Evolutionary relationships between pollination and protective mutualisms in the genus *Macaranga* (Euphorbiaceae)

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Contents

摘要

第 1 章

序論

植物は、植食者に対して様々な防衛形質を進化させてきた。その防衛形質は、植食者との関係ばかりでなく、他の生物との関係にも影響を与えることがある。最もよく研究されているのは物理的・化学的防衛と送粉の関係で、送粉に関わる形質が防衛に利用されたり、逆に防衛に関わる形質が送粉に利用されたりすることで（外適応）、相互の進化に影響する例が知られている。一方、植物の中にはアリと被食防衛共生の関係を結んでいるものが少なくない。アリは植物の防衛に役立つが、植食者だけでなく送粉者も撃退することがある。そこでこの研究では、トウダイグサ科オオバギ属における送粉と被食防衛共生の進化的関係に着目した。オオバギ属のほとんどは、葉の花外蜜腺に誘引されて訪れる不特定のアリによって防衛されているが、一部の種は中空になった幹内に特定の種のアリを住まわせ、極めて強くアリによって防衛される「アリ植物」である。オオバギ属の送粉様式についての研究は少ないが、アリ植物種を中心に、花序の小苞葉の内側で採餌・繁殖を行うアザミウマやカメムシによる送粉が知られている。この論文では、第 2 章でオオバギ属の送粉様式の多様性と進化について、第 3 章ではアリ植物オオバギ属における植物、送粉者と防衛アリの三者関係について調べた結果について述べる。第 4 章では、これらの結果に基づき、オオバギ属における送粉と防衛に関わる形質の進化的関係について議論する。

第 2 章

オオバギ属植物における送粉様式の多様性

第 2 章第 1 節

花序形態の多様性と進化

オオバギ属の一部では、アザミウマやカメムシによる送粉様式が知られている。この送粉様式では、送粉者に蜜のほか、繁殖場所を提供している点で他の多くの植物の送粉様式と異なっている。この送粉様式がどのように進化したのか明らかにするために、この節では花序形態、特に種間で形態差が大きい小苞葉形態の多様性と進化について調べた。まず、植物標本庫の乾燥標本の観察を行い、小苞葉の形態が『蜜腺型』（小苞葉上に円盤状の腺をもつ）、『被覆型』（小苞葉が花を覆う）、『欠損型』（目立つ小苞葉をもたない）の 3 タイプがあることを明らかにした。この花序形態の差異は、送粉様式の違いを反映

していると思われる。次に、分子系統樹にもとづいた最節約形質復元を行った。その結果、オオバギ属の祖先形質は欠損型であると推定された。その後、順序は不明だが蜜腺型と被覆型が起源し、3 つのタイプ間で花序形態のシフトが少なくとも 16 回起こったことが示唆された。アザミウマ媒やカメムシ媒の種は単系統にならなかったこと、それらが含まれる被覆型は少なくとも 4 回進化していることから、このような特殊な送粉様式がオオバギ属内で複数回起源したことが示唆された。どのような生態的・遺伝的要因で送粉様式のシフトが起こったかは、今後の課題である。

第 2 章第 2 節

オオバギ属の姉妹群アカメガシワ属に見られる風虫両媒

風虫両媒（風媒と虫媒を併せ持った送粉様式）をもつ植物は少なく、どのような場合にこの送粉様式が有利になるのかはよく調べられていない。この節では、温帯の森林に生育する先駆植物アカメガシワ(*Mallotus japonicus*)、ボルネオ島熱帯低地林に分布する *Mallotus wrayi* の風虫両媒を報告する。どちらの種においても、花序に網をかけ昆虫を除去しても結実が見られたこと、空気中の飛散花粉が雌花序に到達していたことから、風媒が結実に寄与していることが示唆された。また、体表に花粉の付着した昆虫が雌花序を訪花していたことから、昆虫媒も両種の結実に寄与していると考えられる。アカメガシワと *M. wrayi* はともに風媒と昆虫媒の両方に適した花序形態を持っていることから、風虫両媒は積極的に維持されていると考えられる。先駆植物で風虫両媒が有利なのは、森林の遷移にともなって風の条件が変化するためだと考えられてきた。それに加え、本研究では、アカメガシワでは、雄個体の近くに存在する個体よりも離れて存在する個体で、結実率が花粉量でより強く制限されていたため、遷移にともなう個体群密度の変化もアカメガシワの風虫両媒の維持に寄与する可能性も議論した。

第 2 章第 3 節

***Macaranga sinensis* における小苞葉上の円盤状蜜腺の送粉への寄与**

花蜜は、動物媒の花で最もよく見られる送粉者に対する報酬である。蜜腺は花器官の様々な場所で見られ、形態も多様であることから、花蜜の分泌は被子植物で何度も独立に進化したと考えられる。オオバギ属の花は花弁や花蜜をもたないが、ほとんどの種が植物の防衛者となるアリを誘引する円盤状の蜜腺を葉にもつ。いくつかの種では、葉の相同器官であり花の基部に存在する小苞葉に、葉の蜜腺と似た腺が見られる。この節では、*Macaranga sinensis* において、小苞葉に存在する腺が送粉に寄与するかどうか調べ、蜜腺の起源について議論した。訪花昆虫の観察・採集と、採集された昆虫の体表花粉か

ら、*M. sinensis* は、小苞葉から分泌される蜜を求めて訪れる昆虫によって送粉されることが示唆された。葉と小苞葉から分泌される蜜の糖成分を高速液体クロマトグラフィーで分析したところ、有意な差は見られなかった。また、小苞葉の蜜腺と葉の蜜腺は、見た目や発現場所もよく似ていた。これらの結果から、*M. sinensis* では、葉の蜜腺が花序でも形成されて送粉者を誘引する機能を獲得し、新しい送粉様式の進化につながったと考えられる。

第 3 章
アリ植物オオバギ属における植物・送粉者・防衛アリの三者関係

第 3 章第 1 節
防衛アリが植食者および送粉者に与える影響

アリと被食防衛共生関係をもつ植物では、アリは植物の防衛に役立つが、送粉者をも追い払ってしまうことで送粉を阻害することがある。体内にアリを営巣させ、アリから強く防衛されている「アリ植物」では、葉の花外蜜腺に誘引される不特定のアリに防衛される植物に比べて、アリによる繁殖阻害がより深刻であると考えられる。そこで本研究では、アリが花序に頻繁に訪れるのか、植食者を除去したり送粉を妨げたりするのか調べた。対象としたのは、8 種のアリ植物種と 3 種の非アリ植物種で、いずれもボルネオ島に分布し、送粉様式の分かっていない非アリ植物の 1 種を除いてアザミウマ *Dolichothrips* sp.1、 *Dolichothrips* sp.2 のいずれかによって送粉される。8 種のうち 7 種のアリ植物種では、若い花序か未成熟の果実、またはその両方に食物体を生産していた。アリ植物 3 種で花序に訪れるアリの数を観察すると、食物体を生産している期間、アリの数の増加が認められた。これらの結果から、植物はアリを積極的に花序に誘引していると考えられる。また、花序に食物体をもたない 1 種でアリを花序から除去する実験を行ったところ、アリ除去区では対照区よりも食害が多かったが、送粉者アザミウマの密度は変化しなかった。これらの結果から、花序を訪れるアリは食害を抑える一方で、送粉を妨害しないことが示唆された。

第 3 章第 2 節
アリ植物 *Macaranga winkleri* の送粉者アザミウマによる肛門分泌物を用いた防衛アリの忌避

アリ植物では、アリは植食者だけでなく送粉者も追い払い、送粉を妨害することがある。本研究では、アザミウマ *Dolichothrips* sp.1 によって送粉されるアリ植物 *Macaranga winkleri*

において、送粉者と防衛アリの関係を調べた。まず、さまざまな昆虫に対する防衛アリの行動を調べた。アリは送粉者以外の昆虫には攻撃行動をとることが多かったが、送粉者アザミウマに遭遇した時、特にアザミウマが肛門から液滴を分泌した時には、逃避行動をとることが多かった。さらに、アザミウマの肛門分泌物や、その主成分であるデカン酸を付着させたテフロン片に対する反応を観察したところ、対照区と比べ逃避行動が多く見られた。以上から、送粉者アザミウマは忌避効果のある液滴を肛門から分泌することで、アリからの攻撃を避けていることが示唆された。植物がアリを花序から遠ざける仕組みを持つ例はいくつか報告されているが、送粉者がアリを忌避させる例はこれまで知られていない。オオバギ属では、アリに妨害されにくい送粉者の獲得が、アリ植物の進化に寄与したと考えられる。

第 4 章

総合考察

この研究では、被食防衛共生に関わる形質と送粉に関わる形質が進化の過程で相互に影響したことが示唆された。一部の種でアリを誘引するための葉の花外蜜腺の外適応によって、小苞葉の円盤状蜜腺を求めて訪花する昆虫による送粉様式が進化したと考えられるが、これは、防衛形質が送粉の進化に影響を与えた例だと考えることができる。送粉または防衛と関わる植物形質の外適応による他方の形質の進化は、物理的防衛や科学的防衛でも報告されている。また、アリからの攻撃を受けにくいアザミウマ *Dolichothrips* 属による送粉の獲得によって、アリが送粉を妨害するリスクが減少し、アリ植物への進化が促されたことが示唆されたが、これは送粉様式の変化により、送粉と防衛の間のコンフリクトが解消され、防衛形質の進化が起こりやすくなったと解釈できる。送粉と防衛の間のコンフリクトが植物の進化に与える影響は、これまでほとんど注目されてこなかった。これは、化学的・物理的防衛と送粉の間のコンフリクトが、被食防衛共生と送粉の間のそれに比べて深刻な影響を植物に与えないためだと考えられる。被食防衛共生関係を持つ植物では、蜜という送粉者の報酬ともなりうる分泌物を防衛アリへの報酬としていることと、アリは植物を訪れる動物を区別せず攻撃し、送粉者もアリによって排除されうることから、送粉共生と被食防衛共生は進化的に関係しやすいだろう。このような進化的関係は、アリと被食防衛共生を持つ植物の進化を正しく知る上で重要だと考えられる。

Summary

Chapter 1
General introduction

Plants have evolved various traits for defence against herbivores, and these traits can also affect non-herbivorous plant–animal interactions. The best studied examples of plant defence mechanisms against non-herbivorous animals involve evolutionary relationships between physical or chemical defences and pollination. Evolution by "exaptation" occurs in these relationships, whereby defensive traits acquire new pollination-related functions and pollination-related functions acquire defence-related characteristics. Many plants have biological defence systems in addition to physical and chemical defences. Examples of biological defences include protective mutualisms with ants, in which plants provide ants with food and/or shelter while ants protect the plants from herbivores. In this thesis, to investigate the evolutionary relationships between biological defence and pollination, I focused on the plant genus *Macaranga* (Euphorbiaceae). Most *Macaranga* species have facultative relationships with ants that are attracted to extrafloral nectaries on plant leaves; a few species are "ant-plants", which are inhabited and actively protected by a particular ant species. Pollination by thrips or hemipterans has been reported in a limited number of *Macaranga* species, but details about the pollination systems of most species remain unknown. The diversity and evolution of pollination systems in *Macaranga* is investigated in Chapter 2. In Chapter 3, I focus on the relationships among plants, pollinators and ant guards in ant-plant species. Based on these findings, I discuss evolutionary relationships between plant pollination and defence traits in Chapter 4.

Chapter 2
Diversity of pollination systems in *Macaranga*

Section 2.1
Diversity of bracteole morphology in *Macaranga*

Some *Macaranga* species are pollinated by thrips or hemipterans; the pollination systems in these species are uncommon in that plants offer breeding sites, rather than nectar, to pollinators. To reveal how the pollination system has evolved in *Macaranga*, I examined the diversity and the evolution of inflorescence morphology, focusing on bracteoles because they show considerable variation among species. First, I recorded the inflorescence traits of herbarium materials and recognised three inflorescence types: Discoid-gland, which possess disk-shaped glands on the bracteole surfaces; Enclosing, in which bracteoles cover flowers

(including all the thrips- and hemipteran-pollinated species); and Inconspicuous, in which bracteoles are small, narrow or absent. Second, I investigated the phylogeny of *Macaranga* based on four DNA markers. Information on inflorescence morphology was mapped according to phylogeny, and evolutionary changes in morphology were estimated by the most parsimonious reconstruction, which suggested that the Inconspicuous type was ancestral and that the Discoid-gland and Enclosing types may have occurred later. Inflorescence morphology has shifted among the three types at least 17 times. The known thrips- and hemipteran-pollinated species did not converge into a monophyletic clade, and the Enclosing-type inflorescence was estimated to have occurred at least four times. These findings indicate that pollination systems have changed frequently in *Macaranga*. The ecological and genetic factors driving these shifts will be a subject of future study.

