. More recently, Petoe et al. (2020) noted one species which was the widespread *S. vermicularis* in Lanjak Entimau Wildlife Sanctuary.

2.3 Floral Biology of Arecaceae

Pollination studies in the family are disproportionately skewed towards the Neotropics since the first modern study on *Asterogyne martiana* (H.Wendl.) H.Wendl. ex Drude (Schmid, 1970a, 1970b) with roughly 75% of the studies conducted in that region (Henderson, 2024). A review of pollination systems in Arecaceae by Henderson (2024) revealed that the majority of Arecaceae are cantharophilous followed by apiphily and myophily along with several other pollinator groups. A consensus is that palm pollination strategy consists of two broad categories, inflorescences that are open, elongate and has relatively long lasting anthesis are bee and or fly pollinated palms while condensed, shorter and with brief anthesis are pollinated by beetles. However, there has been increasing evidence of shifts between bee/fly pollination to beetle pollination and vice versa throughout the family (Henderson, 2024). These shifts draw on the significance of morphology on pollination strategy of the members and if we can confidently classify a palm species as one pollination strategy as opposed to the other.

Bees and wasps appear to be rather non-specific generalist pollinators of many palms (e.g., in species of *Licuala* Wurm (Barfod et al., 2003) and *Calamus* (Bøgh, 1996). A wide range of bees and beetles have been observed visiting inflorescences of *Euterpe precatoria* Mart., many of which, together with wind, are predicted to effect pollination (Küchmeister et al., 1997). *Nypa*, at least in New Guinea, is pollinated by drosophilid flies (Essig, 1973). Syrphid flies are responsible for pollinating *Asterogyne martiana* (H.Wendl.) H.Wendl. ex Hemsl. (Schmid, 1970b), whereas bees have been shown to pollinate *Sabal palmetto* (Walter) Lodd. ex Schult. & Schult.f. (Brown, 1976), *Ptychosperma macarthurii* (Becc.) Becc. ex Martelli (Essig, 1973), and *Iriarteia deltoidea* Ruiz & Pav. (Henderson, 1985). Beetle pollination has been observed in *Rhapidophyllum* H.Wendl. & Drude (Shuey & Wunderlin, 1977), *Hydriastele microspadix* (Warb. ex K.Schum. & Lauterb.) Burret (Essig, 1973), *Salacca zalacca* (Gaertn.) Voss (Mogea, 1978), *Bactris gasipaes* Kunth, and *B. porschiana* Burret (Beach 1984), *B. guineensis* (L.) H.E.Moore, and *B. major* Jacq. (Essig 1971), *Cryosophila albida* Bartlett (Henderson, 1985), *Socratea exorrhiza* (Mart.) H.Wendl. (Henderson 1985), *Aphandra* Barfod, and *Phytelephas* Ruiz & Pav. (where the role of scent has been studied in detail by Ervik et al., 1999) and *Attalea* Kunth (Küchmeister et al., 1993). This method of pollination is also suggested by casual observations for many species of *Salacca*, *Daemonorops*, *Pinanga* Blume, *Ceratolobus*, *Manicaria* Gaertn., and *Johannesteijsmannia* H.E.Moore (Dransfield, 1984; Dransfield, 1992; Dufay, 2003; Dransfield et al., 2008; Chan et al., 2011).

Reports by Hartley (1977) that *Elaeis guineensis* Jacq. is anemophilous (i.e., wind pollinated) have been disproved by Syed (1979) who has shown the oil palm to be pollinated by at least twelve different insects in Cameroon, West Africa; among these pollinators is a weevil, *Elaeiodobius kamerunicus*, which has been successfully introduced into western

Malaysia to assist in pollination in oil palm plantations. Thrips (*Thrips hawaiiensis*) had previously been the major pollinator in Malaysia, but hand pollination had been necessary to obtain high yields before the West African weevil increased fruit set. The similarity in the overall form of the rachillae in *Elaeis* and the quite unrelated *Salacca* is noteworthy, as is the similar position of the inflorescences, which are borne down among the protected leaf bases in both genera (Henderson, 2009), especially as weevils are implicated as pollinators of both. Several studies have implicated similar derelomid beetles in the pollination of palms such as *Phoenix canariensis* H.Wildpret (Meekijjaroenroj & Anstett, 2003). Perhaps the most remarkable example is that of *Chamaerops humilis* L. A derelomid beetle species is attracted in large numbers to the inflorescences of *C. humilis*, not by a floral scent but by a scent produced by the leaves that subtend flowers at anthesis. The minute beetles travel down the petioles to the inflorescences where they mate, the females laying eggs in inflorescence axes. The only larvae that develop further are those in the staminate inflorescences, where they eventually pupate and emerge as adults the following spring (Anstett, 1998; Dufay et al., 2003).

A notable pollination system would be that of *Eugeissona tristis* Griff. This species flowers year-round and the buds produced nectars for more than a month which is alcoholic in nature (Henderson, 2024) and nectary unique to its genus (Stauffer et al., 2016). The alcoholic nature of the nectar is hypothesized to be because of fermentation by yeasts inoculated through floral visitors such as drosophilids, nitidulid beetles and various bees (Wiens et al., 2008). The alcoholic nectar mainly attracts the pentailed treeshrew (*Ptilocercus lowii*) which serves as a pollinator along with several other mammalian pollinators (Stauffer et al., 2016). Another great example would be the arecoid palm with subterranean flowers and fruits *Pinanga subterranea* Randi & W.J.Baker. Although knowledge of the existence of this

arecoid palm is widely known by indigenous groups in Borneo but has only been described in scientific literature recently (Kuhnhäuser et al., 2023; Randi et al., 2023). *Pinanga subterranea* has an exclusive reproductive strategy in Arecaceae employing geoflory and geocarpy where development of inflorescence, anthesis, infructescence development and dispersal all occurs belowground (Randi et al., 2023). The geoflory and geocarpy is achieved through a “saxophone growth” of the stem where it grows about 30 cm downwards then upwards which causes the inflorescences to emerge belowground and remains belowground. At present, not much information is available regarding its pollination mechanism aside from a high possibility of insect pollination (Kuhnhäuser et al., 2023). Bearded pigs, *Sus barbatus* have been observed to dig up the underground fruits for consumption and seed collected from the faecal matter has been successfully germinated (Randi et al., 2023). *Eugeissona tristis* and *P. subterranea* in this case serves as outliers as most of Arecaceae are insect pollinated and exhibits relatively poorly diversified morphology and low adaptive significance of inflorescence morphology (Henderson, 2024). However, these views are formed based on largely pollination system studies conducted largely in the Neotropics, if more data is collected in the Southeast Asian region, then we may yet to uncover the other piece of the puzzle such as in *E. tristis* and *P. subterranea*.

Floral attractants in Calameae and the rest of the family are highly similar where the main attractants are scent as floral advertisement and nectar as floral reward. While the floral scent is typically produced from the flowers, it is not the case in the dwarf palm *Chamaerops humilis* L. where the scent produced by the osmophores is located at the sinuses of the palmate leaf (Dufay et al., 2003, 2004). Dufay et al. (2003) found that while the inflorescence does produce floral scent, it is not capable of attracting the main pollinating weevil *Derelomus chamaeropsis* from long distances, however, the scent produced from the leaf

sinus which were more concentrated was able to attract the weevils from long distances and once the weevils have arrived at the plants, the scent from the inflorescence guides the weevils to the inflorescence.

Attracting visitors through floral attractants may be sufficient for many plant species, however members of the family sometimes employ a different strategy to increase the probability of successful pollination. In several genera such as *Attalea* Kunth (Voeks, 2002), *Calamus* (Bøgh, 1996; Kidyoo & McKey, 2012), *Geonoma* Willd. (Olesen & Balslev, 1990; Stauffer et al., 2002) employs a strategy where the pistillate inflorescence exhibits certain characteristics that mimics the staminate inflorescence without offering any substantial floral reward that the staminate inflorescence may have provided to the visitors to attract more pollinators known as Bakerian mimicry. The of mimicry typically involves olfactory and visual imitation of staminate flowers such as colour and production of staminodes. In dioecious plants, staminate plants typically attract more visitors as resource allocation towards reproduction works to produce more floral rewards in the forms of pollen and floral nectar (Bøgh, 1996; Kidyoo & McKey, 2012; Henderson, 2024). The pistillate inflorescence gains advantage by mimicking the staminate inflorescence meant it is not required to allocate as many resources towards attracting visitors but rather allocate the precious resources towards infructescence production to ensure effective dispersal (Kidyoo & McKey, 2012).

Another strategy that may be employed is excess male floral display where number and frequency of flowering of staminate flowers is significantly higher than pistillate flowers (Bøgh, 1996; Chan et al., 2011; Kidyoo & McKey, 2012; Lara et al., 2017; Henderson, 2024). This excess in production of staminate inflorescence ensures availability throughout

for large parts of the year and promotes and abundance and extended presence of floral visitors which can aid in effective pollination (Kidyoo & McKey, 2012; Lara et al., 2017).

2.3.1 Floral Biology of *Calamus*

A better understanding of the genus's floral biology and pollination mechanisms may allow better classification for a genus of this magnitude as up until 1986, the leading view in the scientific literature was that palms were mainly anemophilous (Henderson, 1986). The floral biology of *Calamus* can be considered understudied relative to the size of the genus; species investigated so far included *Calamus caesius* Blume, *C. castaneus*, *Calamus longisetus* Griff., *C. manan*, *Calamus peregrinus* Furtado., *Calamus rudentum* Lour., *Calamus subinermis* H.Wendl. ex Becc., and several species previously belonging to *Ceratolobus* (Dransfield, 1979a, 1979b; Lee, 1995; Bøgh, 1996; Alloysius, 1999; Kidyoo & McKey, 2012; Mohd Rusdi et al., 2022). Flowers with either nocturnal (Bøgh, 1996; Alloysius, 1999) or diurnal anthesis (Lee, 1995; Kidyoo & McKey, 2012).

The phenology of both staminate and pistillate flowers is similar across the genus. In the staminate flowers, anthesis begins with the opening of sepals and petals followed by the anthers emerging to release pollen and the flower dehisces soon after. In the pistillate flowers, the anthesis is initiated by the stigma emerging till the tip of the stigma is recurved and points towards the base of the flower which then slowly turns brown towards the end of the anthesis (Bøgh, 1996; Alloysius, 1999; Kidyoo & McKey, 2012). *Calamus* lacks distinct features to attract specific pollinators but tends to be visited mostly by Hymenopterans such as *Apis* and *Trigona* which are potential pollinators but may sometimes act as pollen thieves as well (Bøgh, 1996; Dransfield, 1979a, 1984; Alloysius, 1999; Kidyoo & McKey, 2012).

Moths have been observed to be potential pollinators during nocturnal visits (Lee, 1995; Alloysius, 1999). Most species of *Calamus* previously placed in *Calospatha*, *Ceratolobus*, and *Daemonorops* were recorded to emit a musty floral odour and were visited by many beetle species which are potential pollinators (Dransfield, 1979a).

Microlepidoptera have been implicated in the pollination of several species of *Calamus* in Sabah, Malaysia (Lee, 1995). Flower visitation occurs at night when the flowers emit a sickly smell. Nectar is produced by the staminate flowers and by the sterile staminate flowers in the pistillate inflorescence; pollen is carried passively from the staminate to the pistillate plant. It is interesting to note that in Thailand, in different species of *Calamus*, Bøgh observed diurnal production of nectar and postulated pollination by bees (Bøgh, 1996).

2.3.2 Floral biology of *Salacca*

Staminate and pistillate inflorescence of *Salacca* occur on separate trees. The staminate rachilla consists of many pairs of staminate flowers while the pistillate ones have a pistillate and a neuter flower (a sterile staminate flower) in each pair (Mogea, 1978). Because the plants are generally dioecious, the pollination is mainly assisted by wind, insects, and humans. A previous study showed that the curculionid beetle is a major pollinator of *Salacca*. Other insects that visited the *Salacca* flowers are stingless bees (Apidae: Hymenoptera) and beetle, *Rhynchophora palmarum* (Coleoptera). The latest beetle species was reported as a pest that damages the flowers (Mogea, 1978). Curculionid beetle, *Nodocnemis* sp. (Coleoptera: Curculionidae) is the main pollinator of Pondoh salacca in Sleman, Yogyakarta (Wagiman et al., 2014). The beetle species also was reported as the

main pollinator of Sumatra salacca (*S. sumatrana* Becc.) (Tanjung et al., 2019; Atmowidi et al., 2021).

2.4 Asexual Reproduction

Most plants that are capable of undergoing asexual reproduction produce clones using vegetative organs (e.g. stolons, rhizomes, tubers, bulbs and roots) and this is where majority of the understandings on clonal plants occurs (Herben & Klimesova, 2020; Martínková et al., 2020; Franklin et al., 2021). It is important to understand the ecology of clonal plants as clonal growth allows certain ecological advantages such as providing insurance when sexual reproduction is hindered (Herben et al., 2014), facilitates in monopolization of space (Zheng et al., 2019), post damage recovery (Martínková et al., 2020), and vegetative dormancy (Ott et al., 2019). Conventional wisdom might lead one to believe that clonal species would have lower genetic diversity owing to lower sexual reproduction and that genetic diversity could decline over time as population narrows down to several large genets. However, instances of genetic erosion are rare and counterintuitively, higher levels of heterozygosity are often observed (Franklin et al., 2021). Genetic erosion does occur in clonal plants albeit with a caveat, Radosavljević et al. (2020) concluded that the erosion of the genetic variability of older patches is likely caused by the inter-genet competition and resulting selection or by a random die-off of individual genets accompanied by the absence of new seedlings establishment. In retrospect, Oborny and Marcsó (2023) observed that the genet's survival probability is strongly influenced by the terminal ramets but is almost unaffected by the internal ones in a broad parameter range.

In clonal species where the ramets remain connected to the parent plant, resource sharing is possible between connected individuals such as in *Alternanthera philoxeroides* (Mart.) Griseb. (Amaranthaceae, You et al., 2014), *Geum reptans* L. (Rosaceae, Pluess & Stocklin, 2004), *Dithyrea maritima* (Davidson) Davidson (Brassicaceae, Aigner, 2004), and *Trillium recurvatum* L.C.Beck (Melanthiaceae, Mandel et al., 2019).

When the ramets are connected to their parents and other ramets, the genet may share resources to increase survivability of the genet as a whole. Gardner & Mangel's (1999) model suggested in clonal plants, sharing of resources between parent and ramet is most beneficial when there's a difference in how well different ramets can grow in their respective environments thus resources can be allocated from better performing ramets to the poorer performing ramets, when the health quality of ramets outweighs the number of ramets, and when mortality probability of ramets is equal throughout the genet as this ensures at least some ramets may survive.

2.5 Single Nucleotide Genotyping

Single nucleotide analysis is used heavily in plant breeding programs mainly to identify the genetic diversity and population structure within the cultivars to improve crop breeds (Yang et al., 2020; Zhang et al., 2020; Pandey et al., 2021; Tomar et al., 2021; Lopes et al., 2022) thus similar methods can also be adopted to identify clones within the population and the resulting clusters that are formed. The single nucleotide polymorphism (SNP) marker was chosen as a molecular marker for this intraspecific genetic diversity study owing to the extensive genome coverage, chromosome-specific location and low cost as compared to

other PCR-based markers as well as being evolutionarily stable due to lower rates of recurrent mutation (Lopes et al., 2020; Yang et al., 2020).

An example of SNPs being to study population genetics was by focusing on the somatic genetic variation of clonal species. Yu et al. (2020) uncovered thousands of SNPs in a population of *Zostera marina* L. that revealed the strength of purifying selection on mosaic genetic variation was greater within than among ramets and that somatic genetic drift during clonal propagation leads to the emergence of genetically unique modules.

CHAPTER 3

PHENOLOGY, POLLINATION BIOLOGY AND BREEDING MECHANISMS OF *CALAMUS LOBBIANUS* AND *CALAMUS PYGMAEUS*

3.1 Introduction

Calamus lobbianus Becc. belongs to the *Calamus conirostris* Becc. (a subgroup of the Sundaland flagellate group) group consisting of seven members (*C. conirostris*, *Calamus convallium* J.Dransf., *Calamus gonospermus* Becc., *Calamus leloi* J.Dransf., *C. lobbianus*, *Calamus pycnocarpus* (Furtado) J.Dransf., and *Calamus spectatissimus* Furtado). This group is delimited by having the rachis of the partial inflorescence thicker than the rachis of the inflorescence distal to the partial inflorescence. Members of the group also tend to have brown or black fruits with non-channelled scales (Henderson, 2020). *Calamus pygmaeus* appears most closely to a group of six flagellates (*C. gaharuensis*, *C. lengguanii*, *C. nematospadix*, *C. nielsenii*, *C. psilocladus*, and *C. pygmaeus*) found across Sundaland indicated by reddish brown rachis and pinnae base, indumentum and small whitish globose fruits with explanate fruiting perianths. Members of this group are morphologically too similar thus species boundary might change with more data (Henderson, 2020).

Studies on the floral biology of *Calamus* covered several groups including the groups of *Calamus albidus* L.X.Guo & A.J.Hend., *Calamus arborescens* Griff., and *Calamus arugda* Becc., in either its natural habitats or plantations (Lee, 1995; Bøgh, 1996; Alloysius, 1999; Hj Abdullah, 2000; Kitching et al., 2007; Kidyoo & McKey, 2012; Mohd Rusdi et al., 2022), however, none has been conducted so far on the groups represented by *C. lobbianus* and *C. pygmaeus*. *Calamus lobbianus* and *C. pygmaeus* while similar in phenology, exhibit stark differences in their floral characteristics and attractants, leading to distinct reproductive

strategies. This contrast will directly be reflected on the visitor assemblage which subsequently should reveal their breeding strategy. Therefore, this study aimed to investigate the floral biology and the pollination mechanism of two non-climbing species, *C. lobbianus* and *C. pygmaeus*.

3.1.1 Problem Statement

So far, there is no study on the phenology and floral biology of the groups representing *C. lobbianus* and *C. pygmaeus* in the natural environment. The differences in the groups represented by the two species mentioned above will provide great insights into the floral biology and pollination biology of the groups they represent.

3.1.2 Objectives

The primary objective of this study was to understand the phenology, pollination biology, and breeding mechanisms of *C. lobbianus* and *C. pygmaeus*. The specific objectives were:

- To determine the phenology and pollination biology of *C. lobbianus* and *C. pygmaeus*.
- To investigate the breeding strategies of *C. lobbianus* and *C. pygmaeus*.

3.2 Methodology

3.2.1 Study Area

This study was conducted at Kubah National Park (KNP) (1°36'45.51"N, 110°11'48.52"E) which is approximately 20 km northwest of Kuching, Sarawak, Malaysia. Populations of *C. lobbianus* and *C. pygmaeus* occur between 200–500 m elev. Kubah National Park comprises largely five vegetation types: lowland mixed dipterocarp forest, kerangas forest, submontane forest, alluvial forest, and secondary forest (Pearce & Geri 2007). Red-yellow podzolic soils cover most of the rocks in KNP, dip-slope podzols (predominantly sandy clay, well-drained, and nutrient deficient) and peaty soil are common on exposed ridges upward from about 300 m elev. (Hazebroek & Kashim, 2000). Kubah National Park faces the Southwest Monsoon (late May-early October) and the Northeast Monsoon (late November-late March) with drier weather intervals between April to May and October to November. The average rainfall in the Kuching – Matang Area is ca. 4200 mm in the year 2020 (Jabatan Meteorologi Malaysia, 2020). Pearce (1992) reported that KNP has the highest diversity of palms in Sarawak with at least 95 taxa of palm species and at least 47 species of *Calamus* within the park and its immediate environs.

Plant individuals were sampled on both sides along the main trail and Selang trail. Individuals were selected based on the presence of senesced inflorescence or infructescence from the previous season. The duration of the whole study lasted from April 2021 to April 2022 (Appendix 1). The number of individuals and flowering individuals were noted. Individuals of *C. pygmaeus* with rooted individuals were noted along the sampling trail. The voucher specimens of both species including the rachillae of both staminate and pistillate partial inflorescences were collected, fixed in alcohol, and deposited at SAR.

3.2.2 Plant Description

Calamus lobbianus (**Figure 3.1**) is a solitary stemless or very short-stemmed and sometimes subterranean, up to 2.0 m tall whereas *C. pygmaeus* (**Figure 3.2**) is a clustered rattan, acaulescent up to 1.5 m tall. The inflorescences of both species have up to six first-order branches (partial inflorescences) distributed along the main axis (terminates with a flagellum in *C. pygmaeus*). An individual stem could produce up to four inflorescences in a single flowering season. In the staminate inflorescences, the flowers are alternately and distichously arranged along the rachillae. The rachillae of pistillate inflorescences bear flowers in dyads which consist of a pistillate flower and a sterile staminate flower.

3.2.2.1 *Calamus lobbianus*

Calamus lobbianus occurs on soil high in organic matter in damp facies of hill forests (**Figure 3.1A**). Most of the individuals were found near the streams under shade and sometimes in open areas with underexposed sunlight. The **stem** reaches c. 0.6 m long with dull green sheaths which are not tubular and open opposite the petiole. The **sheath** is densely armed with triangular spines which are concave at the base proximally, horizontally spreading, yellowish-green and 5-50 mm long (**Figure 3.1B & C**). The **knee**, **ocrea**, and **flagellum** are not present in any of the individuals in the population.



Figure 3.1: A) *Calamus lobbianus*, acaulescent up to 2 meters tall B) Stout irregularly arranged spines on petiole of Morph A C) Thinner irregularly arranged spines on petiole of Morph B D) Greenish pink staminate flowers of Morph A E) Greenish pink pistillate flowers of Morph A F) Powdery white abaxial and glabrous adaxial leaves of *C. lobbianus* leaves G) Maroon staminate flowers of Morph B H) Maroon pistillate flowers of Morph B I) Mature infructescence bunch with top-shaped fruits covered in black and brownish-yellow scales. Scale bars are included.

The **leaf** is ecirrate, c. 140 cm with the petiole up to 82 cm long, armed laterally with yellowish-green horizontal spines, c. 26 mm and rachis c. 30 cm long. The **leaflets** about 20

on each side of the rachis, are regularly arranged but rather distant c. 40*3 cm. The leaflets are lanceolate, with spinules on veins abaxially, the lateral veins approximately parallel, ‘broken’ vein dark green on the adaxial surface, densely chalky-white on the abaxial surface and apical leaflets very briefly joined (**Figure 3.1F**).

The **inflorescence** is short and slender, c. 42 cm long in staminate and 27 cm long in pistillate plants. The **peduncle** is scarcely developed above the point of divergence. The **prophyll** is tubular and persistent, usually splitting only at the apex. The **partial inflorescences** are not stalked, with a pulvinus between the axil of the rachis and the partial inflorescence, with the rachis markedly thicker than the inflorescence rachis distal to the partial inflorescence. The **rachis** of the bracts is tubular and somewhat inflated at the apex, with an oblique opening and a sessile rachilla. The partial inflorescences of both sexes branch into two orders. The **partial pistillate** and **staminate inflorescence** are c. 11.5 cm long and 18.2 cm long respectively. The **staminate sepals** are usually shorter than the petals, cupular, trilobed at the apex, large, fleshy and bi-coloured. The **stamens** are six with uniseriate filaments, inflexed at the apex producing light-yellow powdery pollen. The associated pistillode is opaque and trilobed. The **pistillate inflorescences** comprised of sessile dyads of a pistillate and sterile staminate flower. The calyx, petal and stigma are trilobed. The **fruit** is top-shaped, c. 34*18 mm diameter with the fruiting perianths explanate and covered in c. 15 vertical rows of brownish black, glossy and smooth scales (**Figure 3.1I**). Two morphologically different forms of *C. lobbianus* were found (form A (**Figure 3.1D** and **1E**) and form B (**Figure 3.1G** and **Figure 3.1H**)) with variations in the spines of the petiole and flowers of both sexes. The vegetative and reproductive features of both forms in staminate and pistillate plants of *C. lobbianus* are presented in `

Table 3.1

Table 3.1: Vegetative and reproductive differences between both forms in staminate and pistillate plants of *C. lobbianus*.

	Form A			Form B		
	Male	Female	Sterile staminate	Male	Female	Sterile staminate
Petiole	c. 14.6 mm	c. 9.8 mm	-	c. 11.3 mm	c. 8.9 mm	-
Calyx	light green with a brown margin and white lateral ribs (c. 5.5 mm in height)	light green with white dots, reddish-purple vertical lines, and a brown margin (c. 5.7 mm long)	light green (c. 4.9 mm)	maroon with light maroon lateral ribs (c. 5.2 mm in height)	maroon on the top half, light green on the bottom half with light green dots, and vertical lines (c. 4.9 mm)	maroon on the top half, light green on the bottom half with light green dots, and vertical lines (c. 4.7 mm)
Petals	white on the lateral ribs, green at the distal half, and light green at the proximal half with white dots, internally white at the distal half, and light green at the proximal half (c. 7.1 mm)	light green with white dots, reddish-purple vertical lines, and a brown margin (c. 5.7 mm long)	light green with white dots, reddish-purple vertical lines, and a brown margin (c. 5.5 mm long)	petals are pink with a white margin, and light brown lateral ribs externally, internally light pink (c. 6.8 mm).	Entirely cream white (c. 7.4 mm)	Entirely cream white (c. 7.3 mm)
Sepals	-	yellowish green (c. 6.3 mm)	yellowish green (c. 6.2 mm)	-	maroon on the top half, light green on the bottom half with light green dots, and vertical lines (c. 6.4 mm)	maroon on the top half, light green on the bottom half with light green dots, and vertical lines (c. 6.1 mm)

3.2.2.2 *Calamus pygmaeus*

Populations of *C. pygmaeus* were mostly found in well-irrigated sandy clay soil with a top layer of organic matter (**Figure 3.2A**). At least two populations were found by a stream. The leaf-sheaths of *C. pygmaeus* are not tubular but open opposite the petiole, dull brown, bearing rather few groups of horizontal or slightly reflexed triangular green stout spines, c. 6.1 mm with abundant brown indumentum between spines (**Figure 3.2B**). The flagellum and knee are absent while the ocrea is short, membranous, and soon tattering. The **leaf** is ecirrate, c. 75.5 cm long with the petiole c. 21.5 cm, armed with whorls of horizontal spines near the base but unarmed distally, sparsely covered with reddish-brown or brown indumentum and the rachis (**Figure 3.2D**). The **leaflets** are c. 28 on each side of the rachis, c. 33.5 cm long, unarmed, regularly arranged, linear, with spinules on veins adaxially, the lateral veins approximately parallel, one terminating subapically, leaving a distinct or obscure, adaxial ‘broken’ vein, the bases abaxially sparsely covered with black indumentum (**Figure 3.2E**).

The **inflorescence** emerges between the leaves, slender, and whip-like up to c. 2.5 m long in pistillate plant, and c. 2.7 m long in the staminate plant, peduncle c. 70 cm long in both staminate, and pistillate plants both roots at the apex to generate new plant (**Figure 3.2I**). The **partial inflorescences** (up to four) of the pistillate plant, and staminate plant are c. 5.7 cm long, and c. 6.2 cm long, respectively, which are spread sparsely along the inflorescence, stalked, with a pulvinus in the axil of rachis, and partial inflorescence. The **flowers** are borne on the third order of branching in the staminate plant but on the second order of the pistillate plants. Each partial inflorescence is in c. four units, c. 3.9 cm in length (staminate plants), and c. 3.3 cm in length (pistillate plants); branches further four to six times. The **staminate** rachilla is c. 1.8 cm long, with alternately, and distichously arranged flowers. The calyx is

green with brown tip, c. 1.1 mm; sepal in three, yellowish green; petal in three, cream white, c. 0.8 mm; the pistillode is trilobed but never open, orange; the stamen is c. 2.0 mm with the anther bilobed with powdery yellow pollen, which is dehiscent through a lateral slit, filament white almost opaque (**Figure 3.2F**). The **pistillate** flowers with the calyx green but brown near the margin, c. 0.6 mm long; the petals in three, cream white, c. 1.6 mm; stigma is trilobed, and opaque, almost transparent, c. 1.9 mm. The sterile staminate flowers with the calyx green with brown tip, c. 0.4 mm; the sepal in three, cream white, c. 1.0 mm; the petal in three, cream white; staminode in six, opaque, c. 2.0 mm; the pistillode is trilobed but never open, c. 1.2 mm (**Figure 3.2G**). The **fruit** is (white when mature) round c. 7.3 mm in diameter with the stigma remnant, covered in 11-14 vertical rows of straw-coloured margined scales (**Figure 3.2H**). A summary of plant habits, and floral characters of *C. lobbianus*, and *C. pygmaeus* is provided in **Table 3.2**.



Figure 3.2: A) *Calamus pygmaeus* up to 1.5 m tall B) Acaulescent stem with brown sheaths, and green stout spines C) Aerial roots can sometimes grow in taller specimens D) Reddish-brown indumentum along the rachis E) Black soft spines along the adaxial, abaxial, and pinnae margin F) Staminate in bloom with anthers covered in powdery yellow pollen, and bright orange pistillode G) Staminate flower in bloom with tri-lobed stigma protruding from the pistillate flower H) Globose fruit white scales, and brown margins I) Tip of inflorescence rooting into a new plantlet with the remnant of partial inflorescence still attached. Scale bars are included.

Table 3.2: A summary of plant habits and floral characters of *C. lobbianus*, and *C. pygmaeus* with data presented as an average (range) unit.

	<i>C. lobbianus</i>	<i>C. pygmaeus</i>
Habit	Solitary	Clustering
Stem length (m)	0.6 (0.4–1.2) m	0.6 (0.3–0.9) m
Vertical height (m)	1.5 (1.3–1.8) m	1.2 (0.9–1.7) m
Inflorescence length ♀ (cm)	26.5 (21.3–31.7) cm	2.5 (2.1–2.7) m
Inflorescence length ♂ (cm)	31.5 (27.4–35.6) cm	2.7 (2.4–2.9) m
Partial inflorescence ♀ length (cm)	11.5 (9.7–17.2) cm	5.7 (5.1–6.2) cm
Partial inflorescence ♂ length (cm)	18.2 (15.9–25.5) cm	6.2 (5.5–7.4) cm
Colour of tepals	Light green with brown margin/ Pink with white margins	Cream white
No. of partial ♀ inflorescence	2 (1–3)	4 (3–7)
No. of partial ♂ inflorescence	2 (1–3)	4 (3–6)
No. of rachis per partial ♀ inflorescence	2 (2–3)	5 (4–6)
No. of rachis per partial ♂ inflorescence	2 (2–4)	5 (2–7)
No. of dyads per ♀ rachis	19 (15–30)	7 (5–9)
No. of flowers per ♂ rachis	41 (32–47)	9 (7–13)
Flowering duration of ♀ inflorescence	19 (18–27)	30 (28–33)
Flowering duration of ♂ inflorescence	25 (21–30)	45 (37–51)
Flowering duration of partial ♀ inflorescence	9 (6–10)	7 (6–9)
Flowering duration of partial ♂ inflorescence	8 (7–10)	12 (10–14)

3.2.3 Floral Biology and Floral Visitors

Several days of direct observations were conducted to establish the start, and the end of the anthesis. This was followed by direct observations consisting of four to five hours each day until the entire duration of the anthesis was observed. The total number of flowers per inflorescence was counted. Floral visitors were observed from when flowers were still enclosed in bracts until the end of anthesis. The behaviour of the floral visitors such as interaction with stigma, and anthers, feeding on nectar, and the average duration of visit. The floral visitors were further categorised into primary and secondary visitors based on their interactions with the flowers. The mean number of floral visitors was presented based on the

total duration of a visit for each species. Floral visitors were photographed using camera Canon EOS 200D fitted with a 100 mm macro lens for identification.

3.2.3.1 Floral Availability

A total of 13 individuals (seven staminate and six pistillate) for *C. lobbianus* and 138 individuals (55 staminate and 78 pistillate) for *C. pygmaeus* were included in this study. The observation was carried out from 21 Sept 2021 till 14 January 2022. The sex ratio at two levels (the number of individuals and the number of individuals that flowered during the study period) was tested using binomial tests (Kidyoo & McKey, 2012).

3.2.3.2 Nectar Sampling

The volume and concentration of nectar were measured for both sexes of Form A and B of *C. lobbianus*. The nectar was collected using a 10 µl microcapillary syringe while the concentration was measured using a handheld Brix refractometer model 45-81 (Bellingham + Stanley) at 30-minute intervals. The Brix reading is assumed as sucrose equivalent (Dafni, 1992). Nectar sampling was performed during phenology observations and the number of flowers sampled was dependent on the floral availability (Appendix 1).

3.2.3.3 Pollination Experiments

Two treatments were applied: Bagged flower (T 1)- partial inflorescence was bagged (using 0.5 mm mesh bags) several days before blooming; Free unbagged (T 2)- flowers were left exposed to permit unhindered interactions with floral visitors. The bags were removed

after anthesis and several months later the fruit set was registered. To eliminate any possible effect of inherent variation in the fruit set, a different part of each inflorescence was bagged. One to two individuals from each population were selected; partial inflorescences from four individuals were tagged for *C. pygmaeus* while partial inflorescences from three individuals were tagged for *C. lobbianus*. Further details on the samplings are provided in Appendix 2.

3.3 Results

3.3.1 Phenology

The phenology for both forms of *C. lobbianus* is similar for staminate and pistillate plants. The blooming of the staminate flowers for *C. lobbianus* begins at the distal end of the proximal partial inflorescences. However, the flowers of the remaining partial inflorescences bloom randomly. Two to three staminate flowers of a partial inflorescence bloom per day with one to three-day intervals. The stamen and pistillode extension happen simultaneously during blooming. Pollen extrusion begins between 0500 to 1000 (Day 1) and lasts until around 1300, sometimes 1500 (Day 1). Nectar was exuded from near the base of the petals around 1000 (Day 1) till 1300 on the same day. No odour was discernible even directly above the flowers. The flower dehisces around 1500 (Day 1). A partial inflorescence takes ca. 8 days to finish blooming while the whole inflorescence takes ca. 25 days.

The blooming of pistillate flowers of *C. lobbianus* begins from the distal end of the distal partial inflorescence but the flowering sequence is random within the partial inflorescences. Two to three pistillate flowers of a partial inflorescence bloom per day with one-to-three-day intervals. The stigma begins to emerge around 0800 to 1000 one day before anthesis. By 0400 to 0700 on the following day (Day 1), the female anthesis begins with the

glistening of the surface of the stigma. The stigma fully emerged with the recurving petals by 1200 (Day 1). The stigma dries which indicates the end of the anthesis between 1500 to 1700 (Day 2). In the associated dyad counterpart, sterile male flowers typically open around 0600 (Day 1) and fall off around 1600 on Day 1. However, 17.85 % (5/28) of the staminodes do not open synchronously with their associated pistillate flower. Nectar is exuded from the base of the petals of the sterile male flowers from around 0900 (Day 1) till around 1230 on the same day. The blooming lasts about nine days in a partial inflorescence which takes the inflorescence around 19 days to finish blooming.

The blooming of both sexes of *C. pygmaeus* begins from the distal end of the proximal partial inflorescence but the flowering sequence is random within the partial inflorescences. Several partial inflorescences bloom at the same time in older plants of *C. pygmaeus*. Three to four staminate flowers bloom per day with one to two-day intervals. In male plants of *C. pygmaeus*, the anthesis of the individual flowers begins from 0830 (Day 1) with some flowers opening much later, at ca. 1000. A thin layer of glistening exudate was visible at the base of the petals typically 30 mins after anthesis begins. Stamen and pistillode extension happen simultaneously during blooming. No floral scent was detected throughout the anthesis. Anthesis ends by 1500 (Day 1), and the flower is shed by 1800 (Day 1). The blooming lasts about 12 days in a partial inflorescence which takes the inflorescence around 45 days to finish blooming.

Two to three pistillate flowers of *C. lobbianus* bloom per day with one to three-day intervals with at least one or more staminodes are open within the partial inflorescence. The stigma emerges around 0800 (Day 1). The anthesis begins around an hour later, 0900 (Day 1) with the stigmatic surface appearing receptive and white until 1500 (Day 2). The stigmatic

surface turns brown towards the evening with the corolla and calyx shed. No floral scent was detected. The sterile staminate flower of *C. pygmaeus* opens around 0800 (Day 1), persists until around 1600 (Day 1) and dehisces by 1700 (Day 1). The base of the petals appears to be glistening, likely to be nectar. The sterile male flower blooms randomly, which could be before, during or after the blooming of the associated pistillate flower. The blooming lasts about seven days in a partial inflorescence which takes the inflorescence around 30 days to bloom. The phenological phases are represented in **Figure 3.3**.

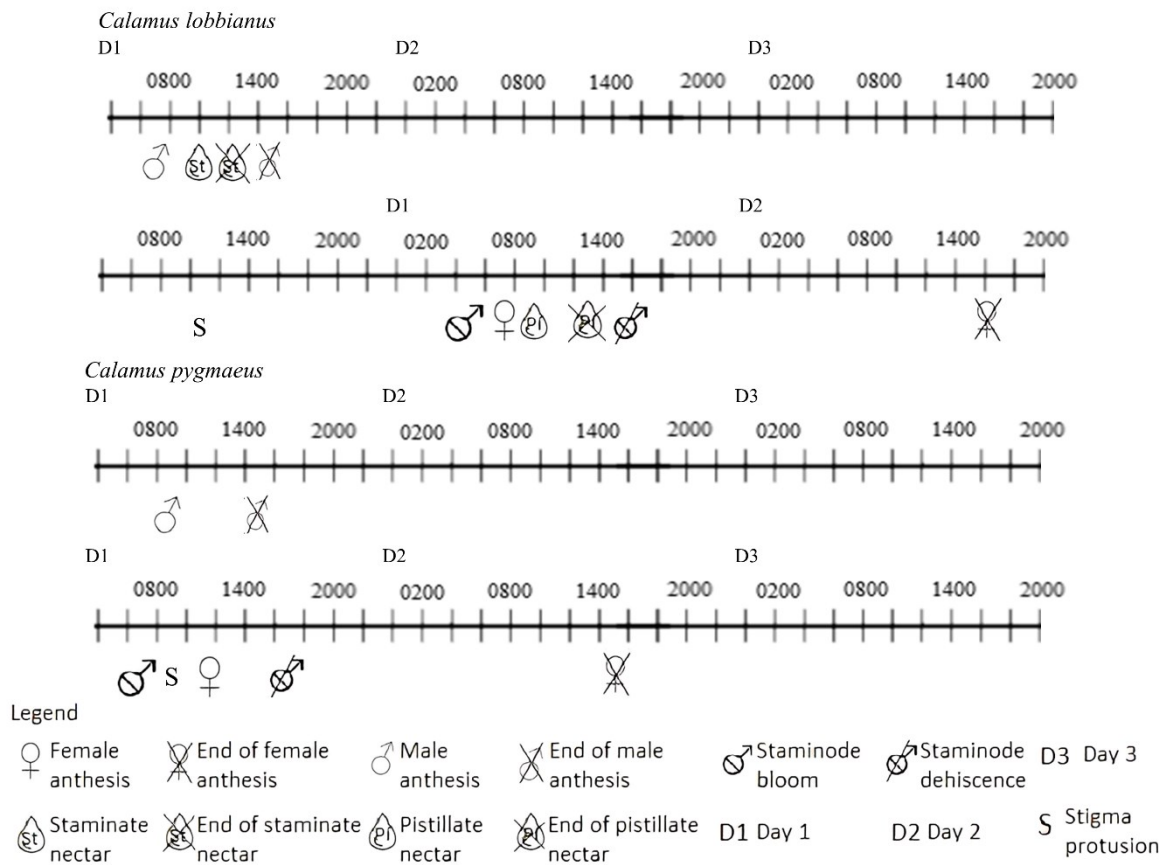


Figure 3.3: A timeline of phenological events for *C. lobbianus* and *C. pygmaeus*.

3.3.2 Vegetative Propagation in *Calamus pygmaeus*

In *C. pygmaeus*, vegetative propagation is achieved via the rooting at the tip of inflorescence (**Figure 3.2I**). *Calamus pygmaeus* displays a population of the same gender within 2 m of each other (2 m is the average length of the inflorescence). Each patch contains 9 to 24 individual plants and sometimes up to 30 individual plants of various developmental stages, with usually one or several individuals in the reproductive stage (Table 3.3) where majority of the individuals within each group consisting of largely the same gender. The rooting at the tip of inflorescence was recorded in 54.54 % of the male individuals and 55.12% of the female individuals (Table 3.3).

Table 3.3: Individuals of *C. pygmaeus* with rooted inflorescences.

		No. individuals	Rooted inflorescence	Rooting percentage (%)
Male	Cluster 2	9	4	44.44
	Cluster 3	10	3	30.00
	Cluster 4	21	15	71.43
	Cluster 7	15	8	53.33
Total		55	30	54.54
Female	Cluster 1	13	9	69.23
	Cluster 5	15	10	66.67
	Cluster 6	14	7	50.00
	Cluster 8	24	14	58.33
	Cluster 9	12	3	25.00
Total		78	43	55.12

3.3.3 Floral Availability and Operational Sex Ratio

The presence of staminate and pistillate inflorescence for *C. lobbianus* and *C. pygmaeus* is presented in **Figure 3.4**. Flowering for both species appears aseasonal throughout the observed period although *C. pygmaeus* blooms more frequently as compared to *C. lobbianus*. The sex ratio of flowering individuals was largely unbiased between male

and female individuals except during October and December of 2021 for *C. lobbianus* where the male bias was significant ($p=0.044$) (Table 3.4).

	September		October				November				December					January	
	W4	W5	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W5	W1	W2
<i>Calamus lobbianus</i>																	
CLMS04M																	
CLMS05M																	
CLMS17M																	
CLMS08F																	
CLMS11F																	
<i>Calamus pygmaeus</i>																	
CLMS02M																	
CLMS03M																	
CLMS06M																	
CLMS01F																	
CLMS07F																	
CLMS16F																	

Figure 3.4: The presence of staminate and pistillate inflorescence for *C. lobbianus* and *C. pygmaeus* from 21/9/2021 until 14/1/2022. W1=Week 1 and so on; voucher code ending with M is a staminate plant, voucher code ending with F is a pistillate plant; the black box represents blooming, and the grey box represents fruiting.

Table 3.4: Binomial test results in the sex ratio of flowering individuals and the operational sex ratio (OSR) expressed as the ratio of flowering staminate to pistillate plants (staminate: pistillate) of *C. lobbianus* and *C. pygmaeus*. ($\alpha=0.05$).

			P values	OSR
October	<i>C. lobbianus</i>	Staminate	0.044	2:0
		Pistillate	0.302	
	<i>C. pygmaeus</i>	Staminate	0.218	6:5
		Pistillate	0.128	
November	<i>C. lobbianus</i>	Staminate	0.302	1:1
		Pistillate	0.265	
	<i>C. pygmaeus</i>	Staminate	0.182	10:11
		Pistillate	0.141	
December	<i>C. lobbianus</i>	Staminate	0.044	2:1
		Pistillate	0.265	
	<i>C. pygmaeus</i>	Staminate	0.182	5:6
		Pistillate	0.154	

3.3.4 Nectar Availability of *Calamus lobbianus*

The volume and concentration of sugar in the nectar varied throughout anthesis for *C. lobbianus* (Form A and B) as shown in **Figure 3.5**. In the male flower of Form A, the volume and concentration started from 1.7 μl and 10.1 % and peaked at 8.1 μl and 11.3 % (ca. 1100 Day 1) (**Table 3.5**). The associated sterile male flower of the pistillate plant produced 2.7 μl amount of 8.8 % (concentration) till peak at 8.0 μl and 12.9 % (concentration) (ca. 0930 Day 1). A similar pattern was observed for both sexes of Form B plants of *C. lobbianus*.

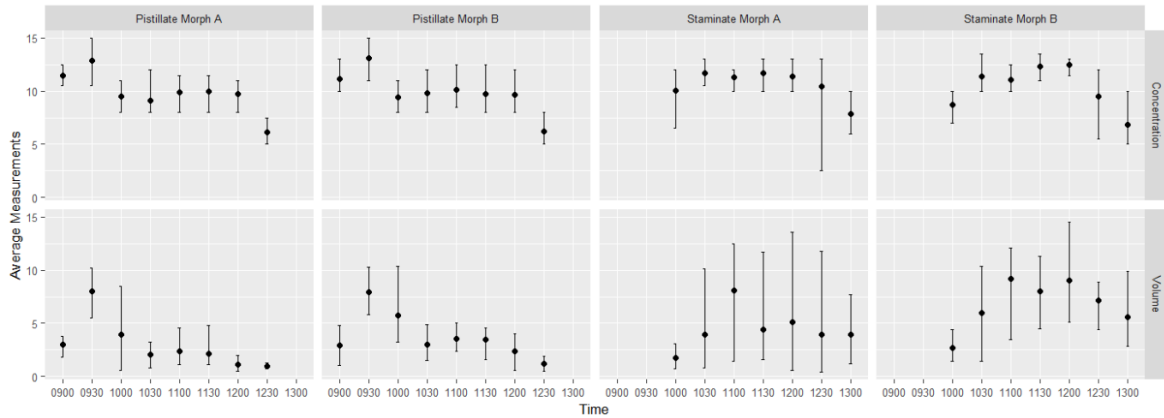


Figure 3.5: Average volume (μl) and concentration (% in Brix) of pistillate and staminate flowers for Morph A and B of *C. lobbianus* through time with error bars represented by maximum and minimum values.

3.3.5 Floral Visitors

The floral visitors observed for *C. lobbianus* were quite diverse and consist of several different taxa, black-headed stingless bee (*Tetragonula melanocephala*) (**Figure 3.6A**), orange stingless bee (*Tetragonula melina*) (**Figure 3.6B**), hover wasp (*Liostenogaster* sp.) (**Figure 3.6C**), potter wasp (*Stenodyneriellus* sp.) (**Figure 3.6D**), black ants (Formicidae) (**Figure 3.6E**), floral mites (Acaridae) (**Figure 3.6F**), fruit flies (*Drosophila* sp.) (**Figure 3.6F**), and spiderhunter (*Arachnothera* sp.) (**Figure 3.6G**). The visiting time was similar for

Table 3.5: Volume (μl) and concentration (Conc.) measured in Brix (%) using sucrose equivalence for staminate (σ) and sterile male (σ^*) plants of *C. lobbianus* for forms A and B.

	900	930	1000	1030	1100	1130	1200	1230	1300
<i>Calamus lobbianus</i> A σ									
Volume (μl)	-	-	1.7 (SE $\pm$ 0.40)	3.9 (SE $\pm$ 1.34)	8.1 (SE $\pm$ 2.12)	4.4 (SE $\pm$ 1.56)	5.1 (SE $\pm$ 1.97)	3.9 (SE $\pm$ 1.81)	3.9 (SE $\pm$ 1.00)
Conc. (% in Brix)	-	-	10.1 (SE $\pm$ 0.78)	11.7 (SE $\pm$ 0.40)	11.3 (SE $\pm$ 0.31)	11.7 (SE $\pm$ 0.44)	11.4 (SE $\pm$ 0.49)	10.4 (SE $\pm$ 1.60)	7.8 (SE $\pm$ 0.61)
<i>Calamus lobbianus</i> A σ^*									
Volume (μl)	3.0 (SE $\pm$ 0.28)	8.0 (SE $\pm$ 0.75)	3.9 (SE $\pm$ 1.19)	2.0 (SE $\pm$ 0.34)	2.4 (SE $\pm$ 0.61)	2.1 (SE $\pm$ 0.57)	1.1 (SE $\pm$ 0.22)	0.9 (SE $\pm$ 0.10)	-
Conc. (% in Brix)	11.5 (SE $\pm$ 0.29)	12.9 (SE $\pm$ 0.68)	9.5 (SE $\pm$ 0.45)	9.1 (SE $\pm$ 0.62)	9.9 (SE $\pm$ 0.54)	10.0 (SE $\pm$ 0.53)	9.9 (SE $\pm$ 0.48)	6.2 (SE $\pm$ 0.38)	-
<i>Calamus lobbianus</i> B σ									
Volume (μl)	-	-	2.7 (SE $\pm$ 0.48)	6.00 (SE $\pm$ 1.24)	9.1 (SE $\pm$ 1.54)	7.9 (SE $\pm$ 1.41)	9.0 (SE $\pm$ 1.62)	7.1 (SE $\pm$ 0.69)	5.6 (SE $\pm$ 1.00)
Conc. (% in Brix)	-	-	8.8 (SE $\pm$ 0.51)	11.4 (SE $\pm$ 0.51)	11.1 (SE $\pm$ 0.35)	12.3 (SE $\pm$ 0.40)	12.5 (SE $\pm$ 0.26)	9.5 (SE $\pm$ 0.91)	6.8 (SE $\pm$ 0.84)
<i>Calamus lobbianus</i> B σ^*									
Volume (μl)	2.9 (SE $\pm$ 0.65)	7.9 (SE $\pm$ 0.74)	5.7 (SE $\pm$ 1.17)	3.0 (SE $\pm$ 0.53)	3.5 (SE $\pm$ 0.49)	3.5 (SE $\pm$ 0.49)	2.3 (SE $\pm$ 0.57)	1.2 (SE $\pm$ 0.21)	-
Conc. (% in Brix)	11.2 (SE $\pm$ 0.46)	13.2 (SE $\pm$ 0.62)	9.4 (SE $\pm$ 0.47)	9.8 (SE $\pm$ 0.71)	10.2 (SE $\pm$ 0.60)	9.8 (SE $\pm$ 0.74)	9.7 (SE $\pm$ 0.63)	6.3 (SE $\pm$ 0.42)	-

the staminate flowers, starting from early morning around 0700 (Day 1) to about 1500 (Day 1) (**Table 3.6**) (**Figure 3.7**).

Both species of stingless bees have similar behaviours where they feed on nectar and collect pollen. Both hover and paper wasps were observed to not actively collect pollen but mainly fed on the nectar, but the potter wasp is larger than the flowers which sometimes allows the anthers to brush against its body. However, it is rare for pollen to be seen stuck onto its head or thorax. A nest of black ants was observed to linger around the inflorescence during the bud formation and bloom to feed on the nectar without contacting the anthers (observed only on one occasion). On several observations, floral mites were seen attached to the visiting insects and carried to another flower throughout anthesis of both sexes. The mites shifted within and between the blooming flowers to feed on pollen and nectar. Fruit flies were always present and occasionally observed to feed on the floral nectar of both sexes. Once, a spiderhunter was observed to visit the flowers around 1200 to feed on the (Day 1) nectar, however, the long beak of the bird did not allow the anther to be in contact with the head and the spiderhunter is thus considered a visitor. Visitors of the pistillate flowers of *C. lobbianus* were similar excluding fruit flies and floral mites. The floral visitors were found during the first day of anthesis (**Table 3.6**). Both species of stingless bees were observed to transfer the pollen onto the stigma and thus are considered as possible pollinators. Both wasps aimed for the nectar in the staminodes and seldom seem to be in contact with the stigma and thus are considered a secondary pollinator. The orange stingless bee (*Tetragonula melina*) (**Figure 3.8A**), black-headed stingless bee (*Tetragonula melanocephala*) (**Figure 3.8B**), and the hover wasp (*Liostenogaster* sp.) (**Figure 3.8C**) visited the flowers of *C. pygmaeus* from 0830 until 1430 (Day 1) for both male and female flowers. The hover wasp and both stingless bees continue to visit on Day 2 from 0830 until 1430 in pistillate plants.

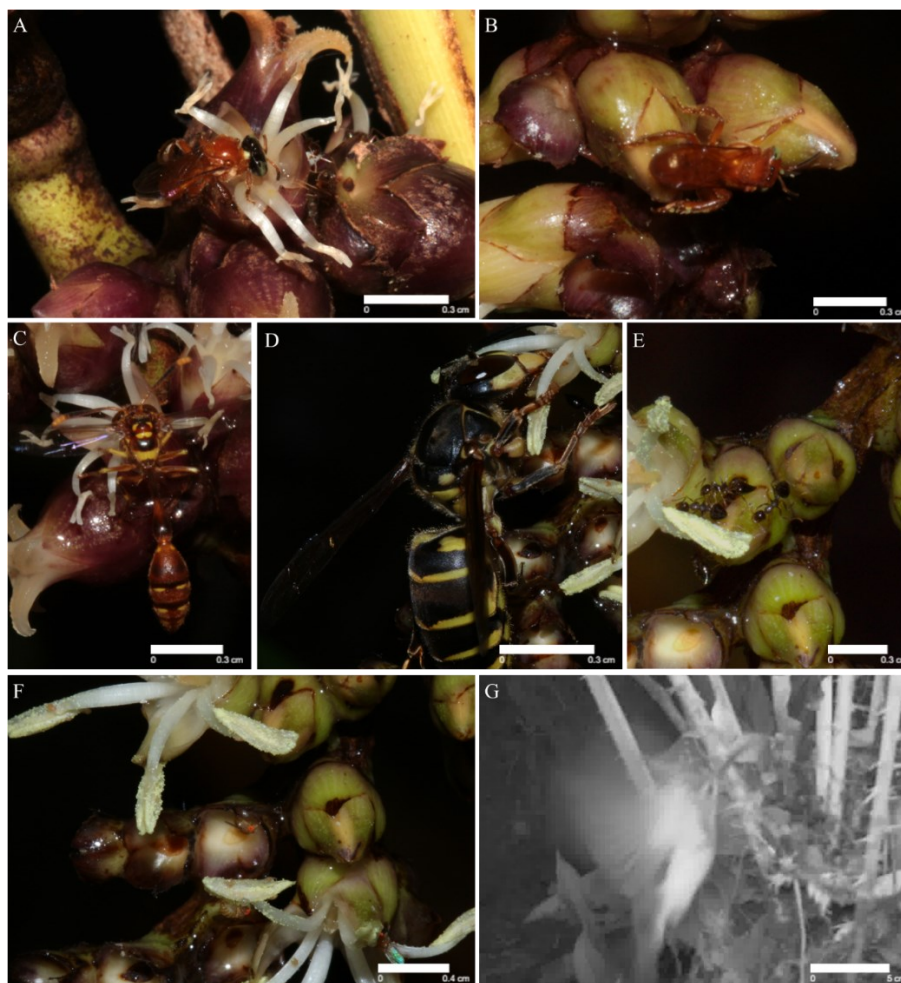


Figure 3.6: Floral visitors of *C. lobbianus*; A) *Tetragonula melanocephala*, black-headed stingless bee B) *Tetragonula melina*, orange stingless bees C) *Liostenogaster* sp., hover wasp D) *Stenodyneriellus* sp., potter wasp E) Unidentified ant species F) *Drosophila* sp., fruit flies, and floral mites G) *Arachnothera* sp., spiderhunter. Scale bars are included.