Section 2.2

Wind and insect pollination (ambophily) in *Mallotus*, a sister group of *Macaranga*

Relatively few flowering plants show ambophily (pollination by both wind and insects), and whether and when ambophily is advantageous has not been well studied. Here, I report ambophily in two dioecious pioneer tree species of genus *Mallotus*, a sister group of *Macaranga*. Pollination of *Mallotus japonicus* and *Mallotus wrayi* was studied in a temperate forest of Japan and a tropical forest of Borneo, respectively. Both species set fruit when flower visitors were excluded, and substantial amounts of airborne pollen reached female trees, indicating the trees were pollinated by wind. Insects may also have contributed to fruit set because insects carrying pollen visited female inflorescences. Because *M. japonicus* and *M. wrayi* exhibit floral characteristics that are adapted to both wind and insect pollination, ambophily may be actively maintained in these two species. Previous studies have indicated that ambophily is advantageous to pioneer plants because of changing wind conditions during forest succession. Fruit set in female *M. japonicus* trees located far from male trees was more pollen-limited than that in trees closer to pollen sources, suggesting that changes in population density during forest succession may also contribute to the maintenance of ambophily in this species.

Section 2.3

Disk-shaped nectaries on bracteoles of *Macaranga sinensis* provide a reward for pollinators

Floral nectar is the most common reward provided by animal-pollinated flowers. Diversity in the position and structure of floral nectaries suggests that floral nectar production evolved repeatedly. Flowers of genus *Macaranga* are apetalous and lack nectar, but many

Macaranga species possess disk-shaped nectaries on their leaves that are sought by ants that defend the plants from herbivory. Similar glands also occur on the bracteoles (modified leaves subtending flowers) in some *Macaranga* species. I investigated whether bracteole glands in *M. sinensis* were involved in pollination. Observation and capture of flower visitors and examination of body pollen on these insects indicated that *M. sinensis* was pollinated by insects foraging on nectar secreted from bracteoles. Analysis and comparison of the sugar composition of nectar from leaves and bracteoles by high-performance liquid chromatography (HPLC) revealed no significant differences; the nectaries were also similar in appearance and position. These results indicated that nectaries on leaves were recruited to inflorescences to serve floral functions and that these nectaries facilitated evolution of the pollination system in *M. sinensis*.

Chapter 3
Interactions among plants, pollinators and guard ants in ant-plant *Macaranga*

Section 3.1
Density of ant guards on inflorescences and their effects on herbivores and pollinators

In protective ant–plant mutualisms, ants are beneficial for plant defence but they often have a negative effect on pollination by deterring pollinators. Interference with pollination is more severe in "ant-plants" (plants that are inhabited and actively protected by ants) than in plants protected by non-specialist ants attracted to extrafloral nectaries. Because little is known about the processes by which ant-plants are pollinated in the presence of ant guards, I examined ant interactions with herbivores and pollinators on plant reproductive organs. Among eight ant-plant and three non-ant-plant *Macaranga* species distributed in Borneo, ten were pollinated by thrips breeding in bracteole chambers on inflorescences (the pollination system in the remaining species could not be determined). Seven of the eight ant-plant species produced food bodies on young inflorescences and/or immature fruits. Food-body production was associated with increased ant abundance on inflorescences of the three non-ant-plant species observed. Exclusion of ants from inflorescences of one species without food rewards resulted in increased herbivory. In contrast, ant exclusion had no effect on the density of pollinator thrips. These results indicated that ants protected the inflorescence from herbivores and did not exclude pollinators.

Section 3.2

Anal secretions of pollinator thrips of *Macaranga winkleri* repel guard ants

In ant-plants, which are actively protected by the resident ants, the ants can negatively affect pollination by excluding pollinators as well as herbivores. In this section, I examine the ways by which the ant-plant *Macaranga winkleri* handles this potential conflict. *M. winkleri* is pollinated by thrips (*Dolichothrips* sp.). I conducted an experiment to categorise behavioural responses of ant guards to diverse insects. Ants were often deterred by pollinator thrips, especially when thrips secreted anal droplets. Conversely, guard ants attacked other types of insects. I then conducted chemical bioassays of guard-ant responses. Ants fled from thrips secretions and their most abundant constituent, n-decanoic acid, more often than they fled from controls. Thus, pollinator thrips probably deter ant attacks by anal secretion of ant-repellent droplets. To my knowledge, this is the first report of pollinators repelling guard ants. The evolution of pollination systems resistant to ant attacks may have predisposed the evolution of ant-plants in *Macaranga*.

Chapter 4

General discussion

This thesis suggests two evolutionary phenomena in which plant traits involved in protective mutualisms with ants might have affected the evolution of pollination, or in which pollination might have affected these mutualisms. The first is that bracteole glands used by pollinators originated by exaptation from pre-existing extrafloral nectaries that attract ant guards on leaves. This may represent a case in which defensive traits affected the evolution of pollination systems. Studies have reported on exaptation of floral traits involved in pollination to physical or chemical defences, and vice versa. The second is that pollination by *Dolichothrips*, which is resistant to ant attacks, might have reduced pollination interference by ants and facilitated the evolution of powerful defences in ants. In this case, changes in the pollination system resolved a conflict between pollination and protective mutualisms with ants and may have resulted in further evolution of the protective mutualisms. The role of conflict between pollination and physical or chemical defences in the evolution of plant traits has not been well studied, possibly because these conflicts are not as severe as those between pollination and defence by ants. Pollination and protective mutualisms with ants are more likely to interact than pollination and physical or chemical defence systems because most plants protected by ants secrete nectar that can also provide a reward for pollinators. In addition, guard ants indiscriminately attack plant herbivores and pollinators. Evolutionary relationships between pollination and protective

mutualisms are important for understanding the evolution of plants that have mutualistic relationships with ants.

Chapter 1
General Introduction

Because herbivores negatively affect the growth and reproduction of plants, plants have evolved various traits for defence against herbivores. The most common defence systems involve physical and chemical mechanisms (Schoonhoven *et al.*, 1998). Physical defences include thick leaves, thorns and trichomes, while chemical defences consist of secondary compounds of plants that are toxic to or repel herbivores. Plants also engage in interactions with animals other than herbivores, such as pollinators and seed dispersers. Traits used for defence can affect not only plant–herbivore interactions but also these other plant–animal interactions. The converse is also true: traits evolved through adaptation to pollinators or seed dispersers may affect plant–herbivore interactions. The best studied examples involve the evolution of traits by "exaptation". In contrast to adaptation, exaptation is a process by which a trait acquires a new function as a by-product of another adaptive trait (Gould & Vrba, 1982). In such cases, plant traits involved in either pollination or defence facilitate the evolution of the others' traits. In the Neotropical vine/shrub genus *Dalechampia* (Euphorbiaceae), pairs of large bracteoles that originally attracted pollinators visually gained a new function (i.e. the protection of floral organs against herbivores by enclosing flowers at night), thus facilitating the evolution of a new defence system (Armbruster, 1997; Armbruster *et al.*, 2009). In some *Cyperus* species (Cyperaceae), bracts that may have originally protected flower buds against herbivory have evolved extrafloral displays that visually attract pollinators by conspicuous colouration (Wragg & Johnson, 2011). In these processes, plant traits involved in pollination have influenced the evolution of physical defence and vice versa. In *Dalechampia*, some triterpenes used as floral defence in basal species are diverted to rewards for particular bee pollinators that collect triterpene resins for nest materials (in the derived species), thus facilitating the evolution of a new pollination system (Armbruster, 1997; Armbruster *et al.*, 2009). In *Cyperus*, monoterpenes originally used for defence are secreted from flowers as odours to attract pollinators (Wragg & Johnson, 2011). These examples illustrate mechanisms by which chemical defences have affected the evolution of pollination systems.

In addition to physical or chemical defence, many plants possess biological defence systems, such as protective mutualisms with ants. To attract ants, plants usually produce extrafloral nectar (nectar secreted from e.g. leaves or petioles) and/or food bodies (nourishing small particles). Because ants are aggressive natural enemies of many herbivores, they can reduce damage to host plants by excluding herbivores (Rico-Gray & Oliveira, 2007). Extrafloral nectaries are found in 2.0–3.6% of flowering plants and have occurred multiple times in

over 100 plant families (Marazzi *et al.*, 2013; Weber & Keeler, 2013). Among the tropical plants called "ant-plants", which provide nesting spaces (domatia) for the ants, some exhibit extremely strong ant defence. Their ant defence is commonly so strong that a lack of ants often causes serious damage and sometimes death to the host plants (Janzen, 1966; Vasconcelos, 1991; Itioka *et al.*, 2000; Heil *et al.*, 2001). Extremely strong ant defence has been observed in some species of *Acacia* (Fabaceae), *Macaranga* (Euphorbiaceae), *Cecropia* (Cecropiaceae), *Barteria* (Passifloraceae) and others (Beattie, 1985; Davidson & McKey, 1993; Rico-Gray & Oliveira, 2007).

Ant guards can both positively and negatively affect plant reproduction. Ants may exclude flower- or fruit-damaging herbivores and thereby promote plant reproduction (Willmer & Stone, 1997). Therefore, some plant species possess extrafloral nectaries on their inflorescences that attract nectar-harvesting ants, which in turn reduce herbivore damage to plant reproductive organs (Oliveira *et al.*, 1999; Falcão *et al.*, 2003; Vesprini *et al.*, 2003; Gaume *et al.*, 2005; Chamberlain & Holland, 2008; Martins, 2009; Hernández-Cumplido *et al.*, 2010; Schmid *et al.*, 2010; Dejean *et al.*, 2011; Subedi *et al.*, 2011). In contrast, ants may interfere with pollination, primarily because they can exclude pollinators from inflorescences (Willmer & Stone, 1997; Altshuler, 1999; Tsuji *et al.*, 2004; Ness, 2006; Willmer *et al.*, 2009). To avoid pollination interference by ants, many plants have evolved mechanisms that deter ants, including repellent chemicals (Janzen, 1977; Guerrant & Fiedler, 1981; Willmer & Stone, 1997; Ghazoul, 2001; Raine *et al.*, 2002; Agarwal & Rastogi, 2008; Junker & Blüthgen, 2008; Willmer *et al.*, 2009), slippery waxy shoots (Harley, 1991), extrafloral nectaries that attract ants away from flowers (Wagner & Kay, 2002; Galen, 2005; Holland *et al.*, 2011) and narrow corollas (Prŷs-Jones & Willmer, 1992; Galen *et al.*, 1999; Galen & Cuba, 2001; Blüthgen *et al.*, 2004). Therefore, conflicts may exist between pollination and protective mutualisms with ants.

While evolutionary relationships between pollination and physical or chemical defence have been studied in several plant groups (Armbruster, 1997; Wragg & Johnson, 2011), those between pollination and protective plant–ant mutualisms have not yet been examined. The question remains as to whether evolution by exaptation, an evolutionary pattern between pollination and physical or chemical defence, also exists between pollination and protective mutualisms. Furthermore, whether the conflict between pollination and protective mutualisms, in which ants interfere with pollination, affects the evolution of pollination systems or protective mutualisms remains unknown.

To address the evolutionary relationships between pollination and defence by ants, I focused on the plant genus *Macaranga* (Euphorbiaceae). This genus is suitable for the objective of this dissertation because some *Macaranga* species are ant-plants that are strongly protected by ants, and the evolution of this interaction with ants has been studied

to some extent. *Macaranga* includes about 260 species distributed in tropical–subtropical regions of Africa, Madagascar, Asia and Oceania (Whitmore, 2008). *Macaranga* exhibits protective mutualisms with ants across the genus (Whalen & Mackay, 1988; Fiala & Maschwitz, 1991; Mackay & Whalen, 1991). Most of the species are protected by ants dwelling out of their bodies. They attract ants through extrafloral nectaries located on adaxial surfaces of leaf lamina and/or leaf margins and sometimes by food bodies (i.e. small particles that contain carbohydrates and proteins) on the leaf surface and/or petioles (Whalen & Mackay, 1988; Fiala & Maschwitz, 1991, 1992a; Mackay & Whalen, 1991; Heil *et al.*, 1998). In contrast, about 30 species distributed in South-East Asia (Borneo, Sumatra and the Malay Peninsula) are strongly protected ant-plants that sustain mainly *Crematogaster* (Formicidae: Myrmicinae) and occasionally *Camponotus* (Formicidae: Formicinae) ants in their hollow stems and offer food bodies from stipules and/or surfaces of new leaves as nourishment to ants (Fiala *et al.*, 1989, 1994; Fiala & Maschwitz, 1992a; Itioka *et al.*, 2000). *Macaranga* ant-plants and the guard ants almost always exhibit species-specific relationships with one another (Quek *et al.*, 2004). The ant-plant species are intensely protected by ants, such that the absence of ants often results in serious damage to the plants (Itioka *et al.*, 2000). Several previous studies have indicated that ant-plants have been independently derived two to four times in part of the derived clade in *Macaranga* (Blattner *et al.*, 2001; Davies *et al.*, 2001; Bänfer *et al.*, 2004).

In contrast to protective mutualisms with ants, little is known about pollination systems in *Macaranga*. Within the genus, about 20 and two species have been reported to be exclusively pollinated by thrips and hemipterans, respectively (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011). Both thrips- and hemipteran-pollinated species possess imbricate bracteoles that cover flowers lacking perianths (Fig. 1.1). The bracteoles harbour trichome- and/or ball-shaped nectaries on the adaxial surface. Because the nectar is secreted from the tufts of trichome-like nectaries or is contained in the ball-shaped nectaries, only insects with a needle-like proboscis can obtain nectar. Pollinators of the thrips-pollinated species are *Dolichothrips* sp. 1 and *Dolichothrips* sp. 2 (Phlaeothripidae) (Fiala *et al.*, 2011). While each thrips-pollinated species is visited by either species of *Dolichothrips*, the pollinator thrips utilize multiple *Macaranga* species. The thrips have not been found in plants other than *Macaranga*. The thrips mate, oviposit and grow during their larval stage in the *Macaranga* inflorescences. *Dolichothrips* sp. 1 spends its pupal stages underground, whereas *Dolichothrips* sp. 2 stays in the inflorescences during pupal stages (Fiala *et al.*, 2011). The pollinators of *M. tanarius* and *M. heynei* are multiple species of Hemiptera belonging to the families Miridae and Anthocoridae. These two *Macaranga* species do not share pollinators. While host specificity of hemipterans is largely unknown, some of the pollinator species are also found in plants other than *Macaranga*. Similar to the thrips pollinators, the hemipteran pollinators oviposit and spend their larval stages in the bracteole

chambers. While the thrips and hemipteran pollination systems are very similar, the evolutionary relationship of the two pollination systems is unknown. Because thrips pollination has only been reported for ant-plant *Macaranga* species, the pollination system may bear some relationship with the evolution and maintenance of ant-plants in *Macaranga*.

In this dissertation, I examine the evolutionary relationships between pollination and biological defence in *Macaranga*. Because pollination systems have been studied in a limited number of *Macaranga* species, I first investigate the diversity of pollination systems in *Macaranga* and its sister genus *Mallotus* in Chapter 2. In Section 2.1, the diversity and evolution of pollination systems are inferred by assessing the inflorescence morphology of species in most infrageneric groups and the phylogenetic relationships thereof. Subsequently, I examine the pollination systems of two *Mallotus* species and one *Macaranga* species in Sections 2.2 and 2.3. The inflorescence morphologies of the two *Mallotus* and the one *Macaranga* species differ from each other and also from those of thrips- and hemipteran-pollinated species. In Chapter 3, I investigate tripartite interactions among plants, pollinators and guard ants. In Section 3.1, I show that the effects of ants on pollinator thrips are not significant. Therefore, I examine how interference with pollination by ant guards is prevented in Section 3.2. Based on the results, I discuss the evolutionary relationships between plant traits for pollination and those for biological defence in Chapter 4.

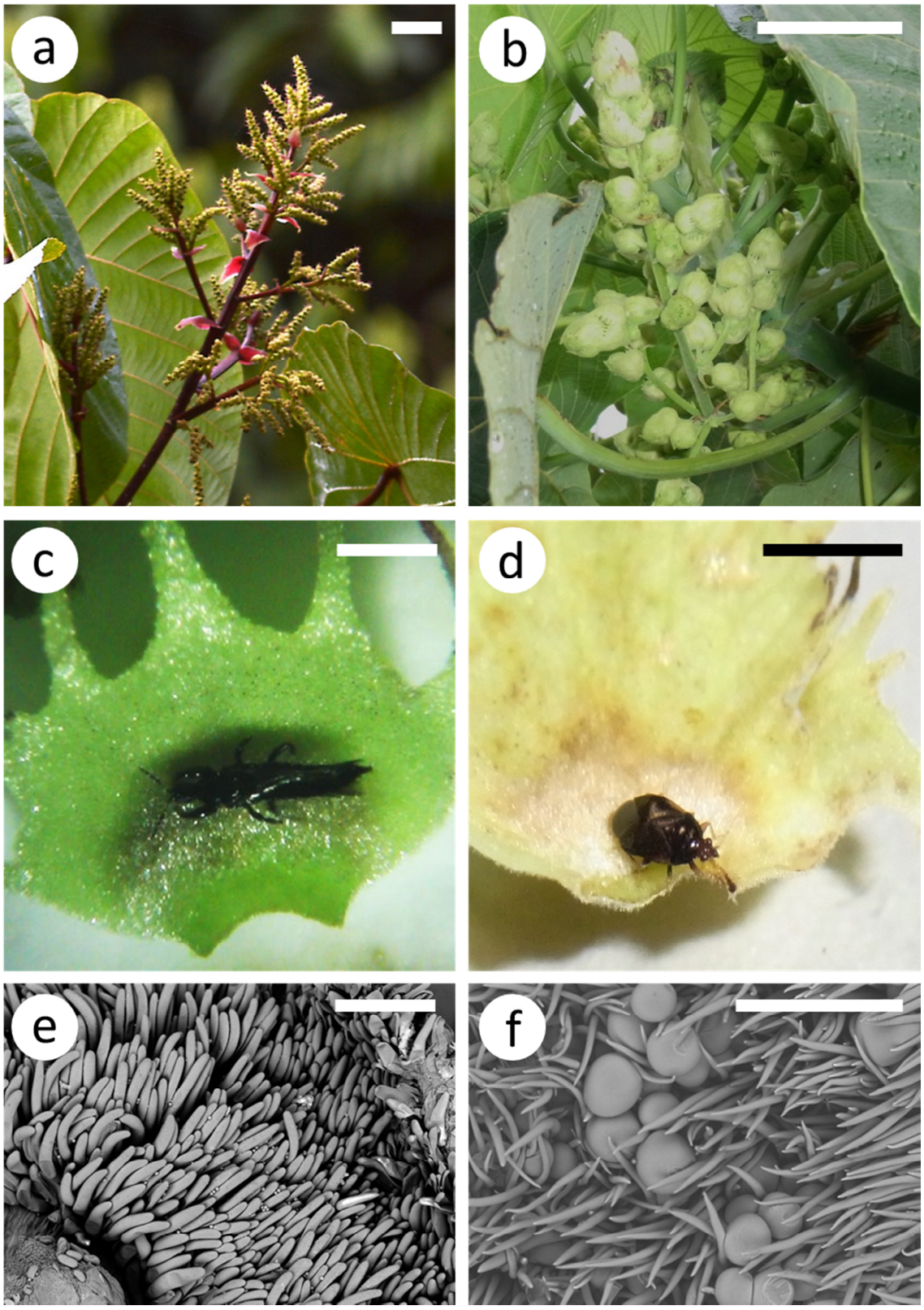

Fig. 1.1 (a, b) Male inflorescences of *Macaranga winkleri* and *M. tanarius*, respectively. (c, d) Pollinators *Dolichothrips* sp. and *Orius atratus* (Anthocoridae) on bracteoles of *M. winkleri* and *M. tanarius*, respectively. (e, f) Trichome-like nectaries and trichome-like and

ball-shaped nectaries on the adaxial surfaces of bracteoles of *M. winkleri* and *M. tanarius*, respectively. Scale bars are 3 cm in (a) and (b), 1 mm in (c), 3 mm in (d) and 100 μm in (e) and (f).

Chapter 2
Diversity of pollination systems in *Macaranga*

Section 2.1
Diversity of bracteole morphology in *Macaranga*

Introduction

Many plant species depend on animals for pollination (Ollerton *et al.*, 2011) and have evolved to provide pollinator rewards to ensure efficient pollen delivery (Fægri & van der Pijl, 1979). While floral nectar and/or pollen grains are commonly offered as rewards for pollinators such as bees, flies, beetles, butterflies and moths, some plant groups offer unusual alternatives. Among these are oil, resin or pheromone precursors for some bees (Dressler, 1982; Armbruster, 1984; Steiner & Whitehead, 1991), ovules for wasps and moths (Weiblen, 2002; Kato *et al.*, 2003; Pellmyr, 2003) and heat for beetles and flies (Thien *et al.*, 2000).

Some species in the genus *Macaranga* offer unique rewards to unusual pollinators, e.g. thrips in the genus *Dolichothrips* and hemipterans. Rewards include breeding sites formed by flower-enclosing bracteoles and trichome-like nectaries located on the adaxial surfaces of the bracteoles (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011). Although bracteoles sometimes attract pollinators (Armbruster *et al.*, 2009), they seldom offer rewards, making this pollination system particularly interesting. However, whether the bracteole chambers of thrips- and hemipteran-pollinated *Macaranga* plants have a similar origin is unclear.

To reveal how the unique pollination systems of the genus *Macaranga* have evolved, I studied the diversity and evolution of the plants' inflorescence morphologies, which often provide useful information for estimating pollination systems (Fægri & van der Pijl, 1979; Proctor *et al.*, 1996). I focused on bracteoles, rather than flowers, because showy petals or calyxes that generally attract pollinators do not exist in *Macaranga* species (Whitmore, 2008), while bracteoles displayed considerable interspecies variation. Pollinator-rewarding bracteoles are rarely found in plants other than those in the genus *Macaranga*. For example, bracteoles of the sister genus *Mallotus* are absent, early caduceus or very tiny (Sierra & van Welzen, 2005; Sierra *et al.*, 2006, 2007; Kulju *et al.* 2007). I first examined variation of inflorescence morphologies among species. Then, I mapped the inflorescence characteristics on a molecular phylogenetic tree and estimated ancestral inflorescence morphologies. Based on my results, I discuss how inflorescence morphologies have evolved in *Macaranga* species.

Materials and Methods

Observation of inflorescence/floral morphologies

I observed the inflorescences of dry specimens of 53 taxa in the genus *Macaranga* (52 species and one variety) in herbaria (K, L, KYO and SAR). I recorded (1) presence/absence of disk-shaped glands on bracteole surfaces, (2) internode distances between adjacent bracteoles and (3) length and (4) width of bracteoles of male specimens and (5) style length in female specimens (Fig. 2.1.1). For each trait, I looked at 2–5 samples from each of 1–5 specimens. For trait (1), I judged disk-shaped glands to be present when at least one bracteole possessed them and I determined that specific taxa possessed the glands if they occurred in at least one specimen. While presence/absence of the glands was consistent in most species, I was unable to detect them in some specimens of *Macaranga denticulata*, which I evaluated these specimens as possessing disk-shaped glands. The shape of the bracteoles which did not possess disk-shaped glands was similar to those in which I detected glands. For quantitative traits (2)–(5), average values were calculated for each specimen and averaged across specimens to obtain species values.

To examine interspecies variation in inflorescence/floral morphologies, I conducted a principal components analysis (PCA) using *Z*-score standardised values of data from the four quantitative traits. Thirty-two taxa in which all four variables were available were included in the analysis. I used the prcomp function of the R statistical package in R 3.0.2 (R Development Core Team, 2013).

The first principal component (PC1) clearly separated species that did not contain disk-shaped glands into two groups, and mainly reflected variations in bracteole length and width (see Results). Therefore, I classified all 53 taxa into three inflorescence types based on presence/absence of disk-shaped glands, and bracteole shape and size (see Results). I also classified two additional species, *Macaranga lamellata* and *Macaranga umbrosa*, having obtained morphological trait data from Fiala *et al*. (2011); however, I did not observe specimens of these species.

Molecular phylogenetic analysis

A molecular phylogeny was constructed based on DNA sequence data on one plastid (*trnL-F*) and three nuclear (ITS, *ncpGS* and *phyC*) markers of 59 taxa in the genus *Macaranga* and species of related genera in the family Euphorbiaceae (*Mallotus japonicus*, *Mallotus paniculatus* and *Cordemoya integrifolia*). Sequence data on 58 *Macaranga* taxa and *C. integrifolia* were acquired from GenBank (Kulju *et al*., 2007) and those of *Macaranga sinensis*, *Mallotus japonicus* and *Mallotus paniculatus* were obtained via the following

procedures. First, DNA was extracted from silica gel-dried leaves following a modified CTAB procedure (Doyle & Doyle, 1987; Okuyama & Kawakita, 2012). Regions were amplified by different primer pairs: *trnL-F* was amplified by c+d and e+f, ITS was amplified by ITS5+ITS4, *ncpGS* was amplified by GSKKf1+GSKKr2 and *phyC* was amplified by PHYC-F+PHYC-R (Kulju *et al.*, 2007). Polymerase chain reaction (PCR) amplifications were carried out in 20-μL reactions using 1.0 μL of the total DNA extract as template. The reaction mixture also contained 1.6 nmol of dNTPs, 4 μL of 5× Ampdirect® (Shimadzu, Kyoto, Japan), 4 μL of 5× Amp Addition-3 (Shimadzu), 8 pmol of each primer and 0.5 U of Ex Taq™ Polymerase (TaKaRa, Otsu, Japan). The PCR program was as follows: initial denaturation step at 94°C for 5 min and 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 7 min. Products were sequenced on an ABI 3100 automated sequencer using BigDye chain termination chemistry (Applied Biosystems, Foster City, California, USA). The primers used in amplification were also used for sequencing.

The sequences were aligned using MEGA 5.05 with manual correction of obvious errors. A majority-rule consensus tree was constructed from 100 replicates of bootstrap analysis under the maximum likelihood criterion in TREEFINDER (March 2011 version). Base substitution models were chosen for each gene separately.

Reconstruction of ancestral inflorescence morphologies

To investigate how inflorescence type has shifted in the genus, I conducted a parsimonious estimation of ancestral inflorescence morphologies using Mesquite 2.74 (Maddison & Maddison, 2010) with a bootstrap consensus tree. I did not use the single maximum-likelihood tree as in the above analysis because many of the basal tree nodes were poorly supported, which may produce biased results in ancestral state reconstruction analyses. Inflorescence morphology types were treated as categorical variables with three states (Fig. 2.1.2; see Results). This analysis assumes that shifts among the three types occur with the same probability.