Both stingless bees usually landed on the anther, collected pollen, and sometimes headed into the cavity to search for nectar. During its visit to the female flower, both stingless bees behaved similarly but remained slightly longer. The hover wasps generally headed into the floral cavity to search for nectar in both male and female flowers. However, owing to the lack of pollen on its body, the hover wasp is considered a secondary pollinator. Several species of ants were observed to visit the flowers on rare occasions but are not considered pollinators. There is no significant difference ($p > 0.05$) in the number of visitors and visit

Table 3.6: Visiting period, mean number, and duration of visit for staminate (♂) and pistillate (♀) plants of *C. lobbianus* and *C. pygmaeus*; value represented as min(average)max for Day 1 (D1) and Day 2 (D2). Significant results between forms of *C. lobbianus* are represented by * (p>0.05).

	Visiting period		Average visitor		Duration		Visitation rate (P value)		Visit duration (P value)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
<i>Calamus lobbianus</i> Form A							T-test results in P value between form A and B			
<i>Liostenogaster</i> sp.	0700-1500 (D1)	0830-1400 (D2)	1(2)3	1(3)5	1.2 sec	4.3 secs	0.47	0.86	0.00*	0.21
<i>Stenodyneriellus</i> sp.	0800-1500 (D1)	0800-1700 (D2)	1(3)5	1(2)5	5.5 secs	6.3 secs	0.45	0.60	0.24	0.14
<i>Tetragonula melina</i>	0600-1500 (D1)	0700-1500 (D2)	3(4)7	2(4)6	5.7 secs	6.1 secs	0.78	0.50	0.08	0.49
<i>Tetragonula melanocephala</i>	0600-1500 (D1)	0700-1500 (D2)	3(5)8	3(5)7	6.3 secs	6.8 secs	0.66	0.60	0.09	0.54
<i>Drosophila</i> sp.	1100-1500 (D1)	-	2(4)6	-	5.6 secs	-	0.55	-	0.02*	-
Floral mite	0500 -1000 (D1)	-	13(15)19	-	31.5 min	-	0.55	-	0.00*	-
<i>Calamus lobbianus</i> Form B										
<i>Liostenogaster</i> sp.	0700-1500 (D1)	0830 (D1)-1400 (D2)	2(2)4	1(2)6	2.8 sec	3.8 secs				
<i>Stenodyneriellus</i> sp.	0800-1500 (D1)	0800 (D1)-1700 (D2)	1(2)4	2(3)5	6.2 secs	5.6 secs				
<i>Tetragonula melina</i>	0600-1500 (D1)	0700 (D1)-1500 (D2)	2(5)6	1(3)6	4.6 secs	6.5 secs				

Table 3.6 continued

<i>Tetragonula melanocephala</i>	0600-1500 (D1)	0700 (D1)-1500 (D2)	2(4)8	2(4) 8	7.3 secs	7.3 secs	
<i>Drosophila</i> sp.	1100-1500 (D1)	-	2(3)6	-	4.5 secs	-	
Floral mite	0500-1000 (D1)	-	11(13) 17	-	27.6 min	-	
<i>Calamus pygmaeus</i>							
<i>Liostenogaster</i> sp.	0830-1430 (D1)	0830 (D1)-1430 (D2)	1(3)4	1(3) 4	1.5 secs	2.5 secs	
<i>Tetragonula melina</i>	0830-1430 (D1)	0830 (D1)-1430 (D2)	2(3)6	1(3) 5	3.6 secs	4.5 secs	
<i>Tetragonula melanocephala</i>	0830-1430 (D1)	0830 (D1)-1430 (D2)	2(4)8	2(3) 7	5.2 secs	6.7 secs	

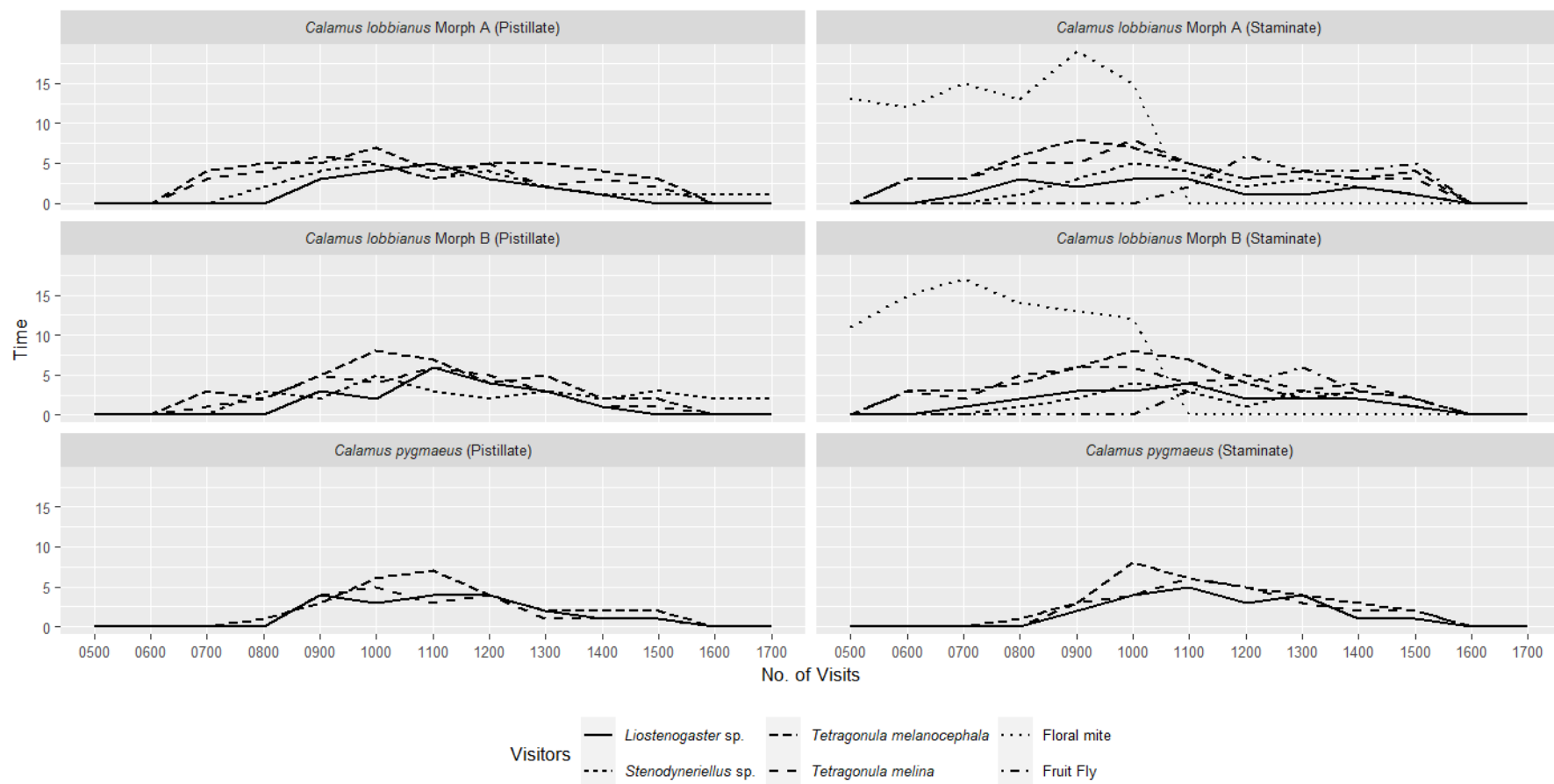


Figure 3.7: Average number for each species of visitor throughout visiting duration for both morphs of *C. lobbianus* and *C. pygmaeus*.

duration on of all visitors except for the visit duration of hover wasps, fruit flies and floral mites between the staminate and pistillate plants of form A and B of *C. lobbianus* (**Table 3.6**). There is also no significant difference on the floral visitors between the different forms of *C. lobbianus*.

3.3.6 Breeding Mechanisms

Pollination experiment on *C. lobbianus* indicates that apomixis is likely to exist as the percentage of fruit set for bagged treatment was lower at 4.77%, 8.33% and 7.68% as compared to unbagged treatment at 21.05%, 19.05% and 16.67% although not statistically significant. However, a seed viability test on apomictic fruit was not performed. The fruit set was recorded in all bagged treatments at three populations which indicates that apomixis is present in *C. pygmaeus* (**Table 3.7**). However, the results showed no significant difference between both treatments for Population 6 and 1 which is likely owing to the pistillate plants showing poor fruiting success as compared to other populations. In Population 5 of *C. pygmaeus*, the fruit set percentage of unbagged treatment was significantly higher than the bagged treatment which differs from the other two populations.

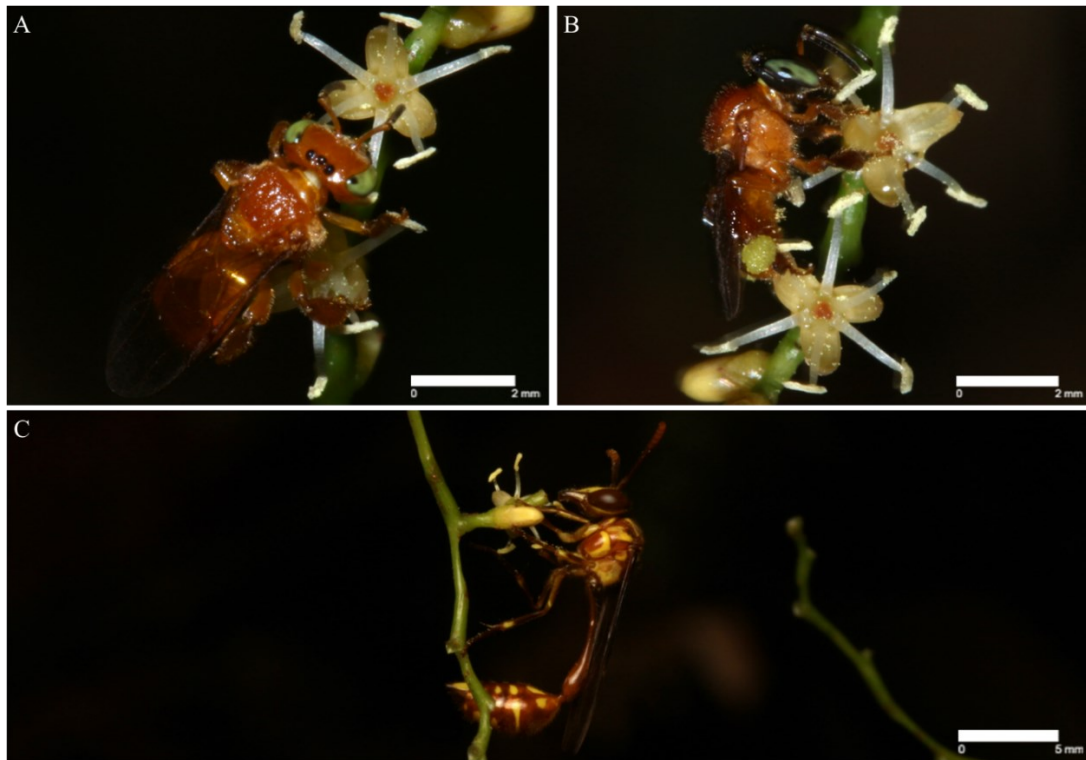


Figure 3.8: Floral visitors of *C. pygmaeus*: A) *Tetragonula melina*, orange stingless bees B) *Tetragonula melanocephala*, black-headed stingless bee C) *Liostenogaster* sp., hover wasp. Scale bars are include

Table 3.7: Results from pollination experiment on *C. pygmaeus* and *C. lobbianus*; T 1=bagged flower, T 2=free unbagged; Chi-square (χ^2) and P-value is calculated between results from T 1 and T 2 where $\alpha = 0.05$.

	T 1	T 1 Fruit Set	T 2	T 2 Fruit Set	Chi-Square	P value
<i>Calamus pygmaeus</i>						
Population 6	2/78	2.56%	6/68	8.82%	2.748	0.0974
Population 5	2/19	10.53%	6/8	75.00%	11.224	0.0008
Population 1	3/12	25.00%	2/14	14.29%	0.2143	0.498
<i>Calamus lobbianus</i>						
CLMS11F	1/22	4.77%	4/19	21.05%	0.7748	0.1245
CLMN08F	1/24	4.17%	4/21	19.05%	0.4489	0.2386
CLMS018F	2/26	7.69%	5/30	16.67%	0.3306	0.3155

3.4 Discussion

3.4.1 Pollinators, Visitors, and Floral Rewards

A wide array of visitor assemblage was reported on *Calamus* mainly Acaridae, Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Thysanoptera (Dransfield, 1979b; Lee, 1995; Bøgh, 1996; Alloysius, 1999; Kitching et al., 2007; Kidyoo & McKey, 2012). However, pollinators of *Calamus* are mainly Hymenopterans such as stingless bees, honeybees, and some Vespid wasps (Lee, 1995; Bøgh, 1996; Kidyoo & McKey, 2012). Lee (1995) reported a wide array of visitors for *C. subinermis* and *C. caesius*, however, most were recorded in very low abundance with the highest recorded from Hymenopterans similar to this study. Some Coleopterans such as nitidulid beetles and weevils are also considered potential pollinators (Dransfield, 1979a, 1979b). The stingless bees are the most possible pollinators for both *Calamus* species in this study with *C. lobbianus* being pollinated by more than one species of stingless bees. The stingless bees were observed to have pollen attached onto their body and transfer it onto the pistillate flowers during their foraging activities. *Liostenogaster* sp. and *Stenodyneriellus* sp. as pollinators in *Calamus* have yet to be reported previously; thus, this is the first record, however, members of this family are known to be pollinators in many other plant families (Bøgh, 1996; Kidyoo & McKey, 2012). The flowers of *C. pygmaeus* are not particularly showy as compared to *C. lobbianus* with relatively small flowers and do not produce scent nor nectar in droplets. The lack of droplets of nectar has also been previously observed in *C. castaneus* (Kidyoo & McKey, 2012). *Calamus lobbianus* on the other hand has large showy flowers and complemented by its production of nectar is capable of attracting a myriad of visitors.

A factor that may attract pollinators is that some palms initially attract visitors with colour vision through the mass flowering of orange-yellow or white flowers without other

major anthecological adaptations (Kiew & Muid, 1989; Listabarth, 2001; Mergos & Yang, 2023), which may serve to confuse the visitors for other species that generally offers rewards with higher nutritional values. The role of the staminate and staminode flowers in this *C. pygmaeus* appears to function as attractants for potential pollinators for pollen transfer which has been observed in several other palms (Uhl & Moore, 1977; Kidyoo & McKey, 2012). The pistillode found on the staminate flowers in the case does not appear to serve any obvious functions apart from being a remnant of a perfect flower. The anthers protrude in all directions and are capable of depositing pollen on any insect of suitable size that crawls around the flowers and the same can be said about the stigma which covers a large area of the pistillate flower. The flowers of *Calamus* have most likely evolved to take advantage of generalist species of pollinators which in this case does not appear seasonal throughout the observation period. The availability of flowers does not suggest seasonality as well since flowers of both staminate and pistillate are available continuously or at the least intermittently throughout the observation period (**Figure 3.4**).

The flowers within one inflorescence often bloom in distinct sequences which may be associated with the pollination mechanism. Basipetal maturation is a process in which the development of plant tissues or organs proceeds from the apex to the base, while acropetal maturation is the opposite process, in which development proceeds from the base to the apex (Dafni, 1992). Henderson (2002) suggested a possible correlation between basipetal floral maturation and beetle pollination while acropetal floral maturation in triads/flowers with bee, fly and, wasp in Arecaceae. The flowering sequence in *C. lobbianus* is acropetal in staminate plants but basipetal in pistillate plants while *C. pygmaeus* is acropetal entirely. *Calamus lobbianus* is likely to be another exception similar to *Licuala peltata* Roxb. ex Buch.-Ham. (Barfod et al., 2003). Basipetal or acropetal maturation may alter the

morphology and physiology of flowers, such as their size, shape, colour, scent, nectar production, and pollen viability, which may also influence pollinator attraction and efficiency. These morphological and physiological differences are directly influenced by the production of secondary metabolites such as phenolics, terpenes, and alkaloids (Taiz & Zeiger, 2010).

It is likely that *C. lobbianus* and *C. pygmaeus* do not strictly rely on the observed primary visitors for pollination but instead have low taxonomic pollinator specificity and are pollinated by a mixed species guild as suggested by Listabarth (2001). Previous pollination works also suggest mixed guild pollination similar to our results (Lee, 1995; Bøgh, 1996; Alloysius, 1999). The observed visitors could be purely owing to their foraging behaviours and nest proximity to the population. Several groups of flies including the fruit flies are always present on the inflorescence, they are, however, generalist of all kinds of flowers and likely of little consequence for pollination in palms, except perhaps in the case of *Geonoma cuneata* var. *sodiroi* in which Borchsenius (1997) suggested that drosophilid flies are the primary pollinator. A more comparative view can be generated by sampling on a larger geographic scale to identify if there is any degree of specialization within the plant-pollinator interactions which is free from the variables generated by local settings which are currently still not available.

3.4.2 Sex Ratio Bias

The operational sex ratio is the ratio of receptive staminate to receptive pistillate flowers in a population at any time (Kidyoo & McKey, 2012). Flowering occurs year-round for both species, thus ideally there should be staminate and pistillate flowers available at all

times, however, pistillate plants in the bloom of *C. lobbianus* occur less frequently compared to staminate individuals. While there are statistically significant biases observed in *C. lobbianus*, it should be noted that the sample size of *C. lobbianus* is small (seven staminate and six pistillate plants) owing to the difficulty in encountering more flowering populations as compared to *C. pygmaeus* (55 staminate and 78 pistillate plants). In *C. lobbianus*, the male-biased scenario provides a ready supply of pollen. The frequent flowering of the staminate plants likely trains the insect visitors to always visit the staminate plants and consequently carry the pollen regardless of the insects visiting the pistillate plants of the same species which in turn increases the likelihood of depositing pollen onto the receptive stigmas. This method works as the main insect visitors were mainly Hymenopterans which have been recorded to develop preferences and repeatedly visit the same plants (Fohouo et al., 2008; Mattu et al., 2012; Sushil et al., 2013). In *C. pygmaeus*, although no sex ratio bias was detected the high number of staminate plants blooming also provided a steady supply of pollen for the pistillate plants. *Calamus castaneus* has also been reported to be aseasonal, flowering through year-round with male plants flowering more frequently compared to females (Kidyoo & McKey, 2012; Mohd Rusdi et al., 2022).

3.4.3 Unique Mode of Vegetative Reproduction in *Calamus*

Currently, there are at least eight species in *Calamus* that is known to be capable of forming new plants at the apex of inflorescences within this genus; four from the Indochinese region (*C. castaneus*, *C. kampucheensis*, *Calamus parvulus* A.J.Hend. & N.Q.Dung, and *Calamus rhabdocladus* Burret) and four from Sunda region (*C. gaharuensis*, *Calamus ingens* (J.Dransf.) W.J.Baker, *C. pygmaeus*, and *Calamus ruptiloides* A.J.Hend.)

(Dransfield, 1992; Henderson, 2020). While this trait is spread across Southeast Asia, it is relatively uncommon within the genus (Henderson, 2020).

The only other genus within Calameae with this trait is *Salacca* (Henderson, 2009). It is probable that this trait was once more widespread within the genus but has since been out-selected and is now a remnant trait. This trait also shows up only in certain populations or individuals and is not common within the species as in the case of *C. castaneus* and *C. rhabdocladus* possibly as a form of mutation (Ruppert et al., 2012; Henderson, 2020; Mohd Rusdi et al., 2022). *Calamus dianbaiensis* C.F.Wei has a similar trait but instead, roots at the shoot apex after growing vertically up to 10 m, bends over, and roots when the shoot touches the ground to produce new plants (Henderson, 2020). The clustering habit observed in *Calamus javensis* Blume where clusters are established through adventitious roots on the aerial stem that can attach to the soil after the stem falls is very similar to the clustering in *C. pygmaeus*.

While the method of clustering differs, they ultimately create a colony of clones within a certain radius. Genetic testing was not performed in this study, however, the individuals within each colony of *C. pygmaeus* were of the same sex and in some cases the tip of the inflorescence was still attached. Watanabe et al. (2006) suggest the clonality in *C. javensis* is very likely a growth strategy to increase the size of the genet rather than a dispersion-propagation strategy to expand the habitat and that the separate clusters are established by seed recruitment with seed obtained from nearby clusters. Given the highly similar clustering pattern, *C. pygmaeus* most likely also evolved to adopt the same strategy.

3.4.4 Apomixis in *Calamus*

In a previous study, it was revealed that *C. longisetus*, *C. peregrinus*, and *C. rudentum* are not capable of apomixis (Bøgh, 1996) while other members of the family (i.e., *Chamaedorea* Wild., *Howea* Becc., *Attalea funifera* Mart. ex Spreng.), showed reduced, and sometimes no fruit set in bagged pollination experiments (Otero-Arnaiz & Oyama, 2001; Voeks, 2002; Savolainen et al., 2006; Rios et al., 2014). It should be noted that while fruit set has occurred in bagged flowers, it is still possible for pollen transfer to occur by smaller insects that may have slipped through the fine mesh or pollen deposited on the wall of the mesh bag which came in contact with the stigma. Both *C. lobbianus* and *C. pygmaeus* are unlikely to undergo apomixis or at least in a reduced form which is in congruence with the other members of the family. While results from the pollination experiments suggest apomictic behaviour, apomixis is a rare trait within the family Arecaceae and is not widespread unlike in large families such as Asteraceae, Poaceae, and Rosaceae (Hojsgaard et al., 2014).

3.5 Conclusions

Calamus lobbianus and *C. pygmaeus* while similar in phenology, exhibit stark differences in their floral characteristics, and attractants, leading to distinct reproductive strategies. This contrast is directly reflected in the visitor assemblage which subsequently reveals their breeding strategy. While both species belong to the same genus, *C. lobbianus* reproduces mainly through outbreeding sexual reproduction while *C. pygmaeus* adopts a unique reproductive approach, combining sexual, and asexual methods by generating plantlets at the inflorescence tip. While this study doesn't delve into it, exploring the impacts

of these diverse reproductive strategies on population genomics and gene flow, especially in *C. pygmaeus*, could yield intriguing insights.

CHAPTER 4

PHENOLOGY, POLLINATION BIOLOGY AND BREEDING MECHANISMS OF *SALACCA VERMICULARIS*

4.1 Introduction

Several members of the genus *Salacca* are widely cultivated in Southeast Asia for their edible fruit, including *S. affinis*, *Salacca sumatrana* Becc., and *Salacca zalacca* (Gaertn.) Voss. While the fruit of *S. vermicularis* is edible, the taste is rather astringent but is still planted on a smaller scale and enjoyed by local communities (pers. obs. 2023). *Salacca affinis* and *S. zalacca* have been reported to be pollinated by curculionid beetles, trigonid bees, and honeybees (Mogea, 1978; Apriniarti et al., 2019; Atmowidi et al., 2021; Siregar et al., 2021), however the floral biology and pollination of *S. vermicularis* has never been studied likely due to its low commercial value, lack of scientific interest, and difficulty in encountering large flowering population as in many pollination studies. The goal of this study was to investigate the floral biology and pollination mechanism of *S. vermicularis*.

4.1.1 Problem Statement

Apart from the commercially important species of *Salacca* such as *S. affinis*, *S. sumatrana* and, *S. zalacca*, little is known about the pollination biology and phenology of the remaining members of the genus. Understanding of the phenology and pollination biology could provide great implications for the conservation of the remaining members of the genus particularly in *S. vermicularis* which has never been studied prior to this study.

4.1.2 Objectives

The primary objective of this study was to understand the phenology, pollination biology, and breeding mechanisms of *S. vermicularis*. The specific objectives were:

- To determine the phenology and pollination biology of *S. vermicularis*.
- To investigate the breeding mechanism of *S. vermicularis*.

4.2 Methodology

4.2.1 Study Area

This study site was conducted at the foothill of Mount Serapi (1°35'19.64"N, 110°13'12.09"E), approximately 20 km northwest of Kuching, Sarawak, Malaysia. The population of *S. vermicularis* is found between 10 m and 100 m elevation. Mount Serapi is dominated by five types of forest: lowland mixed dipterocarp forest, kerangas forest, submontane forest, alluvial forest, and secondary forest (Pearce & Geri, 2007). Mount Serapi is largely covered with red yellow podzolic soil, whereas peaty soil is frequently found on exposed ridges above 300 m elevation. Dip-slope podzols, which are primarily sandy clay, well-drained, and nutrient-deficient, are also common (Hazebroek & Kashim, 2000). Individuals were sampled in an area of about 2 hectares along the main trail to Mount Matang's Sri Mariamman Temple.

4.2.2 Plant Description

Salacca vermicularis (**Figure 4.1A**) is a massive clustering acaulescent palm growing up to 3.5 m typically found in damp sites in lowland dipterocarp forests and alluvial forests (Dransfield, 1984). The **leaves** have pinnae which are dark green adaxially and powdery grey abaxially (**Figure 4.1C**). The **pinnae** are arranged in groups on either side of the rachis proximally and opposite distally with dark brown and triangular spiny petioles, newer petioles closer to the centre are brown, and older petioles are light green (**Figure 4.1D**). The **inflorescence** is axillary, piercing through the subtending leaf sheath.

The **staminate** inflorescence (**Figure 4.2A**) which emerges from tattering bracts, is up to 1 m long, slender, and erect, with up to three orders of branching with the cylindrical staminate rachilla up to 15 cm long, branches up to 25 rachillae in an inflorescence. The **staminate flowers** are surrounded by golden yellow trichomes (**Figure 4.2C**). Each flower is trimerous, sepal membranous, ca. 3 mm long and 2 mm wide, basally united; petals connate in the basal half, red outside, basally dark pink inside, ca. 2 mm long and 1 mm wide; stamens 6, ca. 1 mm long, filament red, anther yellow ca. 1 mm long with yellow powdery pollen. The **pistillate** inflorescence (**Figure 4.2D**) is typically 28 cm in length including the bracts. The **peduncle** is slender approximately 1 cm in diameter at the base, covered by bracts and branches up to three orders. The pistillate rachilla is up to 15 cm long. The bracts are rather tattered and fibrous which envelopes the whole pistillate rachilla during development and slowly tear open when approaching blooming. There are four to five rachillae per inflorescence. The **pistillate flowers** (**Figure 4.2F**) are trimerous with sepals approximately 17 mm long and 15 mm wide, basally united; petals are connate in the basal half, red outside, basally red inside approximately 12 mm long and 10 mm wide. Each pistillate flower is associated with a sterile male flower which comprises six staminodes; each staminode is



Figure 4.1: A) *Salacca vermicularis*, an acaulescent palm growing up to 3.5 m B) Younger petioles closer to the centre are brown, older petioles are light green C) The pinnae are dark green adaxially and powdery grey abaxially D) dark brown triangular shaped spines in rows of two to four along petiole E) sapling of *S. vermicularis*.

around 5 mm long, red. There are ca. 30 pairs of dyads per rachilla. Fruit bunches may develop at varying heights ranging from 0.1 to 1.5 m on different individuals. **Fruit** globose (Figure 4.2G), 4-5 cm long, 4-5 cm in diameter, gradually narrowed towards the base, covered with scales having upturned in ca. 25 rows. A summary of floral characters for *S. vermicularis* is provided in Table 4.1.

4.2.3 Floral Biology and Floral Visitors

Observations on five individuals (totalling 16 rachillae) of *S. vermicularis* were performed between May 2021 and June 2022. Several days of initial observations were conducted to establish the anthetic cycle; then proceeded by direct observations consisting of four to five hours each day until the full duration of anthesis was completely observed.

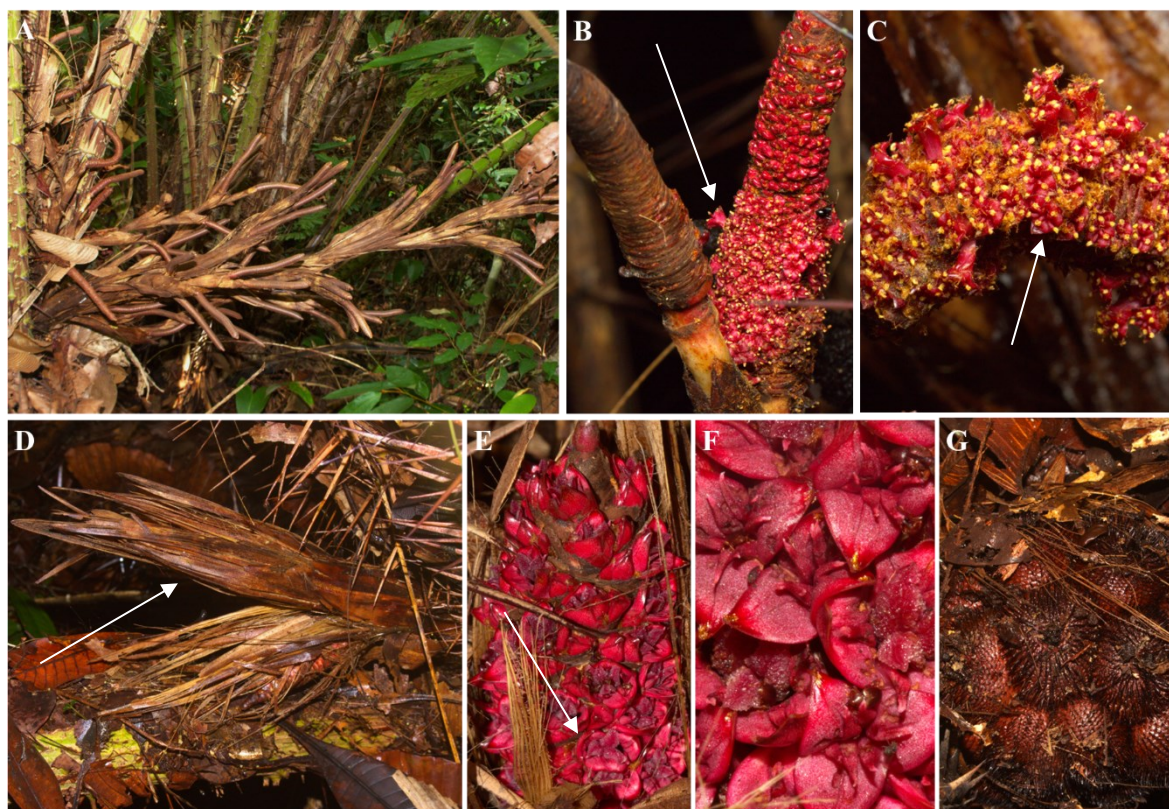


Figure 4.2: A) Staminate inflorescence to 1 m long, slender, and erect, with up to three orders of branching B) Staminate flowers begin blooming from the proximal end of the rachilla C) Staminate flowers with red petals and six anthers surrounded with golden yellow trichomes between flowers D) Pistillate inflorescence enveloped in tattering bracts E) pistillate flowers begin blooming from the proximal end F) flowers are in dyads, pistillate flowers and its associated staminode flower G) matured fruit bunch with fruits covered in long stiff spines.

Table 4.1: Summary of plant habit and floral characters *S. vermicularis* studied.

Characters	
Habit	Solitary
Max stem length (m)	1.5
Vertical height (m)	3.5
Inflorescence length ♀ (cm)	28
Inflorescence length ♂ (m)	1
♀ rachilla length (cm)	15
♂ rachilla length (cm)	15
Colour of petals	Maroon
No. of ♀ rachillae	5
No. of ♂ rachillae	25
No. dyads per ♀ rachilla	60 pistillate flowers

Observations were repeated three times. The values shown represent the average number of visitors on a rachilla. Floral odour was determined through wafting, distance, and scent description. The duration of floral emissions was also recorded.

Behaviour of floral visitors such as interaction with stigma and anther, the average duration of visit, and the mean number of visitors were noted. The number of visitors was counted at two- hour intervals. The floral visitors are determined to be potential pollinators by having the capacity to contribute to pollination via the aforementioned actions. Insect visitors were identified using (Hill & Abang, 2010). Floral visitors were photographed using camera Canon EOS 200D fitted with a 100 mm macro lens for identification. The number of plants observed and the number of inflorescences along with the observational periods for both sexes is shown in Appendix 3.

4.2.4 Floral Availability

Plant individuals bearing remnants of inflorescences and/or infructescence from the previous season were selected. A total of 12 individuals (seven staminate and five pistillate) were included in this study. The sex ratio at two levels (the number of individuals and the number of individuals that flowered during the study period, 15 March 2022 until 23 July 2022) was determined and tested for sex-ratio bias using binomial tests. The operational sex ratio (OSR) is defined as the ratio of fertile males to females (Wehbe & Shackelford, 2020).

4.2.5 Pollination Experiments

The pollination experiments comprised of Bagged Untreated (T 1)- rachillae were bagged three to four days before blooming until the end of anthesis; Open Pollination (T 2)- pistillate flowers were left exposed to permit unhindered animal interactions. Bagging was done using 0.5 mm mesh bags. Two to three rachillae per individual were selected per treatment. Sampling duration and voucher codes are provided in Appendix 4.

4.2.6 Seed Dispersal Agents

Two camera traps (model: Reconyx Hyperfire 2) were deployed at sites (± 2.5 m from a fruiting plant) between 14 March 2022 and 15 July 2022. Active camera days were used to calculate the effort or the total number of camera days where the: Total camera days (TCD) = $\sum c_i d_i$ (1) where c is the active camera operating within a site and d is the number of days (Mohd-Azlan & Engkamat, 2013). The cameras were mounted approximately 30-100 cm from the ground on a tree depending on the height of infructescence with every camera set to capture three-burst shots with no interval after each trigger a 10-second video for each detection, and a 10-second video at 30-minute intervals. Cameras were checked every three to four days for signs of feeding on the fruits and to replace with fresh batteries and memory cards where needed. Images were sorted and identified to a generic level. Animals, where the species could not be determined owing to poor angle or low image quality were not used in the analysis. Animal dispersers were identified using guides provided by Phillipps and Phillipps (2020).

4.3 Results

4.3.1 Flowering Phenology

Several staminate rachillae of an inflorescence may begin anthesis at the same time, however, blooming typically begins from the proximal rachilla towards the younger rachilla at the distal end of the inflorescence following an acropetal development. The rachillae turn from pinkish-white to reddish-brown as they mature. All the flowers within each rachilla bloom within one to two days; flowers at the proximal half of the rachilla bloom first followed by the flowers at the distal half on the next day. The blooming of an inflorescence may take up to 40 days from the opening of the first flower depending on the number of rachillae present, with typically one to two rachillae bloom(s) per day.

The staminate flowers require around 70 days from bud initiation to mature and be ready to bloom. The anthesis of the staminate flowers (**Figure 4.3**) starts at 1200 (Day 1); by 1300 (Day 1), all the flowers are blooming (in a smaller rachilla) ca. 20 mins following the opening of the calyx. The flowers begin to dehisce around 0400 (Day 2) and by 0600 (Day 2) but on rare occasions by 0800 (Day 2), all the flowers dehisced. A very light sweet odour was detectable up to 5 cm away along the rachillae from around 1300 (Day 1) until 0300 (Day 2). The strength and profile remained the same (perceivable through wafting) throughout the emission.

Floral maturation and blooming from the proximal end of the pistillate rachilla towards the distal end following acropetal development. As with the staminate inflorescences, the pistillate rachillae appears pinkish-white during early development to reddish-brown as it matures. As the rachilla matures, the dark red floral buds begin to appear from the bracts at the distal portion of the rachilla. Although flowering begins from the distal

end of the rachilla, the subsequent flowerings begin flowering in a less orderly sequence. All the flowers within each rachilla finish blooming within 24 hours with the whole inflorescence taking up to 15 days depending on the number of rachillae. The pistillate flowers require around 60 days from bud initiation to maturity. *Salacca vermicularis* does not appear to produce any nectar; however, it does produce a thin layer of stigmatic fluid that is present throughout the anthetic period of the pistillate flower and pollen has been observed to adhere to it.

Pistillate anthesis begins at 1700 (Day 1) when the calyx opens to reveal the petals and stigma. All the pistillate flowers in a rachillae will bloom by 0600 (Day 3) when the stigma has fully emerged and recurved. The pistillate flowers were observed to bloom for 20 hours, sometimes up to 40 hours in larger rachillae. The stigma begins to dry around 2000 (Day 3) and by 1000 (Day 4), most flowers have dried up. The sterile male flower blooms within 24 hours after the pistillate flower has fully bloomed, typically around 0800 a day after all pistillate flowers begin anthesis. A very light sweet odour was detectable up to 5 cm away along the rachillae and was present around 0800 (Day 3) until 1500 (Day 3). The strength and profile remained the same (perceivable through wafting) throughout the emission. An infructescence takes ca. 35 days to mature.

4.3.2 Floral Availability

The availability of staminate and pistillate inflorescence is presented in **Figure 4.4**. Flowering appears aseasonal throughout the study duration. The sex ratio of flowering individuals was not significantly unbiased between male and female individuals. However, based on the OSR generated (**Table 4.2**), there is a male bias in flowering for *S. vermicularis* during the three months of observations.

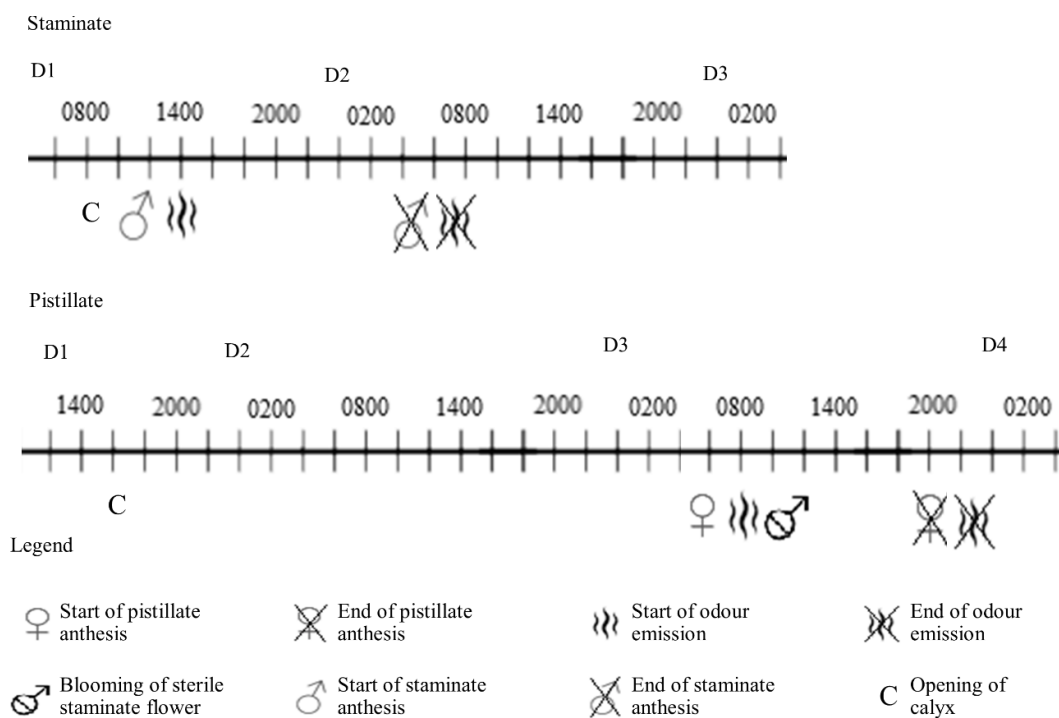


Figure 4.3: A general timeline of phenological events of *S. vermicularis*.

	March			April				May				June					July		
	W3	W4	W5	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4	W5	W1	W2	W3
SLCA001M																			
SLCA002M																			
SLCA003M																			
SLCA007M																			
SLCA008M																			
SLCA019M																			
SLCA010M																			
SLCA004F																			
SLCA005F																			
SLCA006F																			
SLCA011F																			
SLCA012F																			

Figure 4.4: A general timeline of phenological events of *S. vermicularis*.

4.3.3 Floral Visitors

In the staminate flowers, weevils (Curculionidae) (**Figure 4.5A**) arrive first at the start of anthesis at 1200 (Day 1) (**Figure 4.6**) followed by the floral mites (**Figure 4.5A**)

sometimes observed latching onto the weevils. Whenever a weevil stays stationary around a flower, the floral mites would latch onto the carapace of the weevils and would remain attached as the weevils depart. The weevils were also observed to feed on the petals, but the

Table 4.2: Flowering percentage (F %) is the total number of flowering individuals out of total number of individuals expressed in percentage, χ^2 test results to verify if there is any significant bias towards either staminate or pistillate flowering (χ^2 value, P-value where $\alpha \leq 0.05$) and the operational sex ratio (OSR) expressed as the ratio of flowering staminate to pistillate plants (staminate: pistillate) during a particular month.

		F %	χ^2 , P-value	OSR
October	Staminate	41.18	1.974, 0.740	7:2
	Pistillate	16.67		
November	Staminate	23.53	0.202, 0.995	2:1
	Pistillate	16.67		
December	Staminate	52.94	3.932, 0.415	9:2
	Pistillate	16.67		

damage can be considered minimal. Stingless bees, *Heterotrigona* sp. (**Figure 4.5B**) also visit the staminate flowers around 1230 (Day 1). The stingless bees mainly collect pollen. Sciarid flies (Sciaridae) (**Figure 4.5C**) were also seen visiting the staminate flowers from around 1230 (Day 1) till 1900 (Day 1) with similar behaviours as the weevils, however, the sciarid flies were not observed to carry any floral mites. All the floral visitors would remain until floral dehiscence which is around 0400 (Day 2) except for the stingless bees which stop visiting the flowers around 1830 (Day 1). Ants were found to be present throughout the anthesis (**Figure 4.5F**).

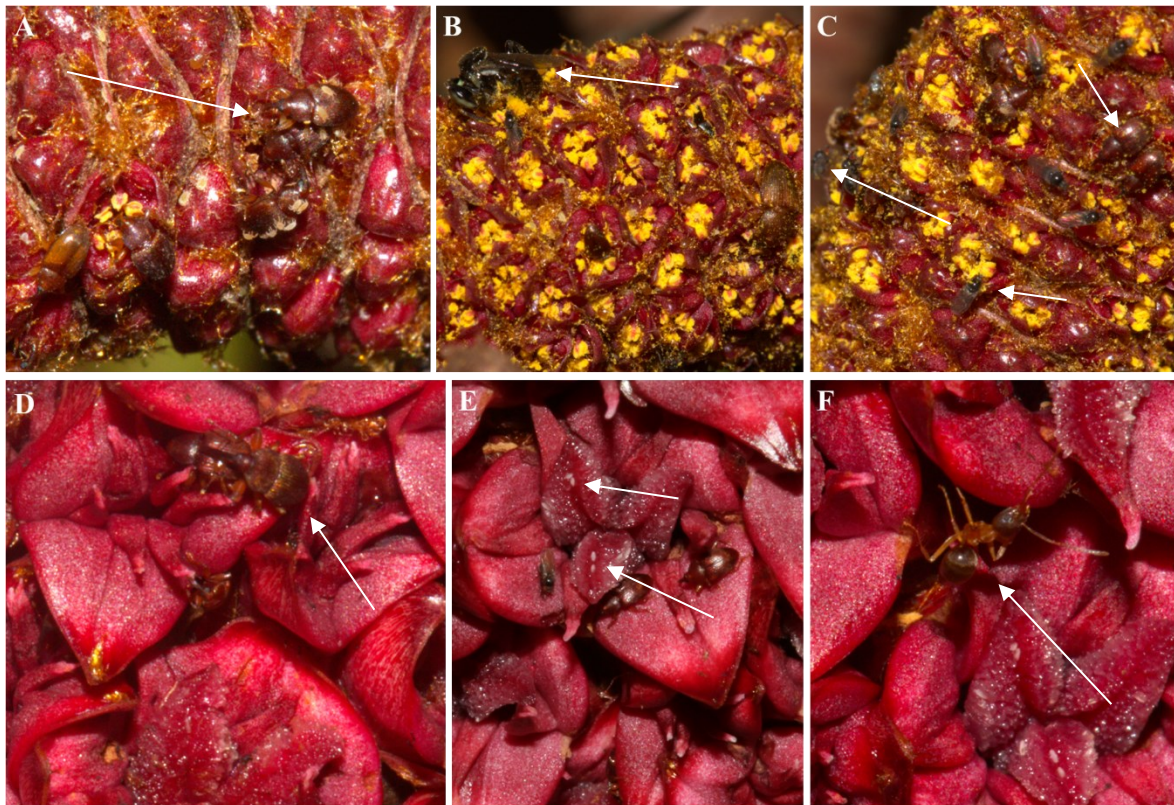


Figure 4.5: A) Floral mites attached to the back of weevils upon arrival at the beginning of the blooming for staminate flowers B) Stingless bee covered in pollen during visit C) Fruit flies, sciarid flies, weevils, and floral mites visiting around the same time on staminate flowers D) Weevils mating on the pistillate flowers E) White weevil eggs deposited around pistillate flowers (indicated by arrows) F) Ants are omnipresent on the flowers.

Table 4.3: Flowering percentage (F %) is the total number of flowering individuals out of total number of individuals expressed in percentage, χ^2 test results to verify if there is any significant bias towards either staminate or pistillate flowering (χ^2 value, P-value where $\alpha \leq 0.05$) and the operational sex ratio (OSR) expressed as the ratio of flowering staminate to pistillate plants (staminate: pistillate) during a particular month.

	♂	♀
Flower mites		
Visiting hours	1200 (Day 1)-0400 (Day 2)	
Mean no. of visitors/hr	25(58)77	
Average duration of visit	48 mins	
Stingless Bees <i>Heterotrigona</i> sp.		
Visiting hours	1230 (Day 1)-1830 (Day 1)	
Mean no. of visitors/hr	2(4)7	
Average duration of visit	3.5 secs	
Weevils (Curculionidae)		

Table 4.3 continued

Visiting hours	1200 (Day 1)-0400 (Day 2)	1500 (Day 2)-1700 (Day 3)
Mean no. of visitors/hr	15(23)27	13(20)25
Average duration of visit	34 mins	39 mins
Sciarid flies (Sciaridae)		
Visiting hours	1230 (Day 1)-1900 (Day 1)	1500 (Day 2)-1900 (Day 3)
Mean no. of visitors/hr	15(19)24	29(36)41
Average duration of visit	16 mins	11 mins
Fruit flies (<i>Drosophila</i> sp.)		
Visiting hours		1500 (Day 2)-1900 (Day 3)
Mean no. of visitors/hr		3(6)11
Average duration of visit		3 mins
Ants (Formicidae)		
Visiting hours	1200 (Day 1)-0400 (Day 2)	1500 (Day 2)-1900 (Day 3)
Mean no. of visitors/hr	11(14)21	6(12)18
Average duration of visit	1 min	2 mins

For pistillate flowers, the weevils arrive first at the start of anthesis around 1500 (Day 2) and remain till 1900 (Day 2). The weevils reappear at around 0600 (Day 3) till 1700 (Day 3). The weevils were observed to move constantly in and out of the flowers, particularly towards the base of the stigma to consume the thin layer of sticky stigmatic fluid. The long snout of the weevil appears to be very efficient in consuming the floral exudate. The weevils were also observed to mate on several occasions (Figure 4.5D) and white substances presumed to be eggs have also been observed (Figure 4.5E). The same feeding behaviour on the petals observed in staminate flowers was also observed. Sciarid flies were also seen regularly visiting the pistillate flowers with the same visiting hours as the weevils on both Day 2 and Day 3. The visiting behaviours of the sciarid flies were very similar to the weevils where the sciarid flies were constantly visiting the base of the flowers. Fruit flies (*Drosophila* sp.) (Figure 4.5C) were observed to visit starting from 1500 (Day 2) towards 1700 (Day 2) and resumed visit from (0900) Day 3 to 1900 (Day 3). It is unsure what attracted the fruit flies as the fruit flies do not seem to head towards the stigma the same way as the weevils

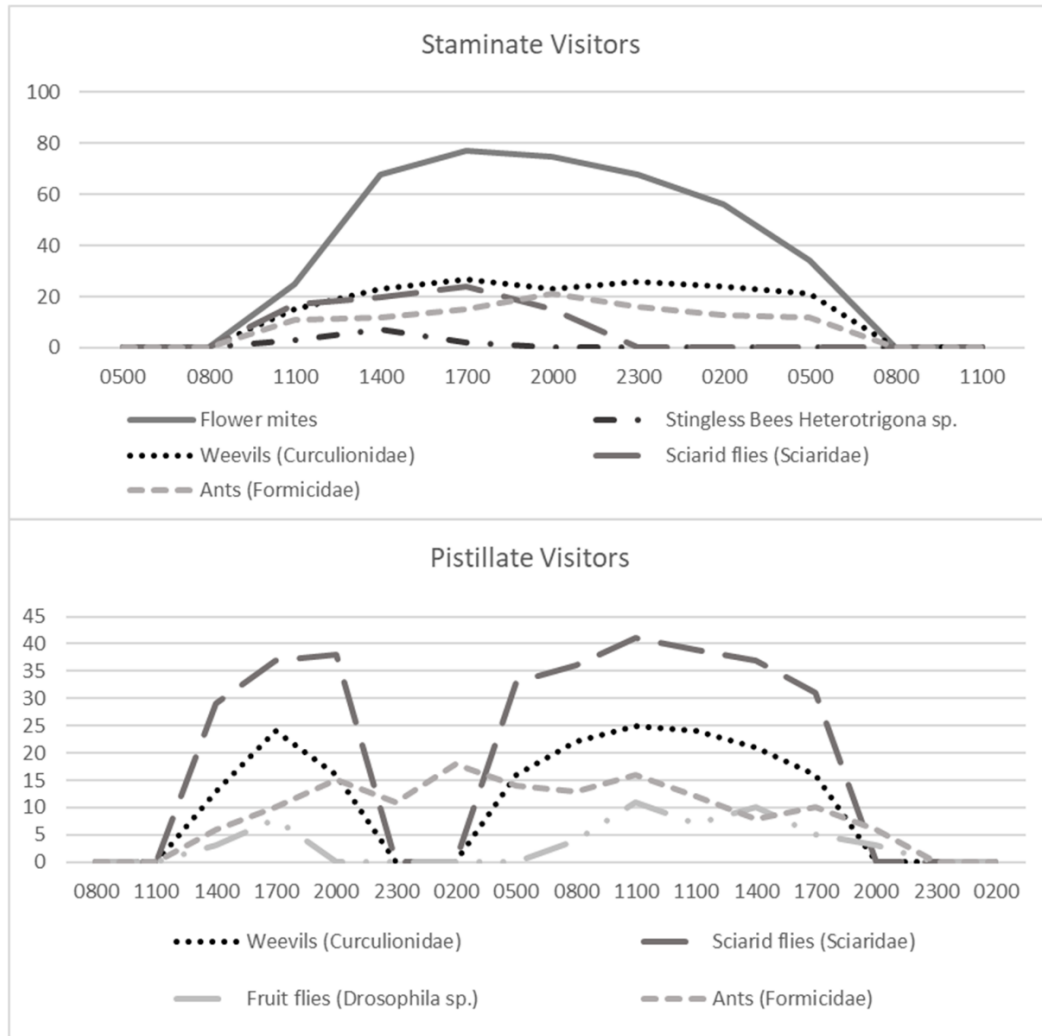


Figure 4.6: Average number for each species of visitor throughout visiting duration.

and sciarid flies. The weevils and sciarid flies are the most likely to be contributing towards pollination as both visitors were observed on the staminate plants and do indeed come into contact with the stigma regularly during visits. Curiously, no mites were observed on the weevils visiting the pistillate flowers. Ants were found to be present throughout the anthesis, feeding on the stigmatic fluid (however, they do not appear to carry any pollen during visits to pistillate flowers).