Results

Observation of inflorescence/floral morphologies

The first and second principal components (PC1 and PC2) of the PCA contributed 54.1% and 24.6% of the total variance, respectively (Table 2.1.1). Bracteole size (length and width) had a positive loading, and style length had a negative loading in PC1 (Table 2.1.1, Fig. 2.1.3). PC1 distinctly separated species not possessing disk-shaped glands into two groups (Fig. 2.1.3): one with relatively large bracteoles and short styles, and one with small

bracteoles and long styles. PC2 mainly represented internode distances between bracteoles and bracteole length, both as negative loadings (Table 2.1.1, Fig. 2.1.3), and some species with disk-shaped glands had extremely low values. The two groups distinguished by PC1 were not represented by PC2.

Based on these results, inflorescence types were classified into three categories using bracteole shape and size and presence/absence of disk-shaped glands: Discoid-gland, Inconspicuous and Enclosing. Style length was not used because I was unable to measure it for many species, mostly due to a lack of specimens containing flowering female inflorescences. The three categories are defined as follows:

(1a) Disk-shaped glands on the bracteole surfaces present ……Discoid-gland
(1b) Disk-shaped glands on the bracteole surfaces absent ……..…. (2)
(2a) Bracteoles very small or narrow (length/width > 1.8), or absent
……..Inconspicuous
(2b) Bracteoles relatively large, enclosing flower clusters…....……..……..Enclosing

All species mainly visited by thrips or hemipterans were of the Enclosing type (Figs. 2.1.3 and 2.1.4), as also observed ant-plant species (Fig. 2.1.4). *Macaranga sinensis*, pollinated by generalist insects attracted to disk-shaped nectaries on bracteoles (Section 2.3), and *Macaranga denticulata* and *Macaranga indica*, whose male inflorescences are mainly visited by generalist insects (bees, flies, wasps and beetles; Fiala *et al.*, 2011) were of the Discoid-gland type (Fig. 2.1.4).

Molecular phylogenetic analysis

The following substitution models were selected for each DNA marker: J3+G for *trnL*, GTR+G for ITS and J1+G for *ncpGS* and *phyC*. The majority-rule consensus tree obtained from bootstrap analysis detected two well-supported basal clades (B1 and B2) and a crown clade, as in Kulju *et al.* (2007), who analysed the phylogeny by the most parsimonious and Bayesian methods (Fig. 2.1.4). Although the crown clade contained many unresolved nodes, it was further roughly classified into three clades (C1, C2 and C3) as in Kulju *et al.* (2007).

Reconstruction of ancestral inflorescence morphologies

All observed species in basal clades B1 and B2 were classified into the Inconspicuous category (Fig. 2.1.4). Conversely, I detected all three inflorescence types in the crown clades. No inflorescence type was determined to be monophyletic. The most parsimonious ancestral state reconstruction indicated that shifts among the three bracteole morphology types occurred at least 16 times within the crown clade.

Discussion

All *Macaranga* species in this study were classified into three inflorescence types based on bracteole morphological characteristics: Discoid-gland, Inconspicuous and Enclosing (Fig. 2.1.2). Because almost all species in *Macaranga* basal clades are of the Inconspicuous type and *Mallotus*, a sister genus, has similar morphologies, the ancestral inflorescence morphology may have been of this type. The Enclosing or Discoid-gland types may have appeared when the crown clade diverged, and inflorescence morphologies may have shifted among the three types at least 16 times in this clade, assuming that shifts among the three types occur with the same probability. Reversion to the Inconspicuous type occurred in the clades at least six times. I also detected shifts between the Enclosing and Discoid-gland types, in which the Discoid-gland type changed into the Enclosing type more frequently than the reverse.

I propose that the three types of inflorescence morphologies are related to different pollination systems. Wind may contribute at least in part to pollination of Inconspicuous-type species. I observed that *Macaranga vedeliana*, classified into the Inconspicuous category (not included in the phylogeny), is wind-pollinated (E. Yamasaki, unpublished data). Furthermore, two *Mallotus* species, whose inflorescences are similar to those of Inconspicuous *Macaranga* species, are pollinated by both wind and generalist insects. The insects are attracted to the pollen and floral nectar of male flowers and occasionally visit female flowers (Section 2.2). Exposed flowers of Inconspicuous-type plants are suitable for dispersing pollen grains into the air and catching airborne pollen. They often also have extremely long (up to 5 cm) styles, which may enable them to efficiently catch airborne pollen. While floral nectar has not been reported in *Macaranga* species (e.g. no nectar was secreted by *M. vedeliana* and *Macaranga coriacea*, classified into the Inconspicuous category; E. Yamasaki, unpublished data), insects may occasionally act as pollinators if pollen-collecting insects visit both male and female flowers by chance.

Species in the Discoid-gland category may be pollinated by insects that forage on disk-shaped nectaries. One Discoid-gland species, *M. sinensis*, was pollinated by generalist insects that collected pollen grains of male flowers and foraged on disk-shaped nectaries of male and female inflorescences (Section 2.3, Yamasaki *et al.*, 2013). Similarly, male inflorescences of two other Discoid-gland species, *M. denticulata* and *M. indica*, are visited by generalist insects such as bees, wasps, flies and beetles, although whether they feed on gland secretions is unknown (Fiala *et al.*, 2011). As in *M. sinensis*, disk-shaped glands on bracteoles of other species may also offer rewards to insects if nectar is secreted. Attracted insects may contribute to pollination because the glands are adjacent to flowers and the anthers/stigmas would likely be touched. This inflorescence type seems to have been

acquired at least twice in the genus, based on the reconstruction of ancestral morphologies. It may have evolved multiple times because of the plants' protective mutualisms with ants (Chapter 4).

Several species containing Enclosing-type inflorescences are pollinated by thrips or hemipterans (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011). Other Enclosing-type species may also be pollinated by small insects because their flower-enclosing bracteoles are likely to prevent relatively large insects from accessing flowers and bracteole chambers. This may explain why shifts from the Enclosing type to the Discoid-gland type are less likely than the reverse: generalist pollinators may seldom access flowers of Enclosing-type species, so disk-shaped glands, which seem to reward generalist pollinators, are not likely to evolve often. Wind pollination is unlikely for this inflorescence type because the bracteoles may interfere with pollen dispersal and with catching airborne pollen. The Enclosing category included ant-plants, many of which are pollinated by thrips in the genus *Dolichothrips* (Moog *et al.*, 2002; Fiala *et al.*, 2011). Because pollinator thrips are resistant to attacks by ant-guards, the evolution of thrips pollination may be related to ant-plant evolution (Chapter 3). The relationship between thrip pollination and ant-plants is discussed further in Chapters 3 and 4.

The Enclosing inflorescence type has evolved at least four times (Fig. 2.1.4) and hemipteran pollination seems to have evolved at least twice. Hemipteran pollination of *Macaranga tanarius* may have evolved independently from that of *Macaranga heynei* or *Macaranga trichocarpa*. However, whether hemipteran pollination of *M. heynei* and *M. trichocarpa* had the same origin is unknown. *Macaranga heynei*, a hemipteran-pollinated species, was included in the clade of thrips-pollinated species, which may also be the case for *M. trichocarpa*. Because hemipteran and thrips pollination systems are similar, they may have the same origin, but the order of evolution is ambiguous in the present study. Past work, looking at the phylogeny of *Macaranga* species and focussing on C1 species, has indicated that the hemipteran-pollinated *M. heynei* may have diverged before the diversification of thrips-pollinated species (Blattner *et al.*, 2001; Davies *et al.*, 2001; Bänfer *et al.*, 2004). Therefore, thrips pollination may have evolved more recently than hemipteran pollination in this clade. However, more extensive molecular phylogenetic analysis is needed to verify this hypothesis.

The present study indicates that unique pollination systems, whereby nectaries outside flowers or bracteole chambers are rewards for pollinators, have evolved multiple times in the genus *Macaranga*. Because mutualistic relationships exist throughout the genus, ants may have contributed in part to the evolution of these unique rewards (Chapter 4). Future studies should focus on the ecological and genetic factors that drove the evolution of *Macaranga* pollination systems.

Table 2.1.1 The proportion of variance and factor loadings of principal components analysis axes using four inflorescence traits.

	PC1	PC2	PC3	PC4
Proportion of variance	54.11%	24.60%	19.11%	2.18%
Internode between bracteoles	–0.20	–0.96	0.02	–0.18
Length of bracteole	0.61	–0.26	–0.35	0.66
Width of bracteole	0.65	0.00	–0.24	–0.72
Style length	–0.41	0.08	–0.91	–0.06

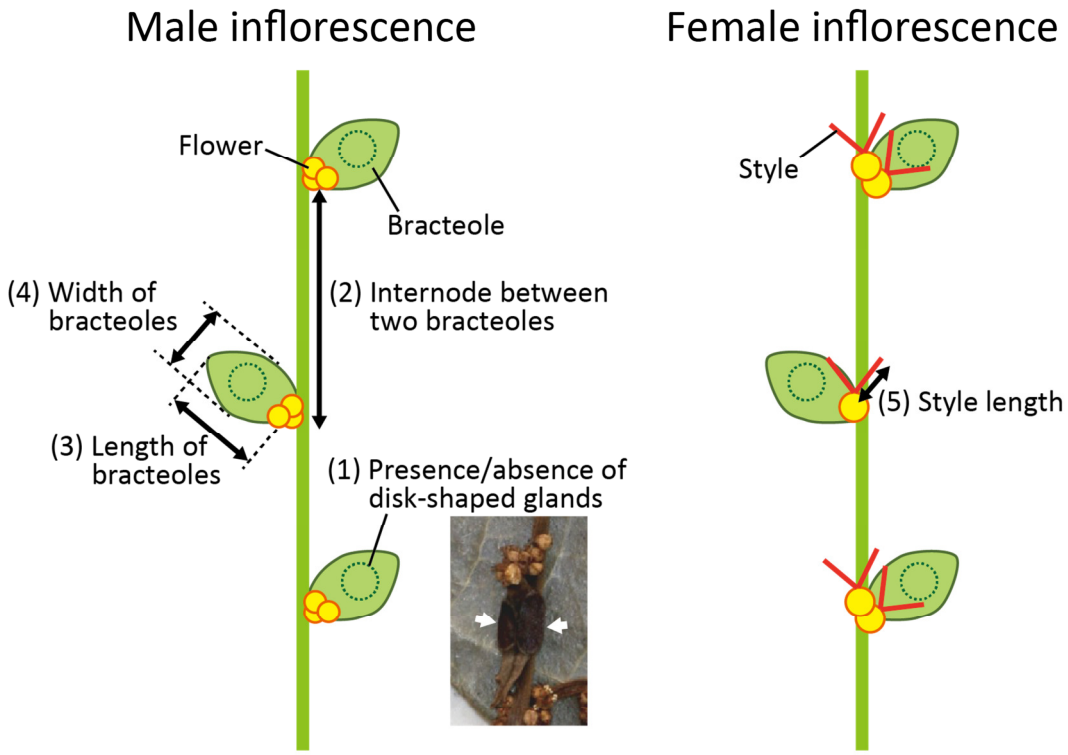

Fig. 2.1.1 The inflorescence/floral traits investigated. See (1)–(5) in the text.

Fig. 2.1.2 Examples of species with the three inflorescence types, categorised based on bracteole morphologies, (a) *Macaranga gigantea* (Enclosing type), (b) *Macaranga sinensis* (Discoid-gland type) and (c) *Macaranga coriacea* (Inconspicuous type), not included in the phylogeny. Scale bar = 1 cm.