4.3.4 Breeding Mechanisms

Treatment 1 gave a 0.00 % fruit set for all replicates while treatment 2 showed 89.24%, 92.09%, and 87.77% for SLCA004F, SLCA005F, and SLCA007F (**Table 4.4**). There is a significant difference between both treatments for all replicates. No apomixis occurs in *S. vermicularis*.

Table 4.4: Results from pollination experiments; T 1=bagged flower, T 2=free unbagged; Chi- square and the P value are calculated between results from T 1 and T 2.

	T 1	Fruit Set	T 2	Fruit Set	χ^2	P value
SLCA004F	0/129	0.00%	141/158	89.24%	226.30	<0.000001
SLCA005F	0/162	0.00%	128/139	92.09%	259.56	<0.000001
SLCA007F	0/143	0.00%	122/139	87.77%	221.21	<0.000001

4.3.5 Seed Dispersal Agents

Asian porcupine (*Hystrix* sp.) (**Figure 4.7A**), Mousedeer (*Tragulus* sp.) (**Figure 4.7C**), and Three- striped ground squirrel (*Lariscus insignis*) (**Figure 4.7D**) are likely the dispersal agents of *S. vermicularis*. A mongoose (Herpestidae) (**Figure 4.7B**) was recorded foraging around but is unlikely to be a dispersal agent owing to its diet. All visitors were only captured on film once except for *Tragulus* sp. (**Table 5**). The total camera days (TCD) was 226 days. Events are defined as visual confirmation on the camera trap whereas evidence of feeding is defined as presence of fruit scraps (**Figure 4.7E**). Post-feeding seed viability tests were not conducted as no faecal samples were collected.

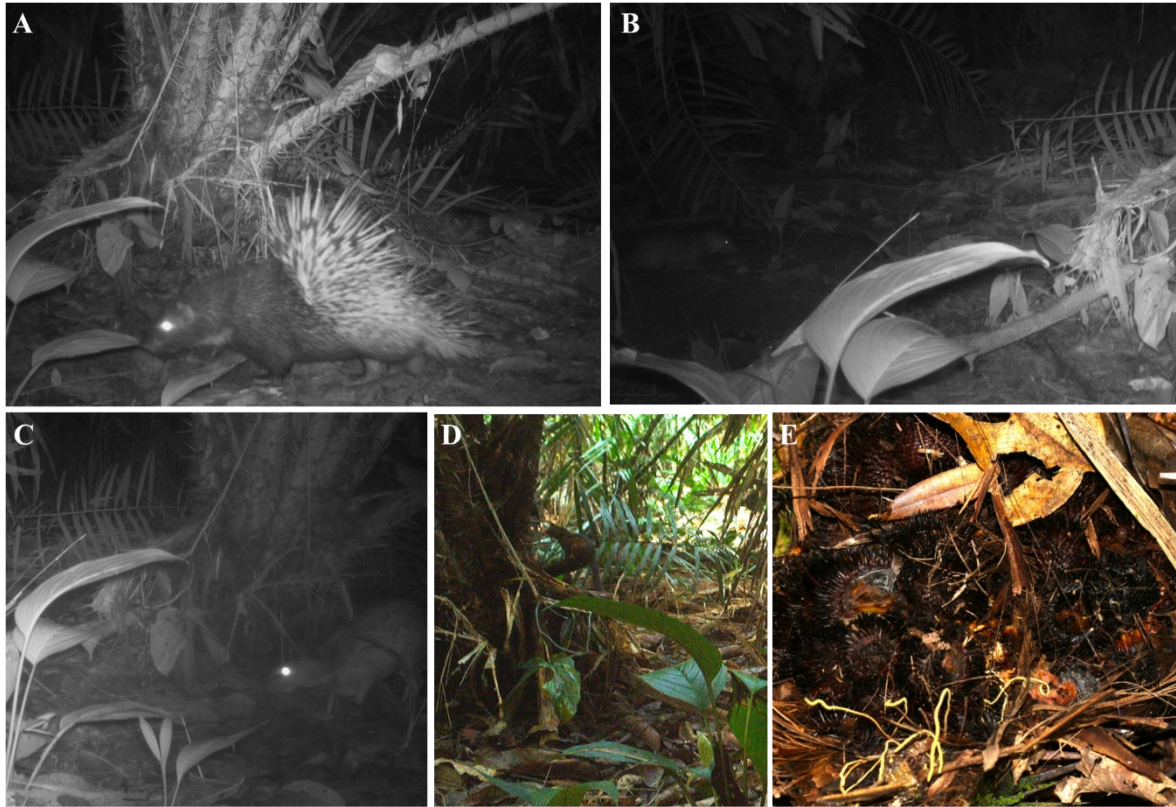


Figure 4.7: A) Asian porcupine (*Hystrix* sp.) B) mongoose (Herpestidae) C) mousedeer (*Tragulus* sp.) D) Three-striped ground squirrel (*Lariscus insignis*) E) Evidence of foraging on the fruits by animal visitors.

Table 4.5: Animal visitors captured on the camera trap from March to June 2022.

	Events	Time of Day Observed	Evidence of feeding
<i>Lariscus insignis</i>	1	1724	Yes
<i>Tragulus</i> sp.	2	0323	Yes
		0224	No
<i>Hystrix</i> sp	1	0232	Yes
Herpestidae	1	0207	No
Sciuridae	1	1134	Yes

4.4 Discussion

4.4.1 Floral Attractants and Visitor Assemblage

Floral visitor assemblage in *Salacca* has been reported to be rather diverse and comprises several orders which include Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera. However, the pollinators are mainly Curculionid beetles (Coleoptera), stingless bees (Apidae), and vespid wasps (Vespidae) (Mogea, 1978; Fisher & Mogea, 1980; Dransfield et al., 2008; Apriniarti et al., 2019; Atmowidi et al., 2021; Siregar et al., 2021). Weevils and stingless bees are among the most reported pollinators for several *Salacca* such as *S. affinis*, and *S. zalacca* (Mogea, 1978; Dransfield et al., 2008; Apriniarti et al., 2019; Atmowidi et al., 2021; Siregar et al., 2021). The presence of sciarid flies and floral mites has yet to be reported as visitors in *Salacca*.

The curculionid beetles have chewing mouthparts and are known to chew on floral parts during their visits, in some of the beetle-pollinated palms have been recorded to have specialised floral parts that allow the beetles to forage without damaging or overconsuming the pollen during visits (Uhl & Moore, 1977; Faegri & van der Pijl, 1979). This behaviour was observed in *S. vermicularis*, as the fleshy petals provide an additional food source for the visiting beetles in preparation for mating. Apart from a food source in the form of fleshy petals, pollen, and stigmatic fluid, the inflorescence of *S. vermicularis* also serves as a form of shelter for the beetles to complete their life cycle by serving as a hatchery for the laid eggs which has also been previously observed in other cantharophilous palms (Barfod et al., 2011).

Although the staminate flowers of *S. vermicularis* were successful in attracting stingless bees, curiously they were not observed to visit the pistillate flowers during the

duration of this study. The contrasting colour of the inflorescence against the darker background coupled with the sweet scent should be ideal for attracting stingless bees as potential pollinators (Kidyoo & McKey, 2012), however, perhaps something is still missing in the pistillate flowers as stingless bees are considered generalist pollinators for this family and maybe poor pollinators (Barfod et al., 2011). There have been several attempts to search for a relationship between floral character and phenology with associated pollinators in palms.

According to Dransfield et al. (2008), beetle pollination is generally associated with protogyny and anthesis in the whole inflorescence occurring over a relatively short period and with the inflorescence usually rather condensed or bearing crowded flowers. By contrast bee pollination seems to be associated more with protandry, anthesis over an extended period, and with the inflorescence laxly branched, or with the flowers individually rather distant. The previously mentioned phenologies does bear some resemblance to *S. vermicularis* in the sense that both the staminate and pistillate flowers are indeed crowded on the rachillae, but the flowering period begins earlier in staminate flowers and flowers marginally longer than the pistillate flowers. Henderson (2002) came to a similar conclusion that beetle-pollinated inflorescences tend to have condensed, unisexual flowers, and enclosed in bracts at anthesis, while bees, flies, and wasps-pollinated inflorescences have elongated, often bisexual flowers, and not enclosed in bracts at anthesis.

The inflorescences of *S. vermicularis* can be rather showy with the large number of flowers within a rachilla, contrasting scarlet red petals against the dark brown colour of the petiolar base and the sweet floral scent. While this species does not produce any nectar, the stigmatic fluid is likely to serve as a form of floral attractant similar to nectar. The

relationship between the floral attractants and rewards against the visitor assemblage of *S. vermicularis* suggests a degree of specificity towards cantharophily but also attracts a generalist assemblage of visitors with varying potential as pollinators. It is important to consider that the visitor assemblage may be dynamic and change due to external factors. Barfod et al. (2011) noted that certain groups such as the bees are opportunists while certain weevils are highly specialised and that it has also been shown that the composition of insect visitors varies through space and time. However, the consequence of these changes may be challenging to detect as it requires long periods of observation and data collection.

4.4.2 Sex Ratio in *Salacca vermicularis*

Flowering occurs year-round in *S. vermicularis* where ideally, there should always be staminate and pistillate flowers available. However, pistillate plants bloom less frequently compared to staminate individuals. It is worth noting that the sample size is small (ten staminate plants and six pistillate plants) owing to the difficulty in encountering large flowering populations. In the case of *S. vermicularis*, the slight male-biased scenario ensures a consistent supply of pollen. Staminate plants frequently flower, which likely allows visitors to consistently visit staminate plants, carrying pollen regardless of whether they visit pistillate plants of the same species. This, in turn, increases the likelihood of depositing pollen onto the receptive stigmas of pistillate flowers when they are available.

As floral reward and fruit production can be reward intensive, there is a greater female bias in species with abiotic pollination and seed dispersal, whereas male-biased sex ratios were associated with biotic pollen and seed dispersal (Sinclair et al., 2011; Field et al., 2013). This is consistent with the reproductive strategy of *S. vermicularis* being slightly

male-biased, biotic pollen dispersal, and relying on animals for seed dispersal. In a study across 47 species of angiosperms, it was found that 46% of the species are male-biased (Barrett et al., 2010). Barrett et al. (2010) also pointed to the earlier onset of male flowering, higher frequency of flowering in males, and higher gender-specific mortality in females could be contributing factors to male bias.

4.4.3 Apomixis in *Salacca*

Salacca vermicularis does not undergo apomixis, however apomixis does occur in *Salacca* albeit rare, as in the rest of the Arecaceae (Hojsgaard et al., 2014). One example of apomictic behaviour in *Salacca* is found in *S. zalacca* (Hutauruk, 1999). Among the Calamoids, (Bøgh, 1996) reported that *C. longisetus*, *C. peregrinus*, and *C. rudentum* are not capable of apomixis while among the rest of the family (i.e. *Attalea funifera* Mart. ex Spreng. *Chamaedorea* Wild. *Howea* Becc.), showed little to no fruit set in breeding mechanism experiments for apomixis tests (Otero-Arnaiz & Oyama, 2001; Voeks, 2002; Savolainen et al., 2006; Rios et al., 2014).

4.4.4 Seed Dispersal and Predation

A great number of palms are adapted for animal dispersal; for example, most members have fleshy mesocarp or a well-developed sarcotesta surrounding the seed and brightly coloured epicarps that are attractive to vertebrates (Dransfield et al., 2008). The varying height of fruit bunches in *S. vermicularis* allows a wider range of animal visitors to have ease of access to the fruits. This feature becomes evident when looking at the three

species of fruit visitors of different heights feeding on different fruit bunches of varying heights with ease. Studies on the exact feeding behaviours of *Tragulus* sp. and *Hystrix* sp. in the wild are still limited to determine the efficacy of their roles as seed dispersers in Arecaceae. However, both species are known to select similar habitats as the occurrence of *S. vermicularis* and are known to feed on forest fruits (Matsubayashi & Sukor, 2005; Taher et al., 2018; Farida et al., 2019; Lemos de Figueiredo et al., 2021). Evidence of scatter-hoarding in *Lariscus insignis* on fruits of *Elais guineensis* observed by Yasuda et al. (2000) suggests it could be an effective seed disperser and reported that *L. insignis* may carry their food cache up to 32 m from the food source with only ca. 19% eaten making this species the most likely to be an effective seed disperser among the three recorded. It is likely the scatter hoarding behaviour similar to *L. insignis* is applicable to the visitors of *S. vermicularis*.

4.5 Conclusions

Staminate flowers began blooming around noon and mostly dehisces by early morning around sunrise the following day while pistillate flowers begin blooming in the early evening, completely blooms by early morning near sunrise on the third day and ends around morning of the fourth day. The floral characters of *S. vermicularis* suggest a primarily outbreeding cantharophilous species intrinsically tied to the life cycle of curculionid beetles, a relationship observed in many species within the family which potentially places this species in a larger plant-pollinator interconnecting framework. Additionally, *S. vermicularis* could potentially be considered as a candidate to be planted in areas of conservation for frugivorous animals as it may provide a steady supply of food sources for members of *Tragulus*, *Hystrix*, and possibly *Muntiacus*.

CHAPTER 5

VEGETATIVE REPRODUCTION AND CLUSTERING IN *CALAMUS PYGMAEUS*

5.1 Introduction

Asexual reproduction exists and is widespread in *Calamus*, however only eight species in the genus is currently known to form plantlets at the apex of the inflorescence with it being an uncommon trait within the genus. This ability to utilise the reproductive organ to undergo asexual reproduction in *C. pygmaeus* may not comply to our understanding of the ecology of clonal plants which largely focuses on clonal plants utilising energy storage and translocation organs such as rhizomes and stolons.

However, the ramets of *C. pygmaeus* are no longer connected to the parents or each other shortly after rooting of the plantlets. This lack of resource translocation would mean a major loss in the benefits of clonal reproduction if compared to most other clonal plant species. This difference in clonal reproduction is also evident when we look at the growth rate of *C. pygmaeus* plantlets. Typically, with the ability to translocate resources between parent and ramet, the ramet may have a steady supply of resources to facilitate the increase in biomass thus increasing survivability of younger ramets. However, in *C. pygmaeus*, the inflorescence typically reaches senescence shortly after the rooting of the plantlets thus cutting off the ability to translocate resources to aid growth. This difference in clonal strategy is thus likely the reason why this form of clonal growth is not more widespread within the genus.

Thus, we decided to investigate if the different clusters of *C. pygmaeus* in Kubah National Park (KNP) are truly ramets and that if the cloning strategy may prove as a viable

growth strategy. To aid in our investigation of its population structure, the method Genotyping by Sequencing (GBS) was used to identify the single nucleotide polymorphism (SNP) within the genome of the samples.

This study utilised two approaches to analyze the population structure of the *C. pygmaeus* observed with one being phylogenetic analysis and the other being Discriminant Analysis of Principle Components (DAPC). The phylogenetic analysis allowed us to not only see how the clusters form but also the natural variation within the subpopulations. The Discriminant Analysis of Principle Components is a multivariate method that does not rely on a particular genetic model with its primary use being identifying and describing clusters of genetically related individuals (Jombart, 2008). These two methods identified clusters with the determination of the relationship among the clusters and the genetic structure within.

5.1.1 Problem Statement

The population of *C. pygmaeus* observed typically contains individuals of the same sex within each population. This observation coupled with the ability to root a plantlet at the apex of the inflorescence leads to the possibility that each subpopulation encountered are a genet containing ramets with the same genetic information. This ability of *C. pygmaeus* to root at the apex of the inflorescence has been previously observed (Dransfield, 1992; Dransfield et al., 2008) however, nothing further has been discussed on its clustering ability and formation of ramets.

5.1.2 Objectives

The primary objective of this study was to understand the population structure of the observed *C. pygmaeus*. The specific objectives were:

- To determine if the observed population of *C. pygmaeus* forms clusters of genetic clones through phylogenetic analysis using SNPs as marker.
- To investigate whether natural variations exist within the population of *C. pygmaeus*.

5.2 Methodology

5.2.1 Study Area

This study was conducted at KNP (1°36'45.51"N, 110°11'48.52"E) which is approximately 20 km northwest of Kuching, Sarawak, Malaysia. Population of *C. pygmaeus* occurs between 200–500 m elev. Kubah National Park comprises largely five vegetation types: lowland mixed dipterocarp forest, kerangas forest, submontane forest, alluvial forest, and secondary forest (Pearce & Geri, 2007). The diversity of palm species in KNP can be attributed to the diverse habitats brought upon by the different rock types, topography, and microhabitats. (Hazebroek & Kashim, 2000).

5.2.2 Sampling Methods

Plant individuals were sampled on both sides along the main trail and Selang trail (**Figure 5.1**). Individuals from 10 clusters of the population were sampled in this study. A group of plants are considered an individual cluster if they are at least 20 m from the next

closest cluster. The sampling was done randomly within each cluster of the population. The number of samples taken from each cluster of population was determined by the total number of individuals where three to five samples were collected from populations with ≤ 10 individuals, ~ 10 samples from populations with ≤ 20 individuals and ~ 15 from ≥ 30 individuals. The aim was to represent at least 40% of the total number of individuals within each cluster. The sampling locations, the number of individuals sampled, and the number of total individuals in each cluster of population are detailed in Appendix 5.

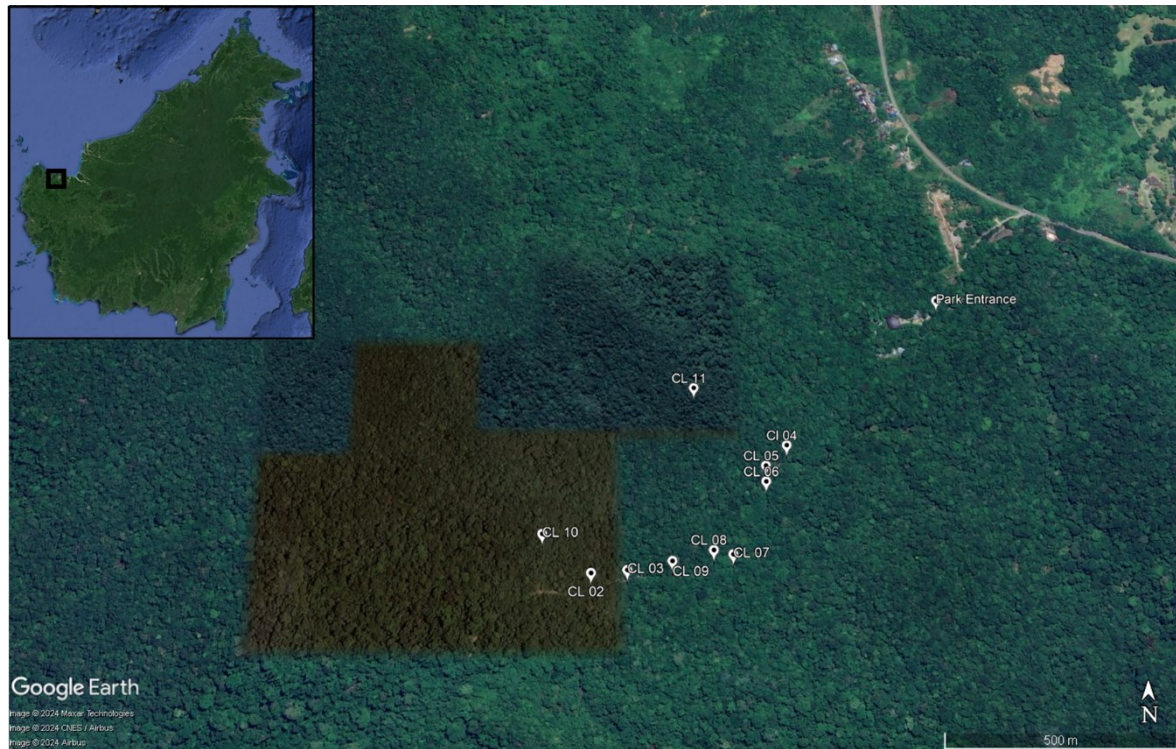


Figure 5.1: Distribution map of the clusters of population sampled for this study. The location of sampling is in Kubah National Park, Sarawak, Malaysia.

5.2.3 DNA Extraction

Total genomic deoxyribonucleic acid (DNA) was extracted from ca. 150 mg of frozen leaf samples for all accessions using DNeasy Plant Pro Kit (Qiagen) according to the manufacturer's protocol. The samples were frozen to -80 °C and lysed using Qiagen Tissue LT according to manufacturer's manual. The extracted genomic DNA was then purified using NucleoSpin gDNA Clean-up by Macherey-Nagel according to the manufacturer's protocol. The purified genomic DNA was then diluted to at least 30 ng/μl concentration with at least 20 μl in volume before being sent for sequencing. 70 samples were sent for next generation sequencing. The quality (A260/A280) of DNA was verified using SpectraMax QuickDrop Micro-Volume Spectrophotometer by Molecular Devices (accepted range was between 1.80 to 2.0) and gel electrophoresis for further visual inspection. The concentration of DNA was verified using Quantus Fluorometer by Promega.

5.2.4 Library Preparation and Sequencing

Genotyping by sequencing was performed on the genomic DNA to obtain the SNPs and insertion and deletion (InDel) sites. The library preparation, sequencing, and variant calling were outsourced to a bioinformatics company Apical Scientific Sdn Bhd. with project ID: NGS14838. The genomic DNA of each sample was digested with restriction enzyme MseI & NlaIII, generating insert size of 240-265 bp. The obtained fragments were ligated with two barcoded adapters that are either with a compatible sticky end with the primary digestion enzyme or Illumina P5 and P7 universal sequence. Followed by several rounds PCR amplification, all the samples were pooled and size-selected for the required fragments

to complete the library construction. Sequencing was performed on the Illumina NovaSeq-PE150 platform.

5.2.5 Sequence Processing

The original image data generated by the sequencing machine were converted into sequence data via base calling (Illumina pipeline CASAVA v1.8.2) and then subjected to quality control (QC) procedure to remove unusable reads. The reads containing the Illumina library construction adapters and contamination, reads containing more than 10% unknown bases (N bases) and reads containing more than 50% of low-quality bases (sequencing quality value ≤ 5) were removed. Sequencing reads were aligned to the reference genome using BWA version 0.7.8-r455 (Li & Durbin, 2009) with parameters ‘mem -t 4 -k 32 -M’. Subsequent processing, including duplicate removal was performed using SAMtools version 0.1.19-44428cd (Danecek et al., 2021). The genome of *Calamus simplicifolius* (Zhao et al., 2018) was selected as reference genome for read mapping.

Variant calling of SNPs and Indels was performed using SAMtools (Danecek et al., 2021) with the parameters as ‘mpileup -m 2 -F 0.002 -d 1000’ with the output stored as VCF files. The resulting SNPs were filtered with the following criteria, reads with mapping quality of less than 20 and reads with number of supports for each SNP that is less than five were removed. The resulting SNPs and InDels were annotated using ANNOVAR version 2013Aug23 (Wang et al., 2010). Individual VCF files were first sorted using BCFtools version 1.10 (Danecek et al., 2021), then compressed using bgzip. The compressed files were then indexed and merged using BCFtools (Danecek et al., 2021). The data from VCF files of SNPs and InDels were visualized using functions create.chromR and chromoqc from vcfr

(Knaus & Grunwald, 2017) package in R version 2023.12.1 Build 402 (R Core Team, 2024) to provide a quick visual overview of the variant data. The visualization includes read-depth, mapping quality, Phred score, and variant count per window.

5.2.6 Phylogenetic Analysis

The SNP data in VCF file format was first converted into PHYLIP format using the Python script `vcf2phyli.py` version 2.0 (Edgardo, 2019). Maximum Parsimony (MP) tree with 1000 bootstraps using the Subtree-Pruning-Regrafting (SPR) search method was generated using MEGA version 11.0.13 (Kumar et al., 2018). Maximum likelihood (ML) tree was generated using RAxML-NG version 1.2.0 (Kozlov et al., 2019). The files were first parsed which checks for file settings and count for alignment patterns using command `'raxml-ng --parse --msa prim.phy --model GTR+G --prefix T2'`. The parsing step reduced tree generation time. Two maximum likelihood trees were generated, first using the nucleotide substitution model GTR with Gamma variation and the second one with the same parameters as above but with 200 bootstraps with commands `'raxml-ng --msa prim.phy --model GTR+G --prefix T3 --threads 2 --seed 2'` and `'raxml-ng --bootstrap --msa prim.phy --model GTR+G --prefix T8 --seed 2 --threads 2 --bs-trees 200'` respectively.

A distance file was first created in R using function `vcf2dist` from package `fasttreeR` (Gkanogiannis, 2024) for the InDel data which calculates a distance matrix between the samples of a VCF file and performs Hierarchical Clustering on this distance matrix. A phylogenetic tree is then calculated with agglomerative Neighbour Joining method (NJ) (complete linkage). The resulting tree is then converted from "character" class to "phylo" class which is subsequently converted into a Newick format file using functions `read.tree`

and `write.tree` from package `ape` (Paradis et al., 2004) respectively. The generated trees are then viewed and edited on TreeViewer (Bianchini & Sanchez-Baracaldo, 2024).

5.2.7 Discriminant Analysis of Principal Components (DAPC)

The number of principal component and number of clusters to retain was obtained using the `find.cluster` function from the `adeigenet` package (Jombart, 2008) in R to identify and describe clusters within the population using the genetic relationships of the data. Genetic clusters were identified using *k*-means, a clustering algorithm which identifies groups (*k*) by maximizing the variation between groups. The lowest Bayesian Information Criterion (BIC) is then utilised as a means to be compared with *k*-means ran sequentially with increasing values of *k*. The optimal clustering solution corresponds to the lowest BIC value indicated by an elbow curve of BIC values as a function on *k*. 70 principal components (PCs) was retained while 10 clusters were selected for the `find.cluster` function. The DAPC analysis is performed using `dapc` function from the `adeigenet` package in R. 30 principal components (PCs) was retained, 10 clusters were selected, and nine discriminant analysis eigenvalues were used for the `dapc` function to obtain a scatter plot. Discriminant analysis was used on the clusters identified in `find.clusters` to describe the relationship between the clusters. Membership probability was analysed based on the retained discriminant functions in the `dapc` object using the `assignplot` function in the `adeigenet` package. Group memberships was used as an indicator of how clear-cut the genetic clusters obtained are and to infer admixed or misclassified individuals.