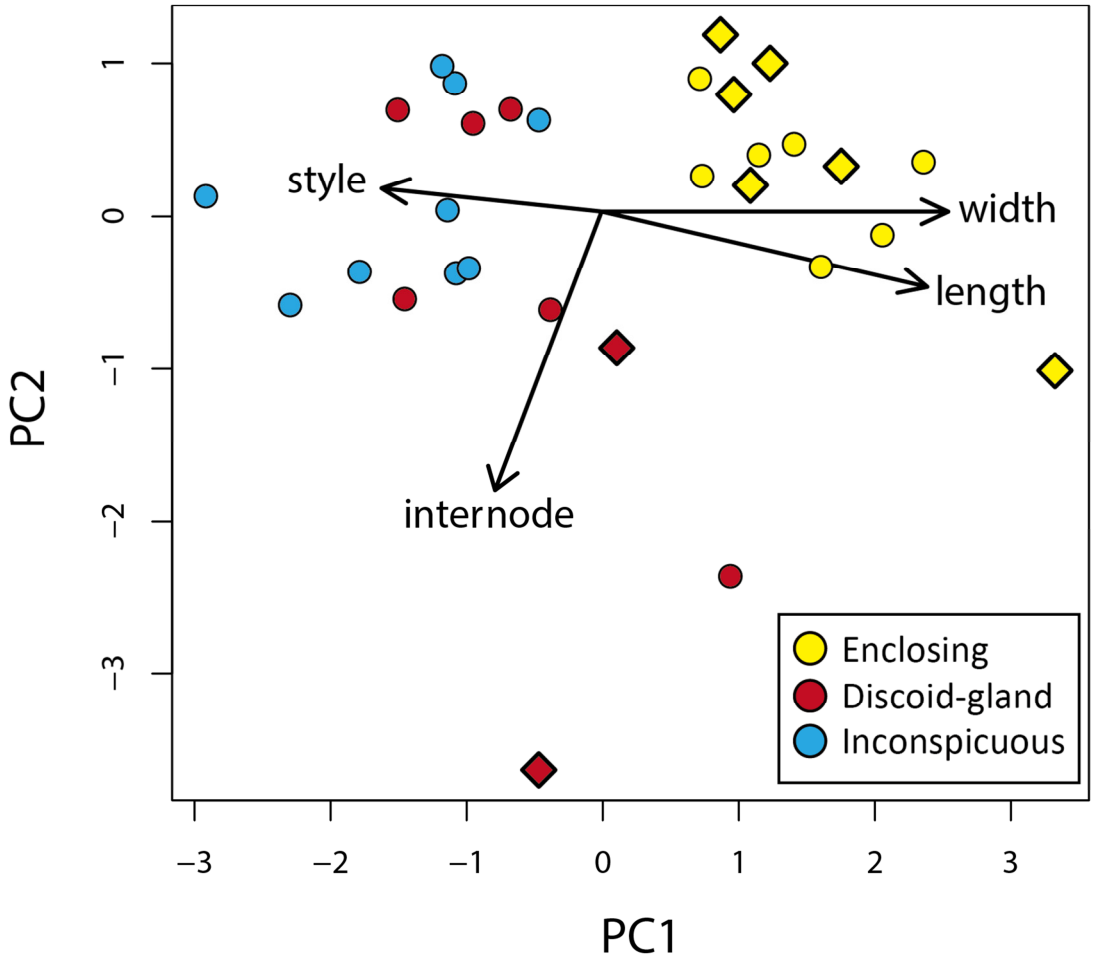

Fig. 2.1.3 Scatterplot of the first and second principal components (PC1 and PC2) of a principal components analysis (PCA) using four inflorescence and floral traits. Different colours indicate inflorescence types (see text for classifications). Species whose main flower visitors are known are indicated by square symbols.

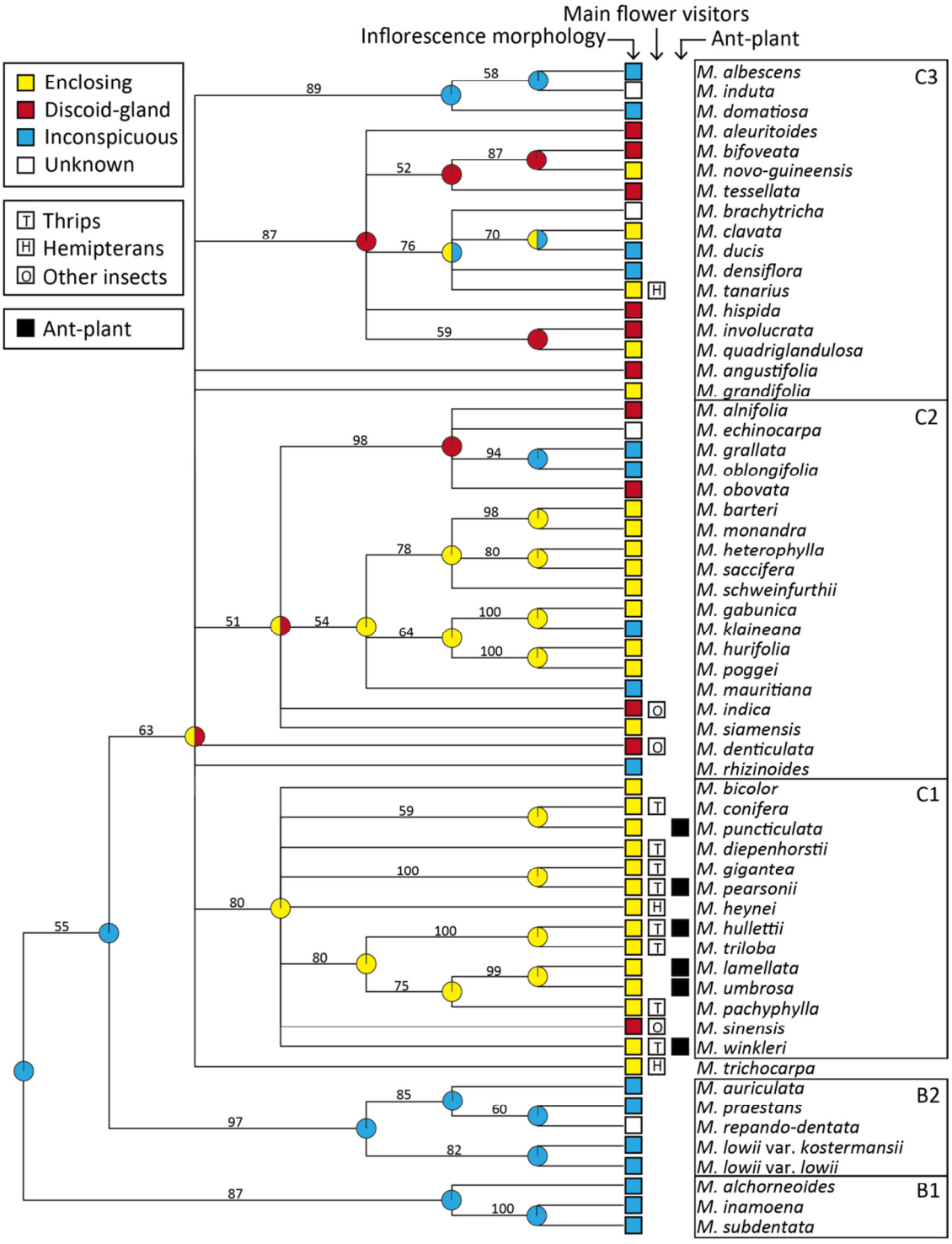

Fig. 2.1.4 A reconstruction of ancestral inflorescence morphologies in *Macaranga* species using maximum parsimony analysis on the consensus tree obtained from bootstrap analysis. Numbers above branches are bootstrap values (>50) and estimated ancestral morphologies are denoted by circles. The main flower visitors and whether the species are ant-plants are shown next to the inflorescence morphology types. Clade grouping (B1, B2, C1, C2 and C3) is according to Kulju *et al.* (2007).

Section 2.2
Wind and insect pollination (ambophily) in *Mallotus*, a sister group of *Macaranga*

Introduction

To transfer pollen grains efficiently from anthers to stigmas, flowering plants have more or less specialised their flowers and/or inflorescences to their pollen vectors (Fægri & van der Pijl, 1979). For example, animal-pollinated (mostly insect-pollinated) flowers are often conspicuous in colour and shape. In addition, they often have adhesive pollen grains and rewards for pollinators such as nectar. Wind-pollinated plants usually produce plenty of powdery pollen and inconspicuous small flowers without nectar. Their stamens and pistils are often exposed outside of the leaf-mass (Fægri & van der Pijl, 1979). Possessing flowers that are suitable for both insect and wind pollination may be costly, because insect- and wind-pollinated plants are expected to allocate resources in different ways; animal-pollinated plants often possess conspicuous petals and/or smell to attract many pollinators, whereas wind-pollinated plants produce large amount of pollen because male reproductive success depends on the number of pollen grains (Fægri & van der Pijl, 1979). In addition, flower characteristics that are suitable for one pollination system often conflict with those for other systems. For example, sticky pollen grains and/or pollinia of many animal-pollinated flowers can be expected to be less likely to be delivered by wind. Only a limited number of plant species are known to employ both wind and insect pollination (ambophily).

Although ambophily is often considered an intermediate condition during a transition to either full wind pollination or biotic pollination (Culley *et al.*, 2002), some studies have suggested that ambophily can be advantageous in environments where conditions favouring either wind or biotic pollination vary spatially and temporally. For example, in alpine regions, populations of effective insect pollinators decline with increased elevation (Warren *et al.*, 1988), whereas wind conditions may be similar along elevational gradients (Gómez & Zamora, 1996). To ensure seed production throughout an elevation gradient, having a wind-pollination system as reproductive insurance may be advantageous for some alpine plants, such as *Hormathophylla spinosa* (Brassicaceae) (Gómez & Zamora, 1996) and some alpine *Salix* species (Salicaeae) (Peeters & Totland, 1999; Totland & Sottocornola, 2001). Other ambophilous plants are pioneer plants adapted to early stages of forest succession; they include *Salix* spp. (Salicaceae) (Tamura & Kudo, 2000; Karrenberg *et al.*, 2002) and *Azadirachta indica* (Meliaceae) (Vikas & Tandon, 2011). Early successional forests are exposed to the wind; thus, wind pollination is suitable for such habitats

(Stellman, 1984; Goodwillie, 1999). However, along with succession, forests gradually become enclosed and wind may diminish within the forests. Reproductive success by wind pollination may decrease, and insect pollination may become relatively more important (Stellman, 1984; Goodwillie, 1999).

In the present study, I examined the pollination system of *Mallotus japonicus* (Euphorbiaceae) in temperate Japan and *M. wrayi* on Borneo Island, Malaysia. *Mallotus* is a genus of ~150 species of dioecious trees or shrubs distributed mainly in palaeotropical regions. Most *Mallotus* species are pioneers, but they occur in various habitats, from secondary forests and riverbanks to the understorey of primary forests (Slik, 2005; Sierra *et al.*, 2007). The physical appearance of inflorescences and flowers of most *Mallotus* species appears to indicate that they are wind-pollinated; the flowers are apetalous and the anthers and stigmas are exposed. However, several studies have reported visitation by insects, such as bees and syrphids, to the male inflorescences of *Mallotus* (Lock & Hall, 1982; Momose *et al.*, 1998; Sierra *et al.*, 2007), and my preliminary study revealed that male flowers of *M. japonicus* and *M. wrayi* produce nectar and that male and female inflorescences of *M. japonicus* have a sweet scent (E. Yamasaki, unpublished data). The goals of this section were to test whether wind and insect visitors contribute to the pollination of the two species and to determine which factors are involved in the maintenance of the pollination system.

Materials and methods

Study species

Trees of *M. japonicus* are distributed in temperate and subtropical regions of eastern Asia. They are dioecious pioneer trees occurring mostly in young secondary forests (Horikawa, 1972). These trees grow up to 10 m in height and become reproductive from ~1 m tall and 2 cm diameter at breast height (DBH). Male trees have several-branched panicles 10–20 cm long (Fig. 2.2.1a). They are formed by tiny apetalous flowers that harbour 60–90 stamens (Sierra *et al.*, 2010). Male flowers secrete flower nectar (0.27 μL per flower with 29% sugar content on average, as determined using a sugar refractometer to assay nectar collected by 0.5-μL microcapillaries; E. Yamasaki, unpublished data). The pollen grains are dry and measure ~23.0 × 25.3 μm in size (Nowicke & Takahashi, 2002). Female inflorescences are composed of non- or several-branched panicles 5–10 cm long formed by tiny apetalous flowers (Fig. 2.2.1b). Each flower has three- or four-branched dry and papillose stigmas. Female flowers do not secrete nectar. Both male and female inflorescences emit similar sweet scents. Flowering occurs almost synchronously within a population and lasts for ~2 weeks. Female flowers open synchronously within an inflorescence, whereas male flowers open sequentially and fall 1–2 days after opening. The fruits mature ~1 month after flowering. Each fruit has three or four locules.

Mallotus wrayi trees are small, up 23 m in height, distributed in Peninsular Malaysia, Sumatra and Borneo (van Welzen & Sierra 2006). They are dioecious trees found widely in primary and secondary forests on the islands. They are reproductive from ~1 m tall and 1 cm DBH. Both staminate and pistillate inflorescences (Fig. 2.2.1c, d) are 5–10 cm long and are rarely branched. Male flowers are apetalous and have 18–40 stamens (Sierra *et al.*, 2010), and the pollen grains are similar to those of *M. japonicus*. They secrete small amounts of nectar (0.04 μL per flower, with 8.6% sugar content on average, E. Yamasaki, unpublished data). Each female flower has one pistil with a three- or four-branched dry and plumose stigma, and does not secrete nectar. I was unable to detect scent from the flowers. Each fruit has three locules. The durations of flowering and fruiting are similar to those of *M. japonicus*.

Study sites

Studies on *M. japonicus* were conducted in June and July 2009, in Seta Park, Otsu, Shiga Prefecture, Japan (34°50' N, 135°50' E). This city park is mostly covered by a young secondary forest. The study area was a bank of a small straight stream (~3 m in width). Wild pioneer plants such as *M. japonicus* and locust trees (*Robinia pseudoacacia*, Fabaceae) stand linearly along the stream banks. Annual mean temperature is 14.9°C, and mean temperatures in June and July were 21.9°C and 25.8°C, respectively (Japan Meteorological Agency, http://www.jma.go.jp/jma/index.html; 27 November, 2012). *M. japonicus* flowers from June to July at the site. Annual total rainfall is ~1500 mm (Japan Meteorological Agency).