5.3 Results

5.3.1 Sequencing Data Analysis

The statistics of sequencing data and quality checks are available in Appendix 6. In total, 41.961 G of raw data from 70 samples were sequenced, with 41.949 G of clean data generated after filtering for low-quality data. What happened to the other samples. Need to mention here. The raw data production for each sample ranged from 397.291 M to 793.514 M, indicating the sufficient amount of data production. As the Q20 and Q30 reached 94.87 % and 86.91 %, respectively, the sequencing quality met the analysis requirements. The GC content of 35.89 % to 41.09 % are also in the normal distribution range, fulfilling the quality standard. The library construction and sequencing procedures were successful and is suitable for downstream processing.

The mapping rates of samples reflect the similarity between each sample and the reference genome. The depth and coverage are indicators of the evenness and homology with the reference genome. With GBS sequencing, tag-related statistics are also calculated. The mapping rate of each sample ranges from 74.08% to 90.09%. The average depth on the reference genome (without Ns) is in 9.72X to 17.76X range, while the more than 1X coverage exceeds 1.55%. The mapping statistics with the reference genome and tag summary are provided in Appendix 7.

5.3.2 SNP and InDel Data

The SNP and InDel detection and annotation data generated from SAMtools and ANNOVAR are in Appendix 8 and Appendix 9 respectively. In total, 31,123,555 raw SNPs were generated from all 70 samples with 713,460 unique SNP patterns identified by RAxML

from 4,429 contigs read in vcfR. In total 2,191,537 raw InDels were generated from all 70 samples from 3892 contigs read in vcfR. Some basic statistics such as depth of each read which represents the number of reads that align each base position in the reference sequence, mapping quality of each read which indicates alignment confidence to a particular region of the reference genome, Phred quality score (Q) of each base call where Q is logarithmically related to the base calling error probabilities and variant count per window where the graph represents the number of variants identified in each window of the genome (the window is a fixed length segment of the genome) for the SNP and InDel data are presented in **Figure 5.2** and **Figure 5.3** respectively.

5.3.3 Phylogenetic Analysis

No outgroup was selected for this analysis as the purpose of this study is to identify clusters and to potentially look at the intra-species diversity thus selecting an outgroup that may be genetically too distant and the compositional heterogeneity may affect the placement of the root and incur erroneous SNPs (Borowiec et al., 2019; Jamil et al., 2019). Most samples from Cluster 7, Cluster 8, Cluster 9, Cluster 10 and Cluster 11 formed distinct clades with high support in MP tree (**Figure 5.4**) and ML tree (**Figure 5.5**) for the SNP dataset. The InDel dataset also exhibit similar clades with the NJ tree (**Figure 5.6**). Members from Cluster 2, Cluster 3, Cluster 4, Cluster 5, and Cluster 6 did not form distinctive clades in any of the trees generated. Sample 0403 formed a very distant branch in the ML and NJ phylograms. The MP and ML from the SNP data both grouped 59% and 60% of the samples into clusters observed in the field respectively while the neighbour joining tree from the InDel tree grouped 56% of the samples into clusters observed in the field. In all the trees

generated, a similar pattern was observed where Cluster 7 and Cluster 8 would form sister clades while Cluster 10 and 11 would form another sister clade.

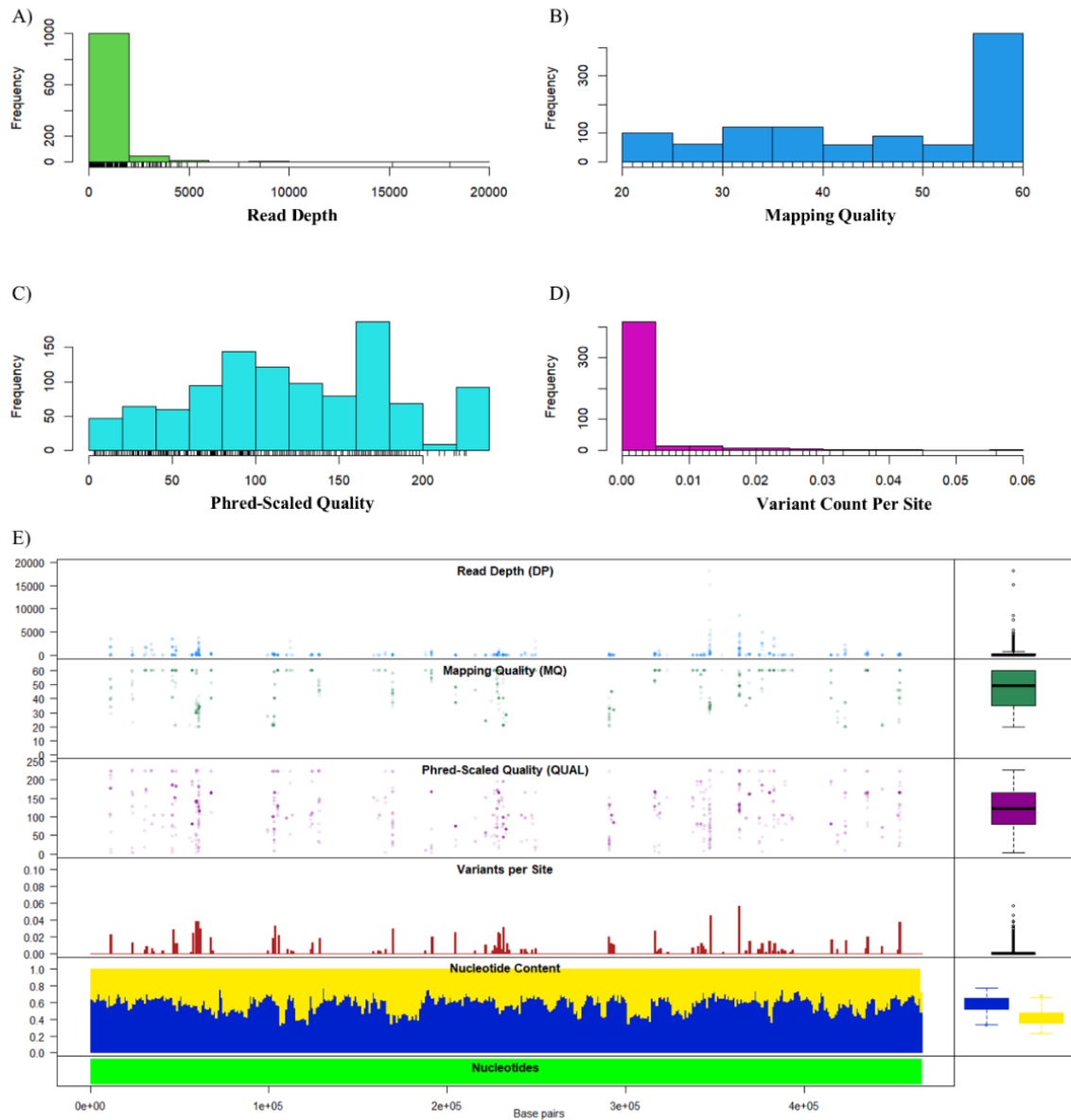


Figure 5.2: SNP statistics A) Read depth graph representing the number of reads that align to each base of the reference genome B) Mapping quality graph presenting mapping quality of each read to the reference genome C) Phred score quality graph presenting quality of each base call D) Variant count per window graph presenting the number of SNPs identified in each window of the genome E) Visualization of read depth, mapping quality, Phred score quality and variant count plots in relation to the reference genome

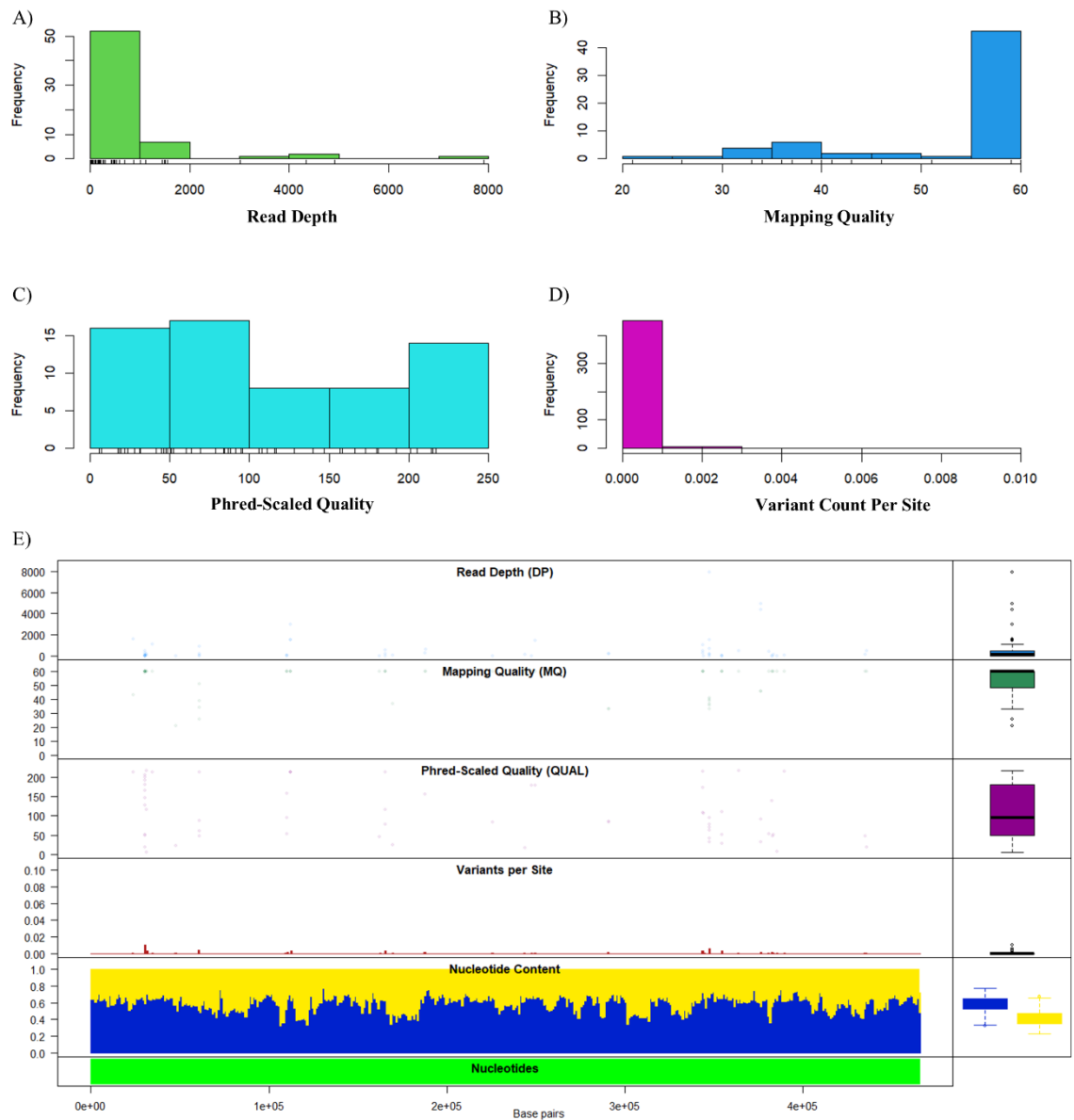


Figure 5.3: InDel statistics A) Read depth graph representing the number of reads that align to each base of the reference genome B) Mapping quality graph presenting mapping quality of each read to the reference genome C) Phred score quality graph presenting quality of each base call D) Variant count per window graph presenting the number of InDels identified in each window of the genome E) Visualization of read depth, mapping quality, Phred score quality and variant count plots in relation to the reference genome

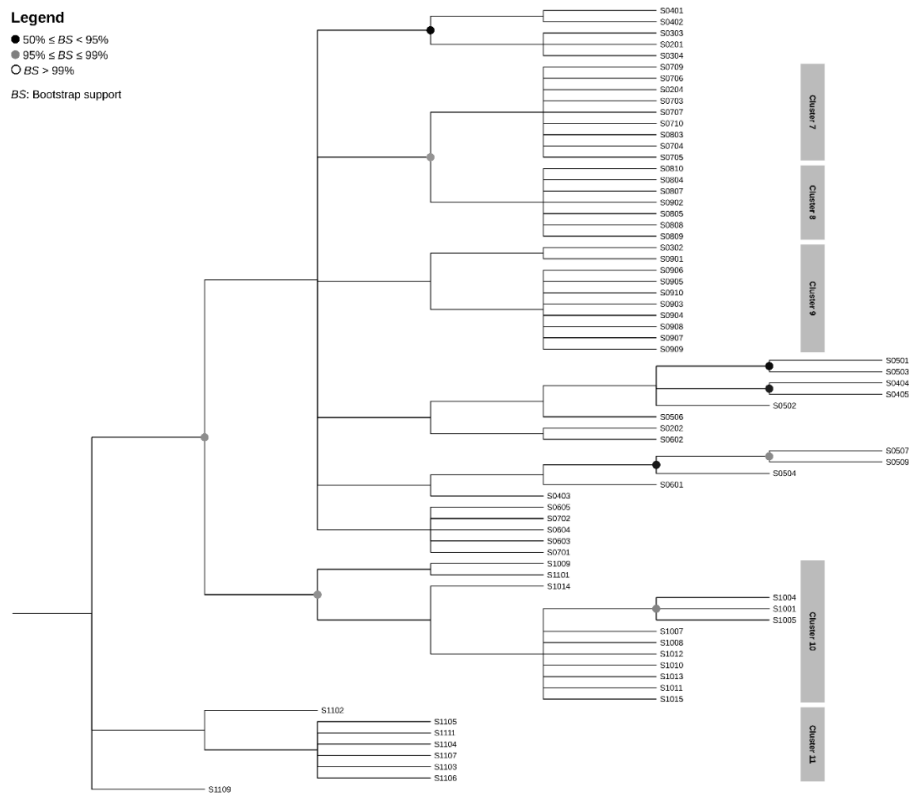


Figure 5.4: Cladogram of Maximum Parsimony with 1000 bootstraps for SNP data. Nodes with support values lower than 50 were collapsed at the leaves. Nodes containing more than three individuals of the same clusters were labeled.

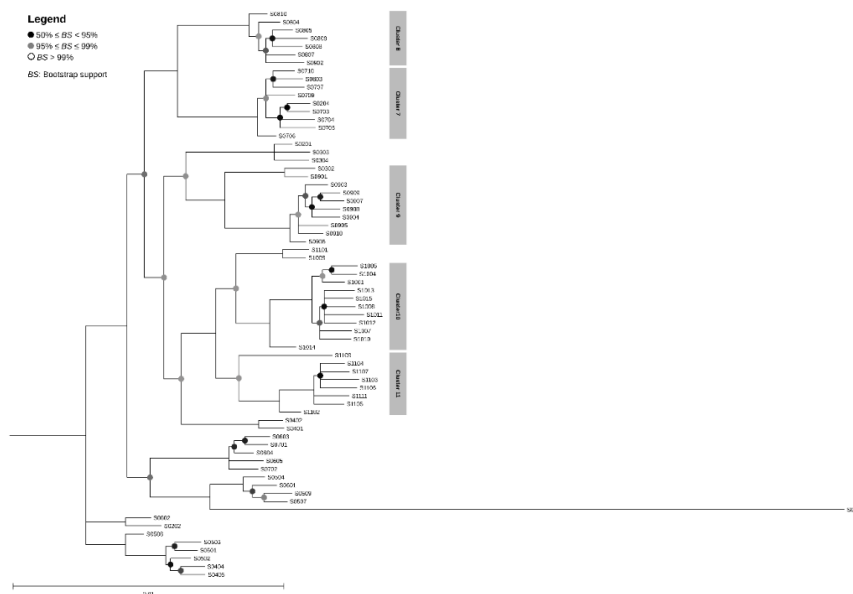


Figure 5.5: Phylogram constructed using Maximum Likelihood with 200 bootstraps for SNP data. Nodes with support values lower than 50 were collapsed at the leaves. Nodes containing more than three individuals of the same clusters were labeled.

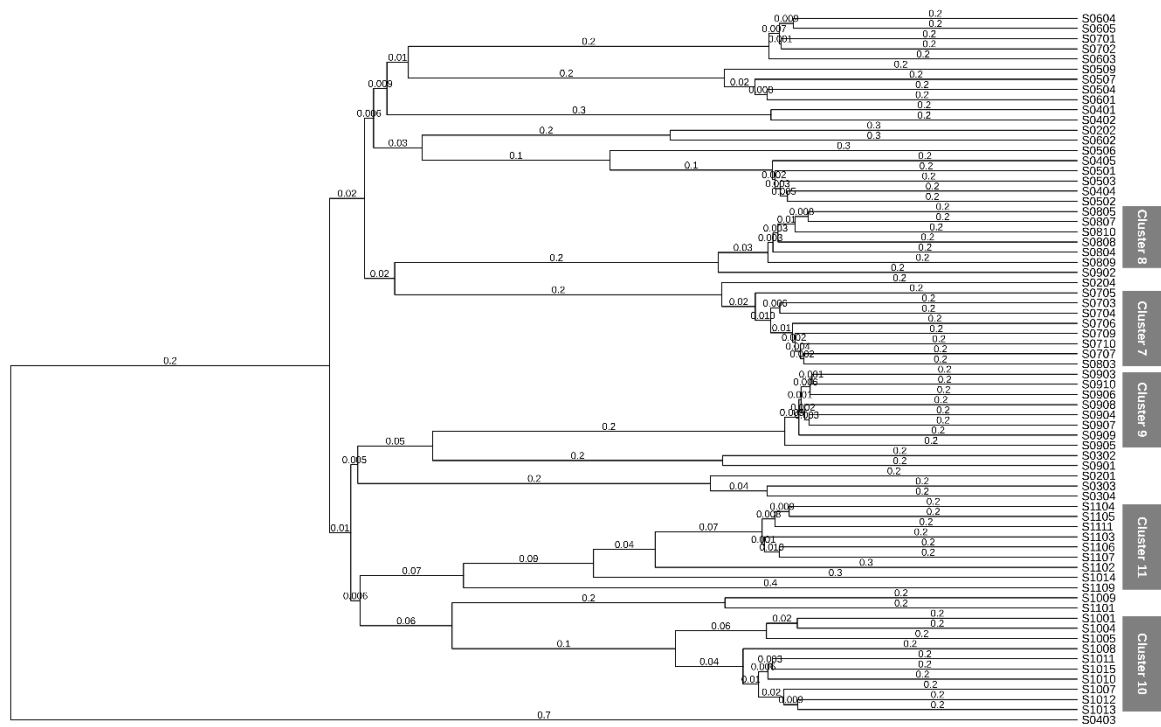


Figure 5.6: Phylogram constructed using hierarchical clustered agglomerative neighbor joining for InDel data. Nodes with support values lower than 50 were collapsed at the leaves. Nodes containing more than three individuals of the same clusters were labeled.

5.3.4 Discriminant Analysis of Principal Components

We can verify the how successful `find.cluster` is in identifying the groups by comparing the inferred groups based on the different values of k , the selected number of clusters and the associated BIC, the group memberships and the group sizes. The resulting table suggests that `find.cluster` (**Figure 5.7**) was moderately successful in identifying the groups by comparing the inferred groups (`infr`) versus the original groups (`ori`) via the presence of a diagonal pattern from the top left to bottom right while some clusters not on the diagonal line indicates misclassification.

The discriminant analysis of principal components (DAPC) assigned 10 groups to the 70 samples (**Figure 5.8**), the groupings formed several clusters that is 64% (Appendix

10) in congruent with the clusters observed in the field. Seven of the groups are clustered towards the origin suggesting high genetic similarity which is expected as they are meant to be the same species. Group 6 which contain only sample S0403 is placed the furthest away from the rest of the clusters indicates unique genetic diversity. Group 5 which mostly contain samples of Cluster 11 observed from the field and Group 8 which contains samples S0302 and S0901 also suggests unique genetic variations. The samples within each group are projected onto each other suggests very low genetic variation within each group based on the SNP data.

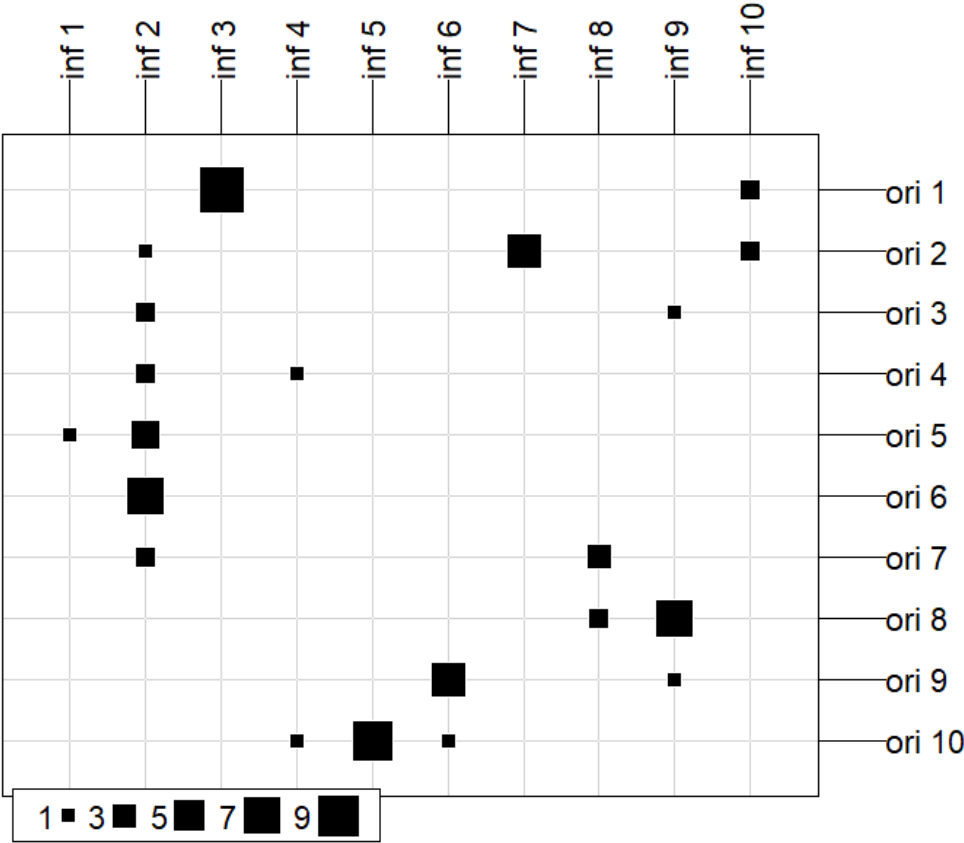


Figure 5.7: A table showing the successfulness of find.cluster in inferring groups via comparison with the original groups. The x-axis represents inferred groups (inf 1 to inf 10), and the y-axis represents original groups (ori 1 to ori 10). The black squares' intensity indicates the count of data points classified into each combination of original and inferred groups.

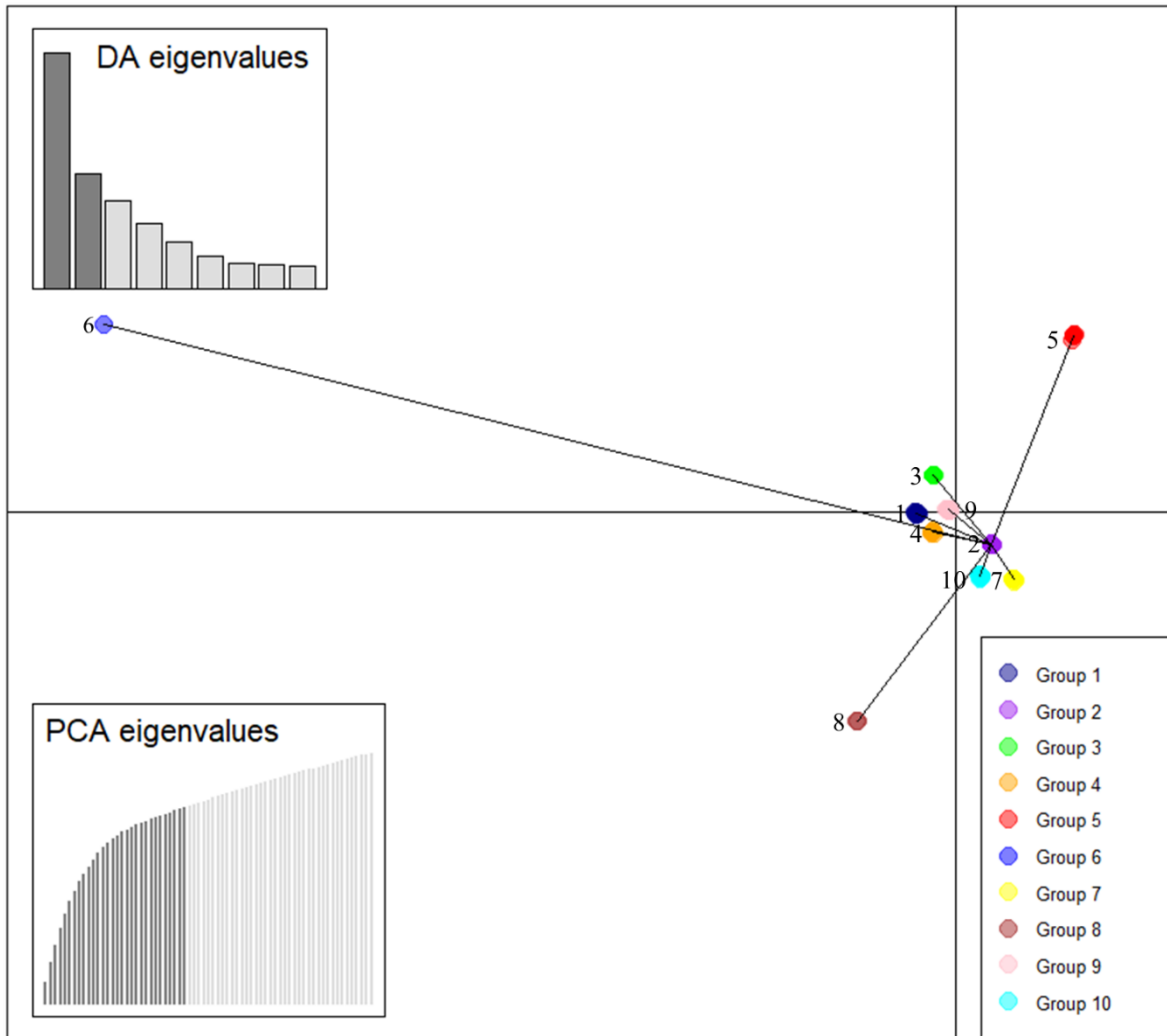


Figure 5.8: Scatter plot from a discriminant analysis of principal components (DAPC). Each group indicates different subpopulation identified by DAPC. The groups contain multiple dots overlapping onto each other due to low genetic variation within the grouping.

The proportion of the successful reassignments based on the discriminant functions of individuals to their original clusters. Clear-cut groupings are indicated by large values while admixed groups are indicated by lower values. The DAPC classifications were consistent to the original groupings indicating no admixed or misclassified individuals (Figure 5.9).

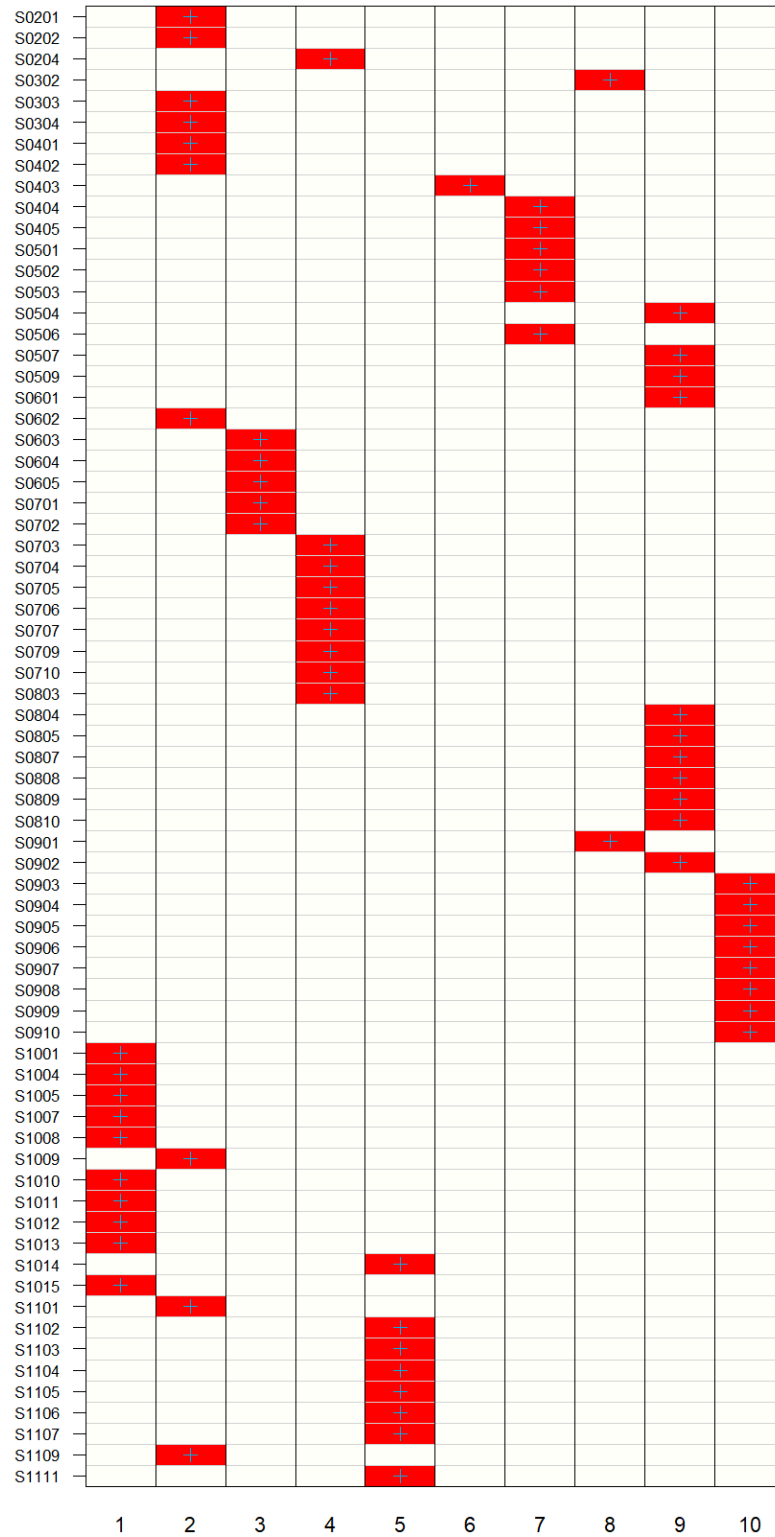


Figure 5.9: Graphical visualization of the information from the DAPC object indicating proportions of succesful reassignment based on the discriminant functions of individuals to their original groups. Heat colors represent membership probabilities (red=1, white=0); blue crosses represent the prior cluster provided to DAPC.

5.4 Discussion

5.4.1 Genetic Clones Within Population of *C. pygmaeus*

The results from the phylogenetic analysis and DAPC analysis suggest that there are subpopulations consisting of genetic clones within the sampled population. Approximately 60% of the sampled population falls into different clusters in both analysis, this tells us that while there are indeed genetic clones forming subpopulations, natural variation indeed exists in certain subpopulations. As previously discussed in Chapter 3, it is highly likely that the different subpopulations are initially formed from seed requirements and later on forms these clusters of subpopulations which would consists of genetic clones' due to the natural ability to undergo asexual reproduction. The natural variation observed can likely be explained by multiple seeds being deposited within the same area during initial seed recruitment. While the subpopulations observed was considered to have the same gender among the individuals, it should be noted that the gender confirmation was through visual inspection on the flowering individuals, gender of sterile individuals were not identified. Gender identification through SNP analysis was not performed due to lack of annotation during variant calling.

The individuals that did not fall into the expected clusters based on both the analysis incidentally were from subpopulations that were less than 15 individuals except subpopulation 5 which had 21 individuals while the individuals that were clustered into different groups in both analyses had number of individuals greater than 25. This difference suggests that it is possible that the natural variation observed in a subpopulation is due to initial seed recruitment from multiple seeds and overtime one particular individual with higher vigour began producing genetic clones with high fecundity that later on dominates the subpopulation eventually causing the subpopulation to end up as one distinct genet as a form of natural selection favouring the genetics of the most fecund individual. Individuals

recruited in favourable locations can potentially have a larger biomass allocation to tissues that are associated with resource uptake (You et al., 2014), this can potentially imply establishment of genets can occur with more ease in locations with higher existing resources to support vegetative growth. Clonality in this case helps in the maintaining and expanding the existing subpopulations while sexual reproduction aids in the recruitment of new genets while still maintaining the genetic diversity (Vallejo-Marín et al., 2010). This habit would be highly similar to the growth strategy of *C. javensis* where clonality presents as a growth strategy to increase the genet size rather than a dispersion-propagation strategy in habitat expansion (Watanabe et al., 2006).

5.4.2 Breeding Strategy of *C. pygmaeus*

In the phylogenetic analysis above Cluster 7 and 8 along with Clusters 10 and 11 form sister clades which showed lower genetic diversity between the two pairs of clusters. This information when combined with their location **Figure 5.1** showed that it is likely that they have a short seed dispersal distance. The shorter seed dispersal distance plays well into the narrative that *C. pygmaeus* uses its asexual reproductive ability to complement its sexual reproduction as a unique breeding strategy. However, if we assume *C. pygmaeus* favours asexual reproduction over sexual reproduction, this can potentially explain the endemism of this species to KNP and its immediate environs as the form of asexual reproduction in *C. pygmaeus* drastically limits its dispersal ability.

While the endemism may initially appear as an evolutionary disadvantage, the ability to establish genets greatly increases the survivability of the genetic variation among the subpopulations and ultimately the species. This breeding strategy also possibly creates a

functional genetic immortality of the most fecund genetic clones. In rare cases, clonality in populations may lead to loss of sexual reproduction and genetic uniformity particularly in species with smaller range (Vallejo-Marín et al., 2010), however this is unlikely based on our current data or at least the subpopulations sampled. Lack of sexual recruitment is typically indicated by dominance of a few large clonal subpopulations (Honnay & Jacquemyn, 2008), however, that is not the case within the subpopulations sampled as indicated by multiple distinct clonal subpopulations even within a small area.

5.4.3 Resource Translocation of the Ramets

In most clonal plants, clonal growth typically involves a large turnover of tissues during the plant lifespan (Herben et al., 2014). Such turnover would incur both energetic and ecological costs, however the inability to translocate valuable resources to its ramets in *C. pygmaeus* brings several important implications on the breeding strategy. The lack of resource translocation would mean that the plantlets resulting from asexual reproduction would need to have functional root and leaves before the senescence of the inflorescence to ensure survivability (Herben et al., 2014) as the plantlets no longer experience the typical benefits of clonal plant growth. This feature is seen when the leaves are the first to develop in the apex of the inflorescence quickly followed by the roots. In some cases, the plantlets can develop mid-air and only touching the ground when they get heavy enough. While they do not receive additional resource to aid growth, the plantlets are fully capable of surviving well before the inflorescence reach senescence. This ability and the fact that the inflorescence is typically 2.5 m long meant that the plantlets do not experience resource competition with its parent plant compared to other forms of vegetative reproduction where

the plantlet remain attached or very close to the parent plant (Mariotte et al., 2012; Saiz et al., 2016).

5.5 Conclusions

The subpopulations sampled are found to consist of genetic clones forming genets with distinct genetic makeup. However, natural variations occur in certain subpopulations particularly in subpopulations with lower number of individuals. The subpopulations begin with initial seed recruitment and in subpopulations with natural variations, it is likely that the variation stems from multiple seeds being deposited within the same location. The genets began to form from the most fecund individual out of the initial seed recruitment as a form of natural selection. Further studies should be conducted to identify the genes responsible for production of plantlets as this could potentially be utilised to create a form of vegetative propagation that removes the typical disadvantages that comes with below ground vegetative propagation.

CHAPTER 6

GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Understanding of floral biology and reproductive strategies of *C. lobbianus*, *C. pygmaeus*, and *S. vermicularis* provides great insight into the complex plant-pollinator network plant biology of Calameae. This information is much needed as Calameae as a whole is still very much understudied despite their commercial importance. *Calamus* and *Salacca* represents groups on the opposite ends of the phylogenetic tree of Calameae, thus this study shed light on the different approach towards their breeding strategy by evolving different floral biology. In *Calamus* flowers are sparser on the rachilla while *Salacca* evolved tightly packed flowers on the rachilla and attracted different groups of insect visitors.

In both species of *Calamus*, anthesis began in the early morning for pistillate and staminate plants, concluding by late noon (staminate plants) but extending till the next day in pistillate plants for both species. Anthesis in staminate flowers of *S. vermicularis* began around noon and persisted until early morning on the second day whilst anthesis in pistillate flowers began around evening and took up to 40 hours to fully bloom. Several insect visitors were observed to be the primary visitors of both *Calamus* species which include two species of *Tetragonula*, a species of *Liostenogaster* sp., and *Stenodyneriellus* sp. In *S. vermicularis*, several insect visitors were observed, namely *Heterotrigona* (Apidae) sp. as well as members of Curculionidae, Sciaridae, Formicidae, and Acaridae. Three species of visitors are likely the dispersal agents of *S. vermicularis*, namely *Hystrix* sp. (Hystriidae: Hystriinae), *Tragulus* sp. (Tragulidae) and *Lariscus insignis* (Sciuridae: Callosciurinae). A mongoose (Herspetidae) was observed foraging nearby. Phylogenetic and DAPC analyses concluded

that the subpopulations of *C. pygmaeus* sampled consist of genetic clones forming genets with distinct genetic makeup between subpopulations, with natural variations occurring in certain subpopulations, particularly those with a lower number of individuals.

A great highlight of this study is the ability of *C. pygmaeus* to form plantlets at the end of the inflorescence providing a highly unique way of asexual reproduction even among Arecaceae which opens up new possibilities in understanding the population genomics of the species.

6.2 Recommendations

Further studies on floral biology, plant-pollinator network on other groups within *Calamus* should be performed as much of it is still unknown to science as it is highly possible that more breeding strategies could be highly unique given the sheer number of members within the genus. The importance of *S. vermicularis* should be highlighted as a possible keystone species to aid in conservation of frugivorous mammals such as *Tragulus*, *Hystrix*, and *Muntiacus*. The genomics of *C. pygmaeus* should also be further studied especially the genes responsible for the unique vegetative reproduction as it may allow exciting possibilities on plant breeding and conservation. Its ability to produce clones that reduce fighting for resources is a great trait to be incorporated possibly with CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology into crops for breeding programmes. It is with great hope that this study will pave the way to further understanding of this great group of plants and to incite the interest of generation to gain better understanding of these palms of Borneo.

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APPENDICES

Appendix 1: Total number of individuals, inflorescences and flowers observed for *C. lobbianus* and *C. pygmaeus*. Code CLMS represents individuals of *C. lobbianus* sampled for nectar volumetric and concentration study (both measurements were taken in tandem).

Species/Code		Form	Individuals	Inflorescences	Flowers	Observational period
<i>Calamus lobbianus</i>	♂	A	3	4	32	29/4/21-8/5/21
	♀	A	2	4	28	3/12/21-26/4/22
<i>Calamus pygmaeus</i>	♂	B	3	4	14	1/4/21-7/5/21
	♀	B	2	3	17	1/4/21-7/5/21
CLMS02M	♂		-	-	16	26/10/21-12/11/21
CLMS03M	♂		-	-	24	3/5/21-10/5/21
CLMS01F	♀		-	-	21	7/12/22-18/12/22
CLMS07F	♀		-	-	19	15/6/22-26/6/22

Appendix 2: Pollination experiments conducted on three separate populations for *C. pygmaeus* and three individuals for *C. lobbianus*.

Species	Pop.	Voucher Code	Unbagged		Bagged	
			Inflor. code	Duration	Inflor. code	Duration
<i>C. pygmaeus</i>	Pop 6	CLMS06F	CT001	17/10/21 - 3/11/21	AP001	3/10/21- 21/11/21
<i>C. pygmaeus</i>	Pop 6	CLMS06F	CT002	3/10/21 - 21/10/21	AP002	6/10/21- 26/10/21
<i>C. pygmaeus</i>	Pop 5	CLMS05F	CT003	15/11/21 -3/11/21	AP003	12/11/21- 3/11/21
<i>C. pygmaeus</i>	Pop 1	CLMS01F	CT004	18/12/21 -6/1/22	AP004	27/11/21- 18/12/21
<i>C. lobbianus</i>	-	CLMS011F	CT005	9/12/21- 21/12/21	AP005	1/5/2022 -19/5/22
<i>C. lobbianus</i>	-	CLMS008F	CT006	13/12/21 - 4/1/22	AP006	2/6/2022 - 9/7/22
<i>C. lobbianus</i>	-	CLMS018F	CT009	18/3/22 – 21/4/22	AP009	26/6/22 - 25/7/22

Appendix 3: Number of plants observed, number of inflorescences observed and observational period for each species on flowering phenology.

	Specimens observed	Total inflorescences observed	Rachillae observed	Observational period
♂	3	3	12	13/5/21-30/6/21
♀	2	2	4	8/3/22 – 30/5/22

Appendix 4: Pollination experiments conducted on *S. vermicularis*.

Voucher Code	Unbagged		Bagged	
	Inflor. code	Duration	Inflor. code	Duration
SLCA004F	CT007	15/3/22 - 16/4/22	AP007	30/5/22 - 7/7/22
SLCA005F	CT008	19/3/22 - 20/4/22	AP008	2/6/22 - 7/7/22
SLCA007F	CT010	22/3/22 - 23/4/22	AP010	20/6/22 - 25/7/22

Appendix 5: Number of clusters, size of clusters (measured in meters (m) at the longest distance), numbers of individuals sampled per cluster, GPS coordinates and elevation (Elev) in meters (m).

Cluster no.	Cluster size (m)	No. individual	Individuals sampled	GPS	Elev. (m)
2	4.01	12	5	1.60686, 110.18952	296
3	6.1	9	5	1.60692, 110.19025	280
4	3.71	10	5	1.60948, 110.19358	220
5	12.7	21	10	1.60905, 110.19313	237
6	3.71	9	5	1.60872, 110.19312	243
7	6.03	34	10	1.60774, 110.19239	263
8	8.95	27	10	1.60732, 110.19200	262
9	14.9	26	10	1.60710, 110.19116	273
10	35.9	38	15	1.60764, 110.18853	319

11	31.2	29	11	1.61071, 110.19168	316
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Appendix 6: Quality check statistics of data sequencing of all samples.

Sample	Raw Base(bp)	Clean Base(bp)	Effective Rate (%)	Error Rate (%)	Q20(%)	Q30(%)	GC Content (%)
S0503	529,561,728	529,410,374	99.97	0.03	97.09	92.06	38.65
S0509	397,290,816	397,132,074	99.96	0.03	95.86	88.81	39.98
S0502	535,143,456	534,997,342	99.97	0.03	96.73	91.12	39.24
S0903	644,787,360	644,640,100	99.98	0.03	97.72	93.38	38.75
S1011	676,321,056	676,102,028	99.97	0.03	98	94.2	41.05
S1101	719,100,576	718,954,772	99.98	0.03	97.31	92.11	40
S0901	667,663,200	667,475,956	99.97	0.03	97.78	93.56	39.14
S0201	714,439,296	714,278,976	99.98	0.03	97.06	91.51	40.27
S1109	666,805,248	666,587,018	99.97	0.03	97.88	93.8	40.9
S0804	531,551,808	531,428,246	99.98	0.03	97.35	92.28	40.45
S1007	756,321,984	756,164,928	99.98	0.03	97.59	92.93	39.91
S0504	464,570,784	464,404,038	99.96	0.03	96.9	91.56	39.31
S0603	455,037,984	454,877,896	99.96	0.03	97.16	92.2	39.59
S0709	557,871,840	557,673,900	99.96	0.03	98	94.21	39.8
S0805	664,292,160	664,134,856	99.98	0.03	96.78	90.85	38.8
S0601	477,465,120	477,310,924	99.97	0.03	95.77	88.6	39.05
S1014	710,701,344	710,494,608	99.97	0.02	98.09	94.45	40.77
S0710	637,761,312	637,554,420	99.97	0.03	97.75	93.38	39.08
S1103	708,818,400	708,586,230	99.97	0.03	97.85	93.69	40.33
S0705	442,429,056	442,233,366	99.96	0.03	96.62	90.91	39.54
S0604	516,891,168	516,736,264	99.97	0.03	96.73	91.05	39.28
S0204	791,701,920	791,489,010	99.97	0.03	97.44	92.43	40.09
S0403	556,797,024	556,607,748	99.97	0.03	96.65	90.96	40.1

S0701	431,210,592	431,087,818	99.97	0.03	97.35	92.75	39.24
S0706	561,577,248	561,399,838	99.97	0.03	97.06	91.54	39.7
S0405	508,856,832	508,720,918	99.97	0.03	95.6	88.19	39.05
S0702	509,647,968	509,475,624	99.97	0.03	96.98	91.72	39.84
S0304	517,379,904	517,247,272	99.97	0.03	96.72	91.16	39.17
S1105	699,122,592	698,907,114	99.97	0.03	98	94.19	40.84
S1001	553,924,224	553,740,330	99.97	0.03	97.84	93.78	39.73
S0810	623,886,336	623,705,712	99.97	0.03	97.72	93.39	39.88
S0303	550,134,720	550,025,898	99.98	0.03	96.53	90.67	38.22
S0501	458,177,760	458,017,166	99.96	0.03	97.41	92.78	39.51
S1008	552,749,184	552,538,828	99.96	0.03	97.66	93.03	41.03
S1010	662,957,568	662,716,978	99.96	0.03	97.87	93.77	41.09
S0904	593,095,392	592,932,066	99.97	0.03	97.57	92.93	39.76
S0906	664,320,384	664,156,930	99.98	0.03	97.72	93.35	39.2
S0404	509,171,328	509,013,222	99.97	0.03	96.62	90.75	39.64
S0703	493,337,088	493,217,154	99.98	0.04	95.31	87.6	39.06
S0808	582,590,304	582,404,640	99.97	0.03	97.3	92.13	40.65
S0803	548,879,904	548,707,036	99.97	0.03	97.69	93.31	39.55
S1005	624,409,632	624,298,768	99.98	0.03	97.69	93.2	37.44
S1104	774,614,016	774,446,852	99.98	0.03	97.28	91.97	39.36
S1106	601,674,336	601,475,638	99.97	0.02	98.17	94.63	41.06
S0704	470,724,480	470,571,136	99.97	0.03	96.21	89.76	39.22
S0506	548,650,656	548,535,446	99.98	0.03	96.47	90.43	38.03
S0302	793,514,016	793,397,206	99.99	0.03	95.1	87.28	38.73
S1015	683,706,240	683,465,684	99.96	0.03	97.98	94.07	41.09
S0605	486,418,752	486,278,236	99.97	0.03	96.71	91.03	39.31
S1009	650,909,952	650,683,630	99.97	0.03	97.58	92.82	40.88
S0602	490,480,992	490,323,374	99.97	0.03	96.61	90.82	39.52
S1013	726,576,192	726,386,182	99.97	0.03	97.84	93.72	40.49

S0907	547,801,920	547,668,458	99.98	0.03	97.69	93.23	38.46
S0809	554,694,624	554,579,842	99.98	0.04	94.87	86.91	39.18
S0902	619,560,288	619,464,254	99.98	0.03	97.54	92.94	35.89
S0905	575,085,888	574,960,296	99.98	0.03	97.75	93.52	38.88
S1004	566,942,688	566,766,296	99.97	0.03	97.81	93.64	39.24
S1111	693,348,768	693,164,954	99.97	0.03	97.5	92.58	39.19
S1102	744,874,272	744,641,018	99.97	0.03	97.72	93.24	40.75
S0401	524,132,928	523,973,552	99.97	0.03	96.42	90.31	39.56
S0908	627,788,736	627,620,988	99.97	0.03	97.22	91.93	39.16
S1107	712,645,920	712,426,000	99.97	0.03	97.93	93.86	41.05
S0909	631,201,536	631,015,870	99.97	0.03	98.03	94.19	39.64
S0507	459,224,640	459,129,724	99.98	0.03	95.99	89.16	38.27
S0807	704,297,664	704,141,988	99.98	0.03	97.62	93.09	38.5
S0910	666,232,416	666,062,960	99.97	0.03	97.78	93.46	39.39
S0202	757,998,144	757,860,882	99.98	0.03	97.78	93.52	39.84
S0707	563,814,720	563,630,862	99.97	0.03	97.8	93.69	39.53
S1012	754,517,952	754,335,162	99.98	0.03	97.87	93.75	40.53
S0402	560,364,768	560,246,680	99.98	0.03	96.78	91.27	38.52

Appendix 7: Mapping statistics with reference genome and tag summary.

Sample	Mapped reads	Total reads	Tag number	Tag 4 number	Mapping rate (%)	Average depth (X)	Coverage at least 1X (%)	Coverage at least 4X (%)
S0503	3106307	3471090	376441	174199	89.49	12.31	1.75	1.03
S0509	2306043	2595778	345548	148112	88.84	9.87	1.62	0.9
S0502	3135326	3529658	357822	173125	88.83	13.03	1.66	1.01
S0903	3808728	4279118	389689	188710	89.01	14.38	1.83	1.1
S1011	4014996	4536180	389037	181373	88.51	15.15	1.82	1.06

S1101	4268195	4813270	429594	198867	88.68	15.29	1.93	1.13
S0901	3970696	4442428	406360	189541	89.38	14.4	1.9	1.12
S0201	4205782	4778948	389763	182545	88.01	16.32	1.77	1.05
S1109	3986690	4488466	393561	179993	88.82	14.91	1.84	1.06
S0804	2621973	3539214	331443	161524	74.08	11.36	1.59	0.96
S1007	4464583	5029938	383571	184730	88.76	17.38	1.76	1.06
S0504	2720594	3064252	344125	162034	88.78	11.62	1.62	0.97
S0603	2682825	3007656	345838	162559	89.2	11.31	1.64	0.97
S0709	3357087	3744980	362843	178376	89.64	13.27	1.74	1.05
S0805	3888817	4382216	416381	189995	88.74	14.1	1.9	1.11
S0601	2745049	3101836	372952	163999	88.5	11.16	1.7	0.98
S1014	4201540	4781422	445565	206347	87.87	14.25	2.02	1.18
S0710	3733234	4232454	402008	187740	88.2	13.64	1.89	1.11
S1103	4159212	4721456	422670	185296	88.09	14.75	1.94	1.09
S0705	2570680	2905110	349771	157273	88.49	10.79	1.64	0.95
S0604	3026325	3416854	356090	171056	88.57	12.62	1.65	1.01
S0204	4706939	5315906	399170	190616	88.54	17.76	1.82	1.1
S0403	3194078	3685924	504560	197114	86.66	9.72	2.24	1.17
S0701	2562604	2867072	322595	160209	89.38	11.48	1.55	0.95
S0706	3329912	3746860	378739	177486	88.87	12.91	1.77	1.05
S0405	2949911	3308086	381607	168760	89.17	11.85	1.72	1
S0702	3009751	3375964	360778	170114	89.15	12.3	1.69	1.01
S0304	3024158	3409558	348120	170458	88.7	12.9	1.62	1
S1105	4149076	4686574	385943	182976	88.53	15.8	1.8	1.06
S1001	3317268	3719026	368539	179805	89.2	12.98	1.76	1.06
S0810	3595976	4178244	376364	183484	86.06	13.83	1.79	1.08
S0303	3172898	3587046	337400	161727	88.45	13.99	1.55	0.95
S0501	2676771	3039478	339443	162298	88.07	11.47	1.62	0.97
S1008	3171402	3678512	370105	164660	86.21	12.54	1.74	0.98

S1010	3893261	4453922	398596	179477	87.41	14.4	1.86	1.06
S0904	3554019	3974538	363699	181690	89.42	14.17	1.73	1.06
S0906	3953017	4453032	376011	189267	88.77	15.3	1.77	1.1
S0404	2955670	3349512	357471	168337	88.24	12.28	1.66	1
S0703	2835783	3217222	357086	163094	88.14	12.14	1.61	0.96
S0808	3232956	3902438	383173	177549	82.84	12.33	1.8	1.06
S0803	3289413	3683836	357066	177517	89.29	13.22	1.71	1.04
S1005	3707079	4114930	371198	178439	90.09	14.65	1.74	1.04
S1104	4563545	5123752	428825	192271	89.07	16.35	1.93	1.11
S1106	3536933	4037840	371638	172526	87.59	13.84	1.76	1.02
S0704	2730618	3060826	360068	162618	89.21	11.31	1.67	0.97
S0506	3176339	3583572	377569	172892	88.64	12.79	1.7	1
S0302	4484229	5111118	451050	189221	87.73	16.36	1.89	1.09
S1015	4048743	4589950	400295	182454	88.21	14.9	1.87	1.08
S0605	2866403	3211804	352211	166535	89.25	12.03	1.65	0.99
S1009	3816305	4357600	444224	189089	87.58	13.04	2.02	1.11
S0602	2873035	3238026	395178	172725	88.73	10.98	1.81	1.03
S1013	4332750	4883260	391696	185903	88.73	16.41	1.82	1.08
S0907	3243087	3617612	375590	178342	89.65	12.6	1.78	1.05
S0809	3152914	3563626	417112	172705	88.47	11.87	1.83	1.02
S0902	3606541	4011136	368009	172415	89.91	14.43	1.71	1.01
S0905	3425505	3835606	344633	174621	89.31	14.26	1.65	1.02
S1004	3340760	3779164	375602	181086	88.4	12.92	1.79	1.07
S1111	4080952	4561914	433242	186448	89.46	14.37	1.97	1.09
S1102	4417262	4977848	458348	205415	88.74	14.75	2.06	1.18
S0401	3061698	3450258	357931	170746	88.74	12.82	1.65	1
S0908	3737956	4174508	397527	186712	89.54	13.96	1.85	1.1
S1107	4192142	4772412	394903	182581	87.84	15.67	1.84	1.07
S0909	3736363	4230908	377047	186929	88.31	14.32	1.8	1.1

S0507	2678218	2991868	338951	155981	89.52	11.85	1.56	0.92
S0807	4181534	4673416	397417	192579	89.47	15.48	1.85	1.12
S0910	4003501	4464504	385127	189718	89.67	15.21	1.81	1.11
S0202	4429122	5055706	395803	187181	87.61	16.78	1.81	1.08
S0707	3329734	3774482	360738	179668	88.22	13.29	1.73	1.06
S1012	4488523	5068460	386798	187096	88.56	17.17	1.8	1.08
S0402	3249117	3682520	346959	171867	88.23	13.97	1.6	1

Appendix 8: Statistics of InDel detection and annotation.