Studies on *M. wrayi* were conducted in October and November 2009, in Lambir Hills National Park, Sarawak, Malaysia (4°20'N, 113°50'E). Temperature exhibited little annual variation, and daily maximum temperature was 32°C (Davies & Ashton, 1999). Annual total rainfall is ~3000 mm (Roubik *et al.*, 2005). Seasonal changes in rainfall are small, but the area irregularly experiences short-term droughts. Such droughts trigger general flowering, during which various tree species flower synchronously (Sakai *et al.*, 2006). The study period coincided with the general flowering season. The area is covered by primary lowland mixed dipterocarp forest, in which trees of *M. wrayi* occur at a relatively low density.

Pollination experiment

I selected five female trees of *M. japonicus* (tagged J1, J2, J3, J4 and J5) at different distances from the nearest males (6, 12, 46, 97 and 101 m, respectively). The female trees were more than 2 m tall and 5 cm DBH, and all were mature. I conducted the following five treatments on each tree: (1) control – three to five inflorescences were tagged and left untouched; (2) insect exclusion – three to five inflorescences were covered with fine nets

(80-μm mesh, Cloth Cabin, Suminoe Teijin Techno, Osaka, Japan), which allowed pollen grains of *Mallotus*, but not insects, to pass; (3) bagged – three inflorescences were covered with paper bags (Grape Bag, DAIICHI VINYL, Fukui, Japan) through which neither pollen nor insects could pass; (4) bagged and hand-pollinated – three inflorescences were covered with paper bags and hand-pollinated while flowering; and (5) pollen supplementation – three to five open inflorescences were hand-pollinated while flowering. I placed bags or nets on the inflorescences for Treatments 2–4 on 11–13 June, and counted the number of flowers for these treatments on 2–4 July. Because all flowers opened almost synchronously, all of the studied inflorescences had not been pollinated before the treatments, and all treated flowers and inflorescences were comparable. No insects were seen on inflorescences when inflorescences were bagged. I counted the number of fruits on the inflorescences on 28 July, when the fruits were still green but fully plump. Fruit set of each inflorescence was calculated by dividing the number of fruits by the number of flowers.

For *M. wrayi*, I selected two reproductive female trees, W1 and W2, for the experiments. Both trees were more than 5 m tall, and DBH was more than 7 cm. I conducted the following treatments on each tree: (1) control – three inflorescences on W1 and 12 on W2 were tagged and left untouched; and (2) insect exclusion – three inflorescences on W1 and 12 on W2 were covered with a fine net before anthesis on 27 September. Since almost all the inflorescences flowered synchronously, all of the studied inflorescences had not been pollinated before the treatments. I counted the number of flowers on each inflorescence on 4 October, and the number of fruits on inflorescences on 2 November. Fruit set was calculated using the same procedure as for *M. japonicus*.

The effects of distance from the nearest male on the fruit set in *M. japonicus* were examined using a generalised linear mixed model (GLMM, function lmer in library lme4) in R 2.14.0 (R Development Core Team, 2010). Because the dependent variables of the two models below were fruit set represented as proportion data, binomial error distribution and logit-link function were chosen. In the first model, the dependent variable was fruit set of the control inflorescences. Distance from the nearest male was included as a fixed term, and the tree individual was modelled as a random effect. In the second model examining effects on pollen limitation, fruit set of inflorescences under the control and pollen-supplementation treatments was the dependent variable. Treatments (control and pollen supplementations) and interactions between treatment and distance as well as the distance to the nearest male were included as fixed effects.

Pollen limitation of individual trees was examined by comparing fruit set of control and pollen-supplementation inflorescences for each tree by using a generalised linear model (GLM) with a binomial error distribution and logit-link function. In this model, fruit set of control and pollen-supplementation inflorescences was included as a dependent variable, and treatment (control and pollen supplementations) was a fixed term.

Monitoring of airborne pollen

For *M. japonicus*, I placed five glass slides (2.6 × 7.6 cm, Micro Slide Glass, Matsunami Glass Industry, Osaka, Japan) layered with petrolatum for 72 h (from 24 to 27 June) on the crown of each of the five female trees used for the pollination experiment. The glass slides were changed every second day. After removal, the number of pollen grains on the glass slides was counted under an optical microscope to calculate the number of pollen grains captured on the slide each day. I distinguished the pollen grains of *Mallotus* from those of other species by their size, colour, ellipsoidal shape and smooth surface.

For *M. wrayi*, I placed five glass slides layered with petrolatum for 42 h (from 30 September to 2 October 2009) on tree W2, and on two additional female trees, W3 and W4. W2 was located near a male tree (distance between the stems < 2 m), and W3 and W4 were located more than 50 m from male trees. The density of airborne pollen was calculated using the same procedure as for *M. japonicus*.

To test whether the number of airborne pollen grains decreases with distance, I fitted a GLMM with a Poisson error distribution and log-link function. In the model, the number of pollen grains caught on a glass slide on 1 day was the dependent variable. Distance from the nearest male was included as a fixed term, and the date when the glass slides were set out was a random effect.

Collection of flower visitors

To investigate whether insects contribute to pollination, I captured visitors to flowers and investigated their body pollen. I captured relatively large flower visitors (mostly dipterans and hymenopterans) with insect nets. For the five female *M. japonicus* trees (J1–J5), 2 h were spent capturing visitors to each tree with insect nets. Visitors to three male *M. japonicus* trees were captured with insect nets during a total of 4 h. I was able to reach 20–30 inflorescences on each tree. Small insects that stayed on flowers (mostly hemipterans and thysanopterans) were captured using aspirators and by sampling inflorescences. At each of the five female trees (J1–J5), 1 h was spent using aspirators to capture insects that stayed on flowers. Five inflorescences from each of the five female trees (J1–J5) and one inflorescence from each of three male trees were sampled, and all insects found on the inflorescences were kept.

For *M. wrayi*, 3 h were spent at each of three female trees and 1 h was spent at each of three male trees to capture flower visitors with insect nets. Although the trees were more than 5 m tall, inflorescences were observed from ~1.5 m, and I was able to reach 10–30 inflorescences on each tree. To capture small insects, 7–26 inflorescences from each of six female trees and three inflorescences from each of three male trees of *M. wrayi* were sampled.

Captured insects were identified to the order level, except for Hymenoptera, which was classified to superfamily. The body pollen of insects captured on female trees was quantified under a stereomicroscope. I investigated whether visitation frequency of each of the six insect orders (see Results) was correlated with the distance from the nearest male by using Spearman's rank correlation tests.

Results

Fruit set

For *Mallotus japonicus*, fruit set of the control inflorescences was 59.7–93.5% and did not significantly change with distance from the nearest male (Fig. 2.2.2, GLMM, $\chi^2 = 0.19$, $P = 0.66$). When insects' access to flowers was excluded by a net, all inflorescences set fruits, although the proportion was much lower than for control inflorescences (Fig. 2.2.2, 14.8–68.4%). In contrast, none of the flowers under the bagged treatment set fruit, whereas the bagged inflorescences with supplemental hand-pollination showed 61.5–100% fruit set. GLMM analysis on fruit set of inflorescences under the control and pollen-supplementation treatments showed that the interaction between the treatment and the distance from the nearest male was a highly significant predictor of seed set ($\chi^2 = 34.75$, $P < 10^{-8}$, Fig. 2.2.2) as was the effect of treatment ($\chi^2 = 5.87$, $P = 0.02$). Fruit set significantly differed between control and pollen-supplementation inflorescences in Tree J2 (GLM, $\chi^2 = 5.01$; $P = 0.03$) and particularly in Trees J3, J4 and J5 ($\chi^2 = 55.73, 66.48, 55.28$; $P < 10^{-12}$) but not in J1 ($\chi^2 = 0.02$; $P = 0.88$).

On *M. wrayi*, fruit set of open and netted inflorescences was 0–58.3% and 0–33.3%, respectively (Fig. 2.2.2).

Airborne pollen

Substantial amounts of pollen of *M. japonicus* and *M. wrayi* were captured by glass slides on all female trees investigated in both species; 138.9 ± 96.1, 79.3 ± 70.8, 21.1 ± 16.5, 13.1 ± 11.2 and 14.3 ± 10.3 pollen grains of *M. japonicus* reached J1, J2, J3, J4 and J5, respectively, per slide per day, and 26.3 ± 27.3, 6.1 ± 4.1 and 0.8 ± 1.3 pollen grains of *M. wrayi* reached W2, W3 and W4, respectively, per slide per day (Fig. 2.2.3). The amount of airborne pollen of *M. japonicus* considerably decreased with distance from a male tree (GLMM, estimated coefficient of distance = –0.03, $\chi^2 = 1842.1$, $P < 10^{-15}$).

Flower visitors

In total, 100 and 111 flower visitors belonging to various orders were collected from female trees of *M. japonicus* and *M. wrayi*, respectively (Table 2.2.1). Female inflorescences were visited less often by insects than were male inflorescences. The most frequently captured

flower visitors during inflorescence collections were thrips (Thysanoptera) on both males and females. These insects stayed on the inflorescences, stuck their proboscises into the filaments or the stigma and sucked the juice. Few of the thysanopterans captured on female inflorescences carried any pollen on their bodies (18% on *M. japonicus* and 1% on *M. wrayi*; Table 2.2.2). The most frequently captured flower visitors during insect-net collections on female inflorescences of both tree species were hymenopterans. Among these, most Vespoidea (family Vespidae) on *M. japonicus* (100%) and Apoidea on *M. japonicus* (family Apidae, Halictidae and Andrenidae) (67%) and *M. wrayi* (Apis dorsata, Apidae) (100%) had large pollen loads (>11 pollen grains; Table 2.2.2), especially on their heads and legs. These insects stayed only for a few seconds on the female inflorescence, whereas they collected both nectar and pollen on males. Some of the other visitors (dipterans, hemipterans, coleopterans and lepidopterans) also had high or low numbers of pollen grains (Table 2.2.2). The numbers of insect visitors and distance from the nearest male tree were not significantly correlated on females of *M. japonicus* (Spearman's rank correlation test, P = 0.08–0.56).

Discussion

The results of the present study suggest that *Mallotus japonicus* and *M. wrayi* are both wind- and insect-pollinated (ambophilous). Both species are wind-pollinated because inflorescences covered by nets set fruits even though all insect visitors were excluded. However, the relative contribution of wind pollination cannot be directly estimated from the results, given the possibility that a portion of airborne pollen was excluded by the extremely small mesh size of the nets. Because inflorescences covered by paper bags did not set fruit, but did when hand-pollinated, these trees do not set fruits by apomixis. The substantial amount of airborne pollen caught on all study trees also supports the effectiveness of wind pollination. In a preliminary experiment using *M. japonicus* during a previous year, all netted inflorescences also set fruits (E. Yamasaki, unpublished data). Possible adaptations for wind pollination include the papillose and plumose stigma, the large amount of dry pollen grains, exposed anthers and stigma and elongated inflorescences of the two species. These species also appear to be insect-pollinated because insects with pollen on their bodies visited female flowers. Because most of the observed body pollen was attached to the heads and legs of flower visitors and these body parts frequently touch the stigma when they land on inflorescences, these insects may be effective pollinators. Male inflorescences of *M. japonicus* and *M. wrayi* attracted insects by nectar and pollen. Male and female inflorescences of *M. japonicus* emitted similar odours and were similar in appearance. Male and female inflorescences of *M. wrayi* are also similar in appearance, although the odour was not as strong as in *M. japonicus*. These characteristics may represent adaptations

for insect pollination. The visitation of insects to male inflorescences of *M. japonicus* and *M. wrayi* may also facilitate wind pollination by scattering pollen grains into the air, as reported for other plant species such as *M. oppositifolius*, *Cravata adansonii* and *Chamaedrea pinnatifrons* (Lock &Hall, 1982; Listabarth, 1993; Mangla & Tandon, 2011).

For both species, the most important pollinator insects appeared to be hymenopterans such as Vespidae, Apidae, Halictidae and Andrenidae, because the visitation rates of these insects were relatively high among all insects captured by insect nets; furthermore, these insects carried high numbers of pollen grains. Hymenopterans travel relatively long distances for foraging (Proctor *et al.*, 1996). In the case of *M. wrayi*, however, whether giant honeybees (*Apis dorsata*) are frequent visitors during every flowering event remains unclear. Because the abundance of giant honeybees increases during the general flowering season at Lambir Hills National Park (Itioka *et al.*, 2001), the abundance and composition of flower visitors may differ when *M. wrayi* flowers during non-general flowering periods. For *M. japonicus*, I conducted flower-visitor collections for two flowering seasons in Seta Park and for one season in each of two other sites in temperate and subtropical areas of Japan (Yasu, Shiga Prefecture, and Okinawa Island). Hymenopterans were always frequent visitors (E. Yamasaki, unpublished data). Many thrips were also observed on *M. japonicus* and *M. wrayi*, but they may contribute little to pollination, as their pollen load and visitation frequency to female inflorescences were very low. Some species of *Macaranga*, the genus most closely related to *Mallotus* (Kulju *et al.*, 2007), are exclusively pollinated by thrips (Moog *et al.*, 2002; Fiala *et al.*, 2011), but this is not the case in the two study species of *Mallotus*.