Sam ple	Upstr eam	Exonic				Intro nic	Splic ing	Downst ream	Upstrea m/ Downst ream	Interg enic	ts	tv	ts/t v	Het rate (%)	Total
		top gain	Stop loss	Synony mous	Non- synonymo us										
S05 03	14,45 0	151	24	3,905	5,213	81,8 51	62	12,716	284	324,9 34	316, 627	126, 963	2.4 93	0.046	443, 590
S05 09	12,77 6	104	17	3,665	4,794	72,3 92	62	11,222	261	287,1 55	283, 011	109, 437	2.5 86	0.04	392, 448
S05 02	14,05 3	126	22	3,790	4,813	78,0 78	54	12,088	281	314,2 50	308, 424	119, 131	2.5 88	0.041	427, 555
S09 03	15,25 2	144	19	4,184	5,515	85,5 12	64	13,355	268	343,7 96	334, 645	133, 464	2.5 07	0.046	468, 109
S10 11	14,74 8	116	18	4,119	5,339	80,9 15	54	12,791	318	324,1 61	318, 244	124, 335	2.5 59	0.041	442, 579
S11 01	15,65 8	136	19	4,242	5,495	87,7 20	67	13,921	319	367,4 14	358, 088	136, 903	2.6 15	0.058	494, 991
S09 01	15,58 1	144	19	4,182	5,559	88,6 29	57	13,676	292	344,1 20	338, 180	134, 079	2.5 22	0.045	472, 259

S02	14,04					81,0				322,4	316,	122,	2,5		439,
01	7	112	19	3,849	5,040	15	57	12,414	313	59	590	735	79	0.041	325
S11	14,65					81,1				323,8	318,	123,	2,5		442,
09	0	109	19	4,108	5,235	14	61	12,748	268	88	788	412	83	0.041	200
S08	13,16					76,0				303,1	298,	115,	2,5		413,
04	1	109	16	3,686	4,808	62	58	11,861	234	28	088	035	91	0.04	123
S10	14,42					80,7				325,2	318,	124,	2,5		442,
07	7	109	16	3,881	5,091	42	58	12,851	309	07	103	588	53	0.041	691
S05	13,47					76,2				302,7	297,	115,	2,5		413,
04	2	104	17	3,666	4,818	35	59	11,949	273	07	709	591	75	0.04	300
S06	13,07					75,8				303,1	298,	114,			413,
03	3	107	19	3,776	4,933	75	49	11,818	240	25	321	694	2,6	0.041	015
S07	14,21					80,5				324,1	318,	123,	2,5		441,
09	1	124	22	4,032	5,220	96	61	13,032	265	37	328	372	8	0.041	700
S08	14,78					86,9				350,3	340,	135,	2,5		476,
05	6	157	21	4,102	5,544	91	66	13,852	274	50	681	462	14	0.048	143
S06	13,43					78,5				314,1	306,	121,	2,5		427,
01	1	134	18	3,779	5,040	12	53	12,076	290	90	069	454	2	0.045	523
S10	16,96					92,1				389,3	379,	144,	2,6		524,
14	7	149	23	4,570	5,865	33	65	14,656	332	99	961	198	34	0.066	159
S07	14,94					85,3				344,8	335,	133,	2,5		469,
10	7	161	23	4,139	5,591	23	64	13,658	282	89	360	717	07	0.046	077
S11	14,75					84,1				340,4	331,	131,	2,5		463,
03	9	161	22	4,261	5,638	65	53	13,729	304	28	611	909	13	0.047	520
S07	13,06					73,9				295,3	290,	112,	2,5		403,
05	3	110	20	3,833	4,845	04	53	11,590	264	51	340	693	76	0.039	033
S06	13,77					77,6				313,0	306,	119,	2,5		425,
04	9	112	18	3,820	4,949	13	49	12,173	242	98	716	137	74	0.041	853
S02	14,48					82,3				332,4	325,	126,	2,5		451,
04	3	123	22	4,086	5,297	19	57	12,865	253	60	745	220	8	0.041	965

S04	14,21					82,8				398,0	380,	138,	2.7		519,
03	0	155	26	4,224	6,070	00	59	13,176	302	54	639	437	49	0.089	076
S07	13,06					74,1				299,4	293,	113,	2.5		406,
01	6	109	19	3,669	4,772	10	49	11,431	204	15	195	649	79	0.039	844
S07	14,14					80,9				324,9	319,	123,	2.5		442,
06	9	116	20	4,062	5,297	90	59	12,860	253	77	351	432	87	0.042	783
S04	13,77					78,8				317,5	309,	122,	2.5		431,
05	4	157	23	3,778	4,959	36	59	12,307	258	99	339	411	27	0.045	750
S07	14,09					78,0				313,2	308,	118,	2.5		427,
02	6	116	19	3,886	5,106	54	51	12,199	256	57	261	779	95	0.041	040
S03	13,32					78,5				312,1	305,	118,	2.5		424,
04	2	108	18	3,686	4,856	55	59	11,877	267	95	972	971	71	0.041	943
S11	14,45					81,3				326,2	320,	124,	2.5		444,
05	0	114	23	4,109	5,309	64	57	12,778	284	43	308	423	74	0.042	731
S10	14,37					82,3				328,0	321,	125,	2.5		447,
01	5	122	19	4,001	5,237	73	54	12,926	311	30	683	765	57	0.043	448
S08	14,62					83,2				332,2	326,	126,	2.5		453,
10	9	121	16	4,055	5,305	02	64	13,190	271	99	551	601	79	0.043	152
S03	12,79					74,3				294,3	287,	113,	2.5		401,
03	6	104	17	3,457	4,495	73	52	11,275	258	31	994	164	44	0.039	158
S05	13,29					75,7				302,6	297,	114,	2.6		412,
01	1	127	21	3,735	4,856	37	55	11,711	266	65	971	493	02	0.04	464
S10	13,55					75,2				303,0	298,	115,	2.5		413,
08	1	113	17	3,948	5,052	09	53	12,002	322	59	016	310	84	0.04	326
S10	14,71					80,3				325,0	318,	124,	2.5		442,
10	5	120	19	4,076	5,276	59	61	12,772	308	40	636	110	67	0.042	746
S09	14,62					82,2				327,5	322,	125,	2.5		447,
04	8	125	19	4,019	5,340	14	64	12,834	256	30	018	011	75	0.042	029
S09	14,98					84,4				337,9	331,	129,	2.5		460,
06	4	136	20	4,079	5,306	12	64	13,220	231	66	017	401	58	0.043	418

S04	13,57					77,4				308,5	303,	117,	2.5		420,
04	8	127	21	3,771	4,830	31	55	12,049	276	44	663	019	94	0.041	682
S07	13,03					74,4				302,3	295,	114,	2.5		410,
03	4	105	20	3,634	4,743	94	51	11,640	240	02	731	532	82	0.04	263
S08	14,28					82,8				329,7	324,	124,	2.5		449,
08	6	119	22	4,019	5,330	04	64	12,994	289	07	717	917	99	0.043	634
S08	14,33					80,1				320,2	314,	122,	2.5		437,
03	6	126	22	3,983	5,175	07	59	12,732	254	36	788	242	75	0.041	030
S10	14,69					82,2				325,2	314,	129,	2.4		444,
05	2	162	15	3,857	5,151	49	57	13,016	254	61	976	738	27	0.045	714
S11	15,10					85,8				345,5	335,	134,	2.4		470,
04	0	159	25	4,147	5,531	47	56	13,557	295	54	414	857	87	0.048	271
S11	13,74					78,5				314,2	309,	119,	2.5		428,
06	9	117	20	3,958	5,184	45	55	12,348	281	45	300	202	94	0.041	502
S07	13,27					75,9				305,3	297,	117,	2.5		415,
04	5	137	22	3,815	5,110	33	61	11,938	238	17	856	990	24	0.042	846
S05	14,09					80,8				332,2	323,	125,	2.5		448,
06	0	134	19	3,620	4,747	62	62	12,433	291	64	257	265	8	0.056	522
S03	14,43					84,5				341,9	331,	131,	2.5		463,
02	4	140	19	3,849	5,145	98	52	13,101	272	45	855	700	19	0.045	555
S10	15,06					81,1				329,5	322,	125,	2.5		448,
15	6	126	21	4,103	5,336	53	55	12,930	311	33	995	639	7	0.042	634
S06	13,69					76,1				307,6	301,	116,	2.5		418,
05	4	107	18	3,755	4,946	40	52	11,807	223	13	755	600	87	0.04	355
S10	15,80					86,0				361,7	353,	134,	2.6		488,
09	6	130	20	4,327	5,635	95	63	13,914	323	05	178	840	19	0.058	018
S06	14,41					82,3				337,6	330,	126,	2.6		456,
02	4	142	18	3,968	5,224	08	63	12,757	236	79	317	492	11	0.056	809
S10	14,71					81,3				328,6	321,	126,	2.5		447,
13	4	114	17	4,020	5,225	77	63	13,062	315	71	572	006	52	0.041	578

S09 07	14,67 1	170	22	4,094	5,508	83,8 36	73	13,121	259	331,7 04	322, 401	131, 057	2.4 59	0.047	453, 458
S08 09	14,06 1	141	19	3,903	5,185	80,9 78	68	12,749	244	331,3 86	322, 674	126, 060	2.5 59	0.047	448, 734
S09 02	13,80 3	179	13	3,619	5,005	81,6 39	74	13,074	247	319,5 29	307, 452	129, 730	2.3 69	0.046	437, 182
S09 05	14,10 0	115	19	3,791	4,954	79,3 77	62	12,453	231	315,3 61	309, 187	121, 276	2.5 49	0.041	430, 463
S10 04	14,94 5	146	16	4,071	5,355	83,3 81	62	12,915	300	332,6 67	324, 593	129, 265	2.5 11	0.045	453, 858
S11 11	15,20 5	187	25	4,253	5,792	86,1 44	62	13,806	291	343,4 52	332, 729	136, 488	2.4 37	0.049	469, 217
S11 02	16,15 8	149	23	4,598	5,930	91,9 91	63	15,097	306	385,8 60	377, 305	142, 870	2.6 4	0.064	520, 175
S04 01	13,49 1	121	23	3,884	5,169	78,2 40	63	12,102	240	312,7 47	306, 967	119, 113	2.5 77	0.041	426, 080
S09 08	14,97 6	139	21	4,182	5,569	85,3 45	67	13,493	274	341,0 17	333, 061	132, 022	2.5 22	0.046	465, 083
S11 07	14,48 7	118	21	4,154	5,393	81,6 68	57	12,945	293	324,8 75	320, 376	123, 635	2.5 91	0.042	444, 011
S09 09	15,00 3	124	21	4,179	5,457	84,6 32	64	13,209	242	337,0 56	331, 076	128, 911	2.5 68	0.043	459, 987
S05 07	13,01 5	127	16	3,463	4,607	74,7 67	53	11,371	286	294,6 84	287, 867	114, 522	2.5 13	0.041	402, 389
S08 07	14,81 6	139	20	4,127	5,466	87,0 81	69	14,019	265	346,7 40	338, 554	134, 188	2.5 22	0.046	472, 742
S09 10	14,82 4	141	21	4,163	5,503	85,3 38	67	13,281	286	340,3 74	333, 115	130, 883	2.5 45	0.044	463, 998
S02 02	14,69 3	162	20	3,993	5,247	83,2 53	53	13,015	275	331,6 23	323, 792	128, 542	2.5 18	0.045	452, 334

S0707	14,367	118	18	3,990	5,236	80,835	58	12,686	238	326,121	319,690	123,977	2.578	0.042	443,667
S1012	14,764	111	17	4,035	5,217	81,209	57	12,818	292	326,913	320,154	125,279	2.555	0.041	445,433
S0402	13,508	120	19	3,742	4,876	77,603	60	12,003	263						

Appendix 9: Statistics of InDel detection and annotation.

Sample	Upstream	Exonic								Intronic	Splicing	Downstream	Upstream/Downstream	Intergenic	Deletion	Het rate (%)	Total
		Stop gain	Stop loss	Frameshift deletion	Frameshift deletion	Non-frameshift deletion	Non-frameshift insertion										
S0503	1,872	4	3	79	270	86	54	9,540	16	1,837	40	17,355	18,934	12,224	0.001	31,158	
S0509	1,699	3	3	77	256	80	57	8,362	13	1,650	33	15,076	16,455	10,856	0.001	27,311	
S0502	1,848	6	3	88	259	89	59	9,212	16	1,768	38	16,982	18,433	11,936	0.001	30,369	
S0903	2,034	4	3	86	295	90	61	9,975	15	1,962	36	18,688	20,262	12,989	0.001	33,251	
S1011	2,030	4	4	102	281	89	67	9,589	16	1,898	43	17,536	19,064	12,600	0.001	31,664	

S1 10 1	2,07 2	4	4	96	280	9 2	61	10,093	15	2,0 02	41	19,172	20,4 72	13, 461	0.002	33, 93 3
S0 90 1	2,09 4	6	3	94	302	9 0	70	10,306	15	2,0 16	45	18,697	20,5 27	13, 212	0.001	33, 73 9
S0 20 1	1,97 2	5	4	103	277	7 9	57	9,481	15	1,8 30	49	17,443	18,9 56	12, 362	0.001	31, 31 8
S1 10 9	2,00 5	7	3	102	294	9 0	68	9,523	17	1,9 17	41	17,488	18,9 54	12, 602	0.001	31, 55 6
S0 80 4	1,79 4	4	3	82	271	8 3	60	8,809	13	1,6 86	30	16,054	17,4 97	11, 396	0.001	28, 89 3
S1 00 7	1,99 6	3	4	101	277	8 4	64	9,697	13	1,8 69	42	17,777	19,3 00	12, 630	0.001	31, 93 0
S0 50 4	1,81 3	4	3	76	265	8 4	59	8,954	14	1,7 18	33	16,192	17,6 10	11, 606	0.001	29, 21 6
S0 60 3	1,76 8	6	3	78	280	8 9	65	8,850	12	1,7 10	39	16,074	17,5 45	11, 430	0.001	28, 97 5
S0 70 9	1,92 9	6	3	92	302	8 9	65	9,562	19	1,9 08	39	17,324	18,9 65	12, 376	0.001	31, 34 1
S0 80 5	2,01 4	8	3	90	295	8 8	61	10,030	15	1,9 53	35	18,587	20,0 64	13, 118	0.001	33, 18 2

S0 60 1	1,77 3	5	3	79	261	8 6	56	9,056	12	1,7 64	31	16,631	17,9 41	11, 819	0.001	29, 76 0
S1 01 4	2,23 8	3	4	114	303	1 0 2	66	10,401	12	2,0 78	47	20,121	21,3 03	14, 189	0.002	35, 49 2
S0 71 0	1,97 1	6	3	94	298	8 0	69	10,089	18	1,9 46	38	18,371	20,0 50	12, 937	0.001	32, 98 7
S1 10 3	2,07 7	2	3	99	285	8 5	65	9,827	18	1,9 45	46	18,198	19,7 60	12, 893	0.001	32, 65 3
S0 70 5	1,75 8	4	3	78	281	7 7	59	8,751	12	1,6 74	40	15,561	17,1 89	11, 112	0.001	28, 30 1
S0 60 4	1,84 1	7	3	83	270	8 2	56	9,146	14	1,7 67	41	16,864	18,2 88	11, 888	0.001	30, 17 6
S0 20 4	2,00 4	4	3	89	287	7 9	64	9,851	16	1,8 99	36	18,047	19,6 74	12, 708	0.001	32, 38 2
S0 40 3	1,76 5	5	4	89	285	8 7	60	8,655	12	1,7 10	28	17,759	18,7 12	11, 750	0.002	30, 46 2
S0 70 1	1,71 9	5	3	86	265	8 1	59	8,711	12	1,6 97	36	16,115	17,5 09	11, 281	0.001	28, 79 0
S0 70 6	1,89 7	6	3	89	292	8 5	66	9,602	17	1,8 84	33	17,183	18,9 12	12, 248	0.001	31, 16 0

S0 40 5	1,80 5	5	3	82	265	8 6	56	9,134	12	1,7 45	35	16,720	18,1 67	11, 783	0.001	29, 95 0
S0 70 2	1,87 5	5	3	93	284	9 6	61	9,093	17	1,7 94	43	16,718	18,2 39	11, 845	0.001	30, 08 4
S0 30 4	1,84 2	6	4	100	266	7 8	62	9,215	14	1,7 65	36	16,712	18,3 22	11, 781	0.001	30, 10 3
S1 10 5	1,98 5	3	3	98	294	8 7	70	9,748	18	1,8 76	42	17,765	19,3 97	12, 593	0.001	31, 99 0
S1 00 1	1,97 0	4	4	99	289	9 3	64	9,797	12	1,8 87	42	17,749	19,3 03	12, 709	0.001	32, 01 2
S0 81 0	2,00 3	5	3	96	300	8 2	58	9,770	17	1,8 56	35	17,751	19,3 28	12, 650	0.001	31, 97 8
S0 30 3	1,74 0	4	4	83	245	6 8	51	8,756	13	1,6 76	32	16,163	17,5 38	11, 300	0.001	28, 83 8
S0 50 1	1,74 3	5	3	81	265	8 1	56	8,950	14	1,6 88	35	16,187	17,6 65	11, 444	0.001	29, 10 9
S1 00 8	1,91 5	4	4	99	257	9 0	62	8,974	10	1,7 49	38	16,158	17,6 26	11, 737	0.001	29, 36 3
S1 01 0	2,00 6	4	4	94	268	9 6	64	9,561	14	1,8 76	38	17,655	19,0 78	12, 606	0.001	31, 68 4

S0 90 4	1,98 6	5	3	91	281	9 0	64	9,677	13	1,8 76	30	17,751	19,3 15	12, 553	0.001	31, 86 8
S0 90 6	2,02 6	4	3	88	300	8 8	60	10,023	15	1,9 41	32	18,480	20,1 97	12, 864	0.001	33, 06 1
S0 40 4	1,86 8	4	3	81	280	9 0	60	9,054	14	1,7 29	41	16,606	18,0 90	11, 743	0.001	29, 83 3
S0 70 3	1,74 1	6	3	79	268	7 7	60	8,868	16	1,6 71	37	15,959	17,4 80	11, 307	0.001	28, 78 7
S0 80 8	1,96 0	8	3	100	304	8 7	65	9,566	17	1,8 73	36	17,431	19,0 08	12, 444	0.001	31, 45 2
S0 80 3	1,90 1	6	3	89	289	8 5	68	9,445	12	1,7 97	36	17,061	18,7 21	12, 074	0.001	30, 79 5
S1 00 5	1,93 1	4	4	96	269	8 0	47	9,761	12	1,8 86	35	18,013	19,5 83	12, 556	0.001	32, 13 9
S1 10 4	2,02 3	3	3	99	273	8 2	62	9,977	16	1,9 74	45	18,566	20,0 67	13, 058	0.001	33, 12 5
S1 10 6	1,95 2	2	3	104	279	8 4	64	9,265	14	1,7 84	42	16,974	18,4 66	12, 105	0.001	30, 57 1
S0 70 4	1,76 9	7	3	93	274	7 6	62	8,927	16	1,7 15	32	16,028	17,6 25	11, 380	0.001	29, 00 5

S0 50 6	1,82 4	4	3	92	256	7 3	51	9,194	12	1,7 66	38	17,687	18,7 77	12, 224	0.001	31, 00 1
S0 30 2	1,94 8	4	3	83	272	7 7	58	9,812	14	1,9 34	41	18,315	19,8 68	12, 694	0.001	32, 56 2
S1 01 5	2,04 1	4	4	102	288	9 0	67	9,610	13	1,8 90	43	17,792	19,2 16	12, 733	0.001	31, 94 9
S0 60 5	1,81 1	6	3	89	260	8 7	58	8,890	12	1,7 41	38	16,444	17,8 43	11, 597	0.001	29, 44 0
S1 00 9	2,13 8	4	4	98	283	9 5	61	9,895	20	1,9 82	46	18,645	19,8 93	13, 380	0.002	33, 27 3
S0 60 2	1,95 9	5	4	95	276	9 7	63	9,376	10	1,8 24	33	17,441	18,7 32	12, 453	0.001	31, 18 5
S1 01 3	2,01 6	3	4	104	276	8 9	68	9,716	14	1,8 99	44	17,874	19,3 52	12, 758	0.001	32, 11 0
S0 90 7	1,92 4	5	3	83	295	8 8	63	9,738	13	1,8 93	28	17,844	19,4 90	12, 489	0.001	31, 97 9
S0 80 9	1,88 4	5	3	82	275	8 4	62	9,243	11	1,8 12	31	16,970	18,4 62	12, 002	0.001	30, 46 4
S0 90 2	1,78 8	5	3	87	254	7 3	48	9,514	11	1,8 55	23	17,789	19,2 94	12, 158	0.001	31, 45 2

S0 90 5	1,93 6	4	3	80	270	8 7	55	9,354	13	1,8 11	27	17,240	18,7 62	12, 119	0.001	30, 88 1
S1 00 4	2,00 7	4	4	99	284	9 9	60	9,884	17	1,8 86	38	17,917	19,4 99	12, 801	0.001	32, 30 0
S1 11 1	2,05 3	2	3	107	281	8 2	59	9,991	17	1,9 53	45	18,477	19,9 75	13, 097	0.001	33, 07 2
S1 10 2	2,16 1	5	4	105	307	9 5	71	10,453	14	2,0 50	49	19,812	21,0 45	14, 083	0.002	35, 12 8
S0 40 1	1,78 4	4	4	95	276	8 1	58	9,251	11	1,7 77	30	16,700	18,1 48	11, 925	0.001	30, 07 3
S0 90 8	2,00 9	3	3	86	295	8 8	64	10,022	14	1,9 63	35	18,407	20,0 38	12, 952	0.001	32, 99 0
S1 10 7	2,03 2	3	3	105	299	9 1	62	9,681	16	1,9 04	49	17,575	19,1 12	12, 709	0.001	31, 82 1
S0 90 9	2,06 0	4	3	86	302	8 8	63	10,031	14	1,9 52	33	18,326	20,0 56	12, 908	0.001	32, 96 4
S0 50 7	1,68 7	4	2	70	237	6 6	45	8,607	12	1,6 34	37	15,834	17,0 88	11, 148	0.001	28, 23 6
S0 80 7	2,02 6	7	3	97	300	8 9	65	10,118	17	1,9 96	38	18,730	20,3 58	13, 131	0.001	33, 48 9

S0 91 0	2,03 6	3	3	96	300	8 6	62	9,950	17	1,9 62	38	18,452	20,0 21	12, 985	0.001	33, 00 6
S0 20 2	2,01 4	5	4	89	283	8 6	54	9,731	12	1,9 27	44	18,188	19,6 00	12, 838	0.001	32, 43 8
S0 70 7	1,90 2	5	3	84	288	8 1	69	9,612	17	1,8 50	38	17,387	19,0 60	12, 280	0.001	31, 34 0
S1 01 2	2,05 9	4	4	103	272	8 5	63	9,812	17	1,9 01	44	17,937	19,5 14	12, 790	0.001	32, 30 4
S0 40 2	1,76 0	4	4	95	262	7 8	55	9,205	14	1,7 34	28	17,094	18,3 48	11, 986	0.001	30, 33 4

Appendix 10: Inferred grouping by DAPC and original cluster assignment of each sample.

Samples	Initial clusters	DACP Grouping
S0201	CL 2	2
S0202	CL 2	2
S0204	CL 2	4
S0302	CL 3	8
S0303	CL 3	2
S0304	CL 3	2
S0401	CL 4	2
S0402	CL 4	2
S0403	CL 4	6
S0404	CL 4	7
S0405	CL 4	7
S0501	CL 5	7
S0502	CL 5	7
S0503	CL 5	7
S0504	CL 5	9
S0506	CL 5	7
S0507	CL 5	9
S0509	CL 5	9
S0601	CL 6	9
S0602	CL 6	2
S0603	CL 6	3
S0604	CL 6	3
S0605	CL 6	3
S0701	CL 7	3
S0702	CL 7	3
S0703	CL 7	4
S0704	CL 7	4
S0705	CL 7	4
S0706	CL 7	4
S0707	CL 7	4
S0709	CL 7	4
S0710	CL 7	4
S0803	CL 8	4
S0804	CL 8	9
S0805	CL 8	9
S0807	CL 8	9
S0808	CL 8	9
S0809	CL 8	9

S0810	CL 8	9
S0901	CL 9	8
S0902	CL 9	9
S0903	CL 9	10
S0904	CL 9	10
S0905	CL 9	10
S0906	CL 9	10
S0907	CL 9	10
S0908	CL 9	10
S0909	CL 9	10
S0910	CL 9	10
S1001	CL 10	1
S1004	CL 10	1
S1005	CL 10	1
S1007	CL 10	1
S1008	CL 10	1
S1009	CL 10	2
S1010	CL 10	1
S1011	CL 10	1
S1012	CL 10	1
S1013	CL 10	1
S1014	CL 10	5
S1015	CL 10	1
S1101	CL 11	2
S1102	CL 11	5
S1103	CL 11	5
S1104	CL 11	5
S1105	CL 11	5
S1106	CL 11	5
S1107	CL 11	5
S1109	CL 11	2
S1111	CL 11	5