Insect pollinators visited not only male, but also female inflorescences, even though female inflorescences did not possess any rewards such as nectar or pollen; these insects may have been deceived by the smell and/or appearance of female inflorescences similar to those of males. The African species *M. oppositifolius* may also be pollinated by various bees and flies that are deceived by smell and appearance (Lock & Hall, 1982). This type of insect pollination might occur broadly in *Mallotus*. Although insect visitation to female inflorescences has not been confirmed, visitation of bees and flies has been reported for *M. griffithianus*, *M. penangensis*, *M. brevipetiolatus* and *M. paniculatus* (Momose *et al.*, 1998; Corlett, 2004; Sierra *et al.*, 2007).

Given that floral characteristics adapted for both wind and insect pollination can be recognised in both species, ambophily in *M. japonicus* and *M. wrayi* may be actively maintained because of several advantages of this pollination system, in contrast to either accidental maintenance or a possible transitional state of the two species. In some pioneer plants, ambophily is considered a strategy to accommodate changing wind conditions during different stages of forest succession (Stellman, 1984; Goodwillie, 1999). In addition, I propose that changes in population density also contribute to the maintenance of

ambophily. Population densities of pioneer plants such as *Mallotus* species change as forest succession progresses; densities are high in early successional forests and gradually decrease as late successional plants colonise the forests (Pacala, 1996; Guariguata & Ostertag, 2001). Several studies have reported that in wind-pollinated plants, pollen limitation increases rapidly with increases in distance from a pollen source (Levin & Kerster, 1974; Steven & Waller, 2007; Vandepitte *et al.*, 2009; Hesse & Pannell, 2011). In *M. japonicus*, I also found that the amount of airborne pollen rapidly decreased with distance from the pollen source. I observed pollen limitation only in trees without males in their vicinity, which may be attributable to short-distance pollination by wind. Interestingly, fruit set of control inflorescences itself did not change with distance. One possible explanation may be varying resource availability for fruit production among trees; female trees far from males might have suffered from pollen limitation in previous years and accumulated more resources, thus setting more fruits when pollen was supplemented. In contrast, pollen limitation does not strongly depend on distance from a pollen source in insect-pollinated plants (Schulke & Waser, 2001; de Jong *et al.*,2005 ; Albrecht *et al.*, 2009). In *M. japonicus*, insects with ample body pollen, primarily hymenopterans, visited the inflorescences regardless of distance from a pollen source.

Although the data presented here are still preliminary, the results may indicate that the effectiveness of wind and insect pollination may differentially depend on population density, which has rarely been examined in ambophilous plants. Ambophily has been documented only in ~10 genera, most of which were thought to be either wind- or insect-pollinated before close investigation (Culley *et al.*, 2002). Ambophily may thus be more common than currently thought (Culley *et al.*, 2002). Further studies may reveal that ambophily is an important mechanism to ensure reproduction for plants experiencing unstable habitats.

Table 2.2.1 Visitation frequency of flower visitors on *M. japonicus* and *M. wrayi*. Means ± standard deviation (variation among trees) are shown.

		M. japonicus		*M. wrayi*	
Collection method	Taxon of insects	Female	Male	Female	Male
Insect net	Hymenoptera				
(per tree per hour)	Vespoidea	0.8 ± 0.6	1.8 ± 1.9	0.1 ± 0.2	0.3 ± 0.6
	Apoidea	0.6 ± 0.9	3.3 ± 1.3	0.3 ± 0.0	16.6 ± 9.7
	Chalcididea	0.1 ± 0.2	0.3 ± 0.6	0.0	0.0
	Tenthredinoidea	0.0	0.0	0.0	0.0
	Ichneumonidea	0.2 ± 0.4	0.4 ± 0.8	0.0	0.3 ± 0.6
	Diptera	0.8 ± 0.6	1.8 ± 1.6	0.0	0.3 ± 0.6
	Lepidoptera	0.1 ± 0.2	0.0	0.0	0.0
	Number of trees	5	3	3	3
Aspirator	Thysanoptera	8.4 ± 4.0	-	-	-
(per tree per hour)	Diptera	1.0 ± 1.2	-	-	-
	Hemiptera	0.8 ± 0.8	-	-	-
	Coleoptera	0.2 ± 0.4	-	-	-
	Number of trees	5			
Sampling of whole	Thysanoptera	1.0 ± 0.5	18.3 ± 9.0	1.0 ± 1.1	7.7 ± 3.2
inflorescences	Diptera	0.1 ± 0.1	0.3 ± 0.6	0.0	0.0
(per inflorescence)	Hemiptera	0.1 ± 0.3	8.0 ± 5.0	0.3 ± 0.2	0.2 ± 0.4
	Coleoptera	0.2 ± 0.4	0.0	0.0	0.0
	Number of trees	5	3	6	3

Table 2.2.2 Proportion of flower visitors collected on female inflorescences with no (0 pollen grains, indicated by "-"), small (1–10 pollen grains, "+"), or large (>11 pollen grains, "++") pollen load.

	M. japonicus				*M. wrayi*			
	-	+	++	*N*	-	+	++	*N*
Thysanoptera	0.82	0.18	0.00	57	0.99	0.01	0.00	73
Hymenoptera								
Vespoidea	0.00	0.00	1.00	8	0.00	1.00	0.00	1
Apoidea	0.00	0.33	0.67	6	0.00	0.00	1.00	3
Chalcididea	0.00	0.00	1.00	1	-	-	-	0
Ichneumonidea	0.00	1.00	0.00	3	-	-	-	0
Diptera	0.27	0.55	0.18	11	-	-	-	0
Hemiptera	0.50	0.38	0.13	8	0.78	0.22	0.00	32
Coleoptera	0.00	1.00	0.00	5	1.00	0.00	0.00	2
Lepidoptera	0.00	1.00	0.00	1	-	-	-	0

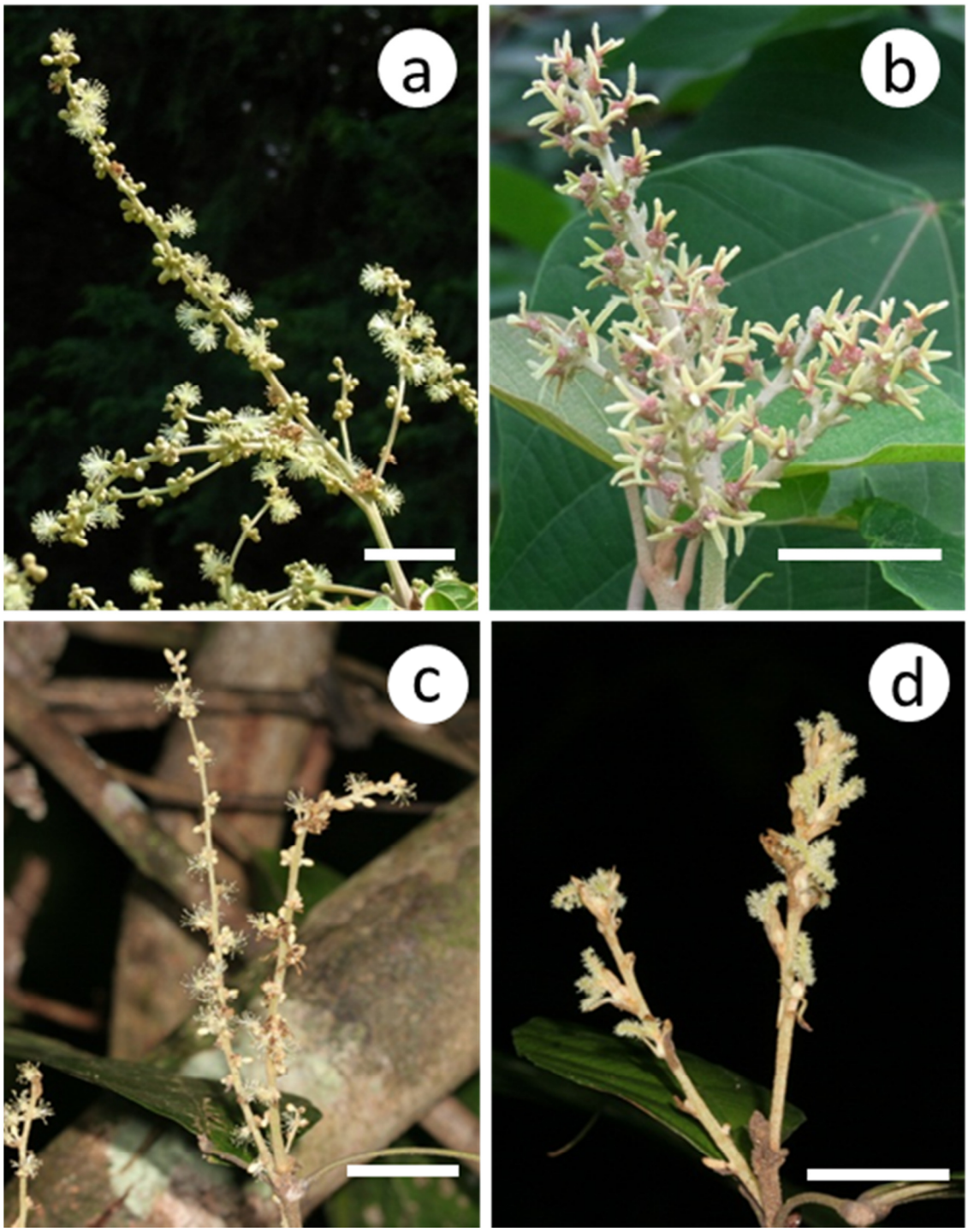

Fig. 2.2.1 (a) A male inflorescence of *Mallotus japonicus*. (b) A female inflorescence of *M. japonicus*. (c) A male inflorescence of *M. wrayi*. (d) A female inflorescence of *M. wrayi*. Scale bars indicate 3 cm in (a), (b), and (c) and 1 cm in (d).

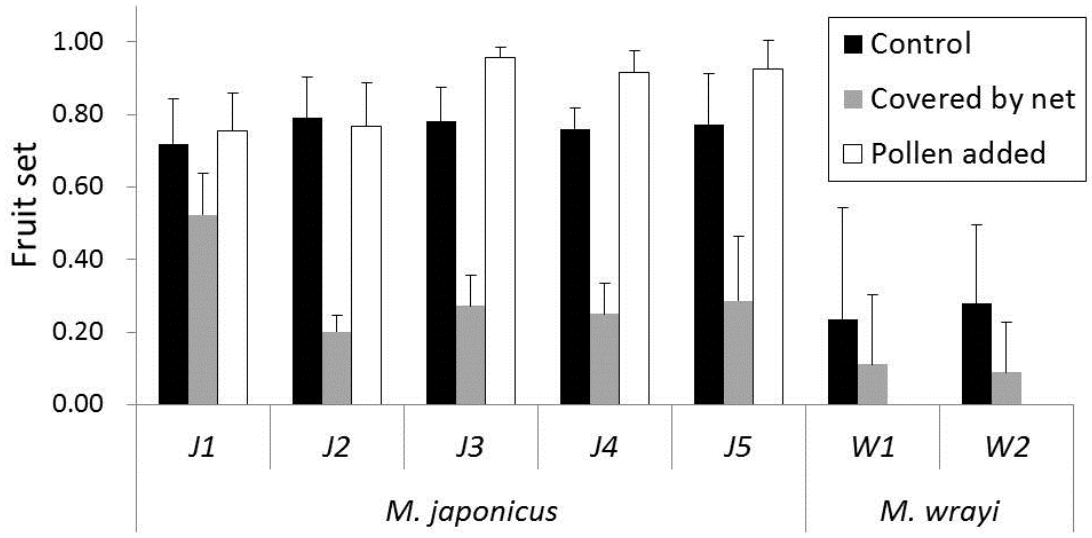

Fig. 2.2.2 Fruit set (number of fruits per number of flowers) of *Mallotus japonicus* and *M. wrayi*. Columns show control inflorescences, inflorescences covered by nets, and pollen-supplemented inflorescences as indicated. Vertical bars represent standard deviation. Labels are the IDs of female trees.

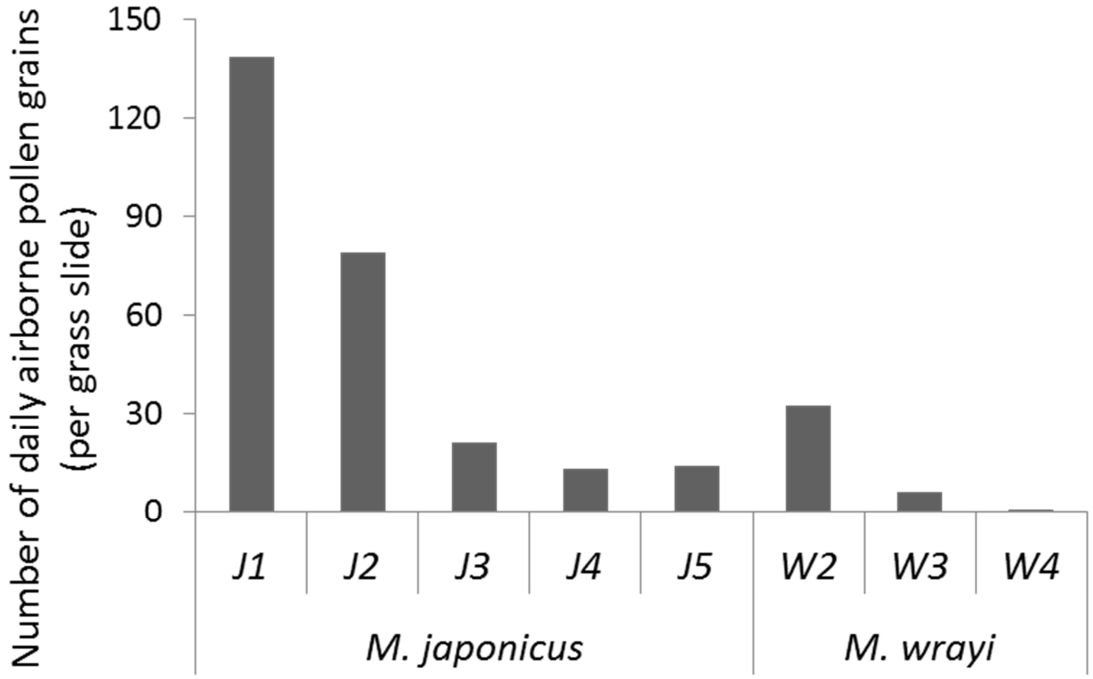

Fig. 2.2.3 Mean number of airborne pollen grains on glass slides placed on female trees each day.

Section 2.3
Disk-shaped nectaries on bracteoles of *Macaranga sinensis* provide a reward for pollinators

Introduction

Nectar is arguably the most common floral reward provided by angiosperm flowers (Proctor *et al.*, 1996; Bernardello, 2007). Floral nectar can occur in various parts of the flower, ranging from receptacles to petals, sepals, pistils, and stamens. The diversity in the position and structure of floral nectaries suggests that the ability to produce floral nectar has evolved many times in angiosperms; however, most floral nectaries are ancient, and thus tracing the evolutionary origin of floral nectar is difficult in most angiosperm lineages (Lee *et al.*, 2005; Bernardello, 2007). Studies on more recent occurrences of floral nectar may thereby enhance our understanding of floral nectar origin in angiosperms.

Macaranga has apetalous flowers formed in racemes at the base of leaves. To date, no species have been reported to produce nectar from flowers. In turn, many *Macaranga* species produce foliar nectar, which is sought by ants, which in turn deter herbivores from feeding (Whalen & Mackay, 1988; Mackay & Whalen, 1991; Fiala & Maschwitz, 1991). Most *Macaranga* species possess disk-shaped nectaries on the adaxial surface of their leaves, usually with two or more nectaries that occur at the base of lamina or petiole insertion (Davies, 2001; Whitmore, 2008; Table 2.3.1, Appendix 2). Additionally, about one-quarter *Macaranga* species also have similar disk-shaped glands on the bracts and/or bracteoles of inflorescences (Whitmore, 2008; Table 2.3.1, Appendix 2). According to the literature, 13 of 19 infrageneric groups of *Macaranga* include species with and without disk-shaped glands on bracts/bracteoles (Davies, 2001; Whitmore, 2008; Table 2.3.1). To date, their function has not been studied.

In this study, I investigated whether the disk-shaped glands on inflorescences of *Macaranga* contribute to pollination by identifying the pollination system of *M. sinensis*, which has disk-shaped nectaries on the bracteoles. The pollination system of the genus *Macaranga* is largely unknown, although thrips pollination and hemipteran pollination have been reported in some species (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011; see Chapter 1 for more information). Because the nectar of thrips- and hemipteran-pollinated species is enclosed in ball-shaped nectaries or may be secreted within the dense tufts of the trichome-like nectaries (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011), only insects with needle-like elongated proboscises can access nectar. Since species with disk-shaped glands on the bracts/bracteoles lack such bracteole chambers (Whitmore, 2008; Fiala et al., 2011), they are unlikely to have such pollination systems. If

the disk-shaped glands play an important role in attracting pollinators, it may provide an example where plants, which had once lost nectaries for pollinators, regained floral nectar by co-opting the nectaries originally developed for ant guards.

Materials and Methods

Study species

Macaranga sinensis is a dioecious small tree distributed on Lanyu Island and the Philippines Archipelago. The species has broadly truncated leaf lamina with disk-shaped nectaries near the petiole insertion and at the apical end (Whitmore, 2008) (Fig. 2.3.1a, b). Inflorescences are racemous and up to 14 cm in length. Flowers are apetalous and stamens and pistils are exposed to the outside. Both male and female flowers are subtended by paddle-like bracteoles, which possess one to five disk-shaped glands on the adaxial surface (Fig. 2.3.1c–f).

Study site

Fieldwork was conducted 7–11 June 2011 and 23–27 May 2012 on Lanyu Island, located about 80 km southeast from the Taiwan mainland (22°01' N, 121° 57' E). This island lies in the tropical monsoon climate. The study site was characterized by secondary forests with a low canopy height (~10 m) and located on a wind-exposed slope.

Capturing and observing flower visitors

To identify pollinators of *M. sinensis,* I captured flying insects with insect nets on flower visits. Since few insects were seen in the afternoon and nighttime, I focused our observations in the morning (0530–1200 hours). In total, 10 h and 13.5 h were spent capturing insects on male and female flowers, respectively. All collected insects were identified by taxonomic order, and hymenopterans were further identified to family. Insects were checked for the number of pollen grains on their body under a binocular microscope.

Sugar composition of nectar

To compare the sugar composition of nectar from leaves and bracteoles, I collected nectar from two male and four female trees. I enclosed leaves and inflorescences in the same mesh bags to prevent insects from consuming nectar. Up to 20 flower clusters and ten leaves were enclosed in each of the bags. After 24 h, I removed the bags and collected nectar with 1-μL and 5-μL microcapillary tubes. The collected nectar was diluted with 10 or 20 μL distilled water and subjected to high-performance liquid chromatography (HPLC) analysis. Five microliters of the sample was injected to the HPLC system equipped with a pump (LC-6A; Shimadzu, Kyoto, Japan), a column (Wakosil, 5 NH_2, φ 4.6 mm × 150 mm; Wako Pure

Chemical Industries, Ltd., Osaka, Japan), a column oven (CTO-10A; Shimadzu), and a refractive index detector (RID-10A; Shimadzu). The temperature of the column oven was 40°C, and 75% acetonitrile was used as an eluent at a flow rate of 1 mL·min^{-1}. Each sugar was identified by retention time and then quantified from a standard curve using an authentic sugar.

I assessed eventual significant difference in the composition of nectar secreted from leaves and male or female inflorescences using a Steel–Dwass test since the data on sucrose were skewed negatively and deviated significantly from normal distribution (goodness of fit test, $\chi^2 = 7.95$, df = 2, $P = 0.02$). The amount of each sugar (fructose, glucose, and sucrose) was compared between different nectary locations (leaves, male inflorescences, or female inflorescences).

Airborne pollen

To examine the possibility of wind pollination, I monitored airborne pollen. I placed three petrolatum-coated glass slides (2.6 × 7.6 cm) on the branches of each of the five female trees located less than 5 m from the nearest male tree. The glass slides were collected 24 h later and the number of pollen grains was counted under an optical microscope.

Results

Flower visitors

In total, nine and eight species of insect were captured on male and female flowers, respectively (Fig. 2.3.2). The most frequently captured insects on both male and female inflorescences were Colletidae bees (Hymenoptera). All of these bees belong to the genus *Hylaeus*, and 95.8% of them had more than ten pollen grains on their bodies. Forty-five percent of the other visitors also carried more than ten pollen grains. The flower visitors licked disk-shaped glands on the bracteoles of male and female inflorescences or collected pollen grains from male flowers (Fig. 2.3.3). While foraging for nectar, the flower visitors often touched anthers or stigmas.

Composition of nectar

Nectar secretion started 2–3 days before anther dehiscence and continued while plenty of pollen grains remained on the stamens within male inflorescences. On female inflorescences, nectar secretion started 2–3 days before stigmas opened and recurved. It continued while the stigmas were fresh, and ceased as the stigmas turned brown. The bracteoles fell off from the plants by the time fruits matured. On average (± SD), 0.5 (± 0.3) and 1.0 (± 0.1) μL of nectar were secreted per day from female and male inflorescences, respectively, with 10–20 nectaries. More than 3 μL of nectar was secreted on average per day from a leaf. Nectar

secreted from male and female inflorescences and leaves was mainly composed of fructose and glucose (Table 2.3.2). No significant difference was observed in the relative amount of fructose, glucose, and sucrose included in the nectar (Steel–Dwass test, $P > 0.05$).

Airborne pollen

The density of *M. sinensis* pollen grains was 0.24 ± 0.20 grains cm^{-2}·day^{-1}. Pollen grains of other plants were also seen on the slide glass (0.41 ± 0.39 grains cm^{-2}·day^{-1}).

Discussion

Observations of insect visitors suggest that the disk-shaped glands on bracteoles of *Macaranga sinensis* play major roles in pollination. Insects belonging to a variety of taxa visited the inflorescences of *M. sinensis*, foraging for nectar and/or pollen grains. Since the disk-shaped nectaries exist at the tip of the bracteoles and the bracteoles are located just at the base of the flowers, many insect visitors had to hold onto the flowers to lick the nectar (Fig. 2.3.3a). Pollen grains may move from anthers to insect bodies or from insect bodies to stigmas at these times. Ants were often seen visiting disk-shaped nectaries on inflorescences, but they may contribute little to pollination because their travel range is much narrower than that of flying insects (Peakall *et al.*, 1991); since *M. sinensis* is dioecious and male and female flowers are located on separate trees, ants are not likely to be effective pollinators. Furthermore, ants' smooth integument, frequent grooming, and antimicrobial secretion from their metapleural glands may also prevent ant pollination (Peakall *et al.*, 1991; Dutton & Frederickson, 2012). Wind is also thought to contribute little to pollination because the amount of airborne pollen reaching female *M. sinensis* trees was found to be very small. In *Linanthus parviflorus* (Polemoniaceae) and *Mallotus japonicus* (Euphorbiaceae), which are pollinated by both wind and insects, 16.65–51.44 grains cm^{-2}·day^{-1} and on average 4.21 grains cm^{-2}·day^{-1} are captured by the same procedure as the present study (Goodwillie, 1999; Yamasaki & Sakai, 2013; Section 2.2). *M. sinensis* is a rare example of an angiosperm with nectaries outside of the flowers that contribute to pollination.

I hypothesize that the existence of disk-shaped nectaries on leaves to attract ant guards has facilitated the evolution of disk-shaped nectaries on bracteoles for pollination. Disk-shaped nectaries on leaves and bracteoles may be homologous because both nectaries are very similar in shape and located on the adaxial surface, and secrete nectar with almost the same sugar compositions. Since disk-shaped nectaries on leaves exist in most *Macaranga* species but disk-shaped nectaries on bracts/bracteoles are not seen in the basal groups (Section 2.1), the origin of disk-shaped nectaries on bracts/bracteoles may be more recent than nectaries on leaves. Two evolutionary scenarios of disk-shaped nectaries on bracteoles are possible. First, inflorescences may have had bracteoles without disk-shaped nectaries

initially, and a series of genes related to shaping disk-shaped nectaries on leaves may have become newly expressed on bracteoles. Second, leaves with disk-shaped nectaries might have appeared within inflorescences as bracteoles as well, and the blade might have degraded, so that only the nectaries remained. However, narrowing the two possibilities is difficult.

Table 2.3.1 Summary of the number of total species and species that are described to possess disk-shaped glands on (1) leaves, (2) bracts/bracteoles, and (3) both leaves and bracts/bracteoles in Davies (2001) and Whitmore (2008). I considered the term "glands" in the literature as disk-shaped glands, while more specific terms "granular glands," "conical glands," and "gland-tipped" were not considered disk-shaped. Detailed information on each species is available in Appendix 2.

Infrageneric group	Total species	Disk-shaped glands		
		Leaf	Bract/ bracteole	Both leaf and bract/bracteole
African group	25	16	1	1
Angustifolia	13	12	2	2
Bicolor	6	4	1	1
Brunneofloccosa	19	12	8	7
Coniferae	5	1	0	0
Coriacea	6	1	0	0
Denticulata	6	3	2	2
Dioica	24	22	13	13
Gracillis	7	7	2	2
Javanica	13	11	9	9
Longistipulata	19	12	13	9
Mappa	21	12	5	1
Mauritiana	1	0	1	0
Oblongifolia	10	7	0	0
sect. *Pachystemon*	25	11	0	0
sect. *Pruinosae*	9	2	1	1
sect. *Pseudorottlera*	15	12	0	0
Tanarius	14	4	1	1
sect. *Winklerianae*	2	0	0	0

Table 2.3.2 Sugar composition of nectar produced from inflorescences and leaves. Mean sugar concentration (μg/μL) ± SD are shown.

	Inflorescences		Leaves
	Male	Female	
Fructose	55.6 ± 12.3	35.8 ± 21.8	44.2 ± 17.8
Glucose	47.2 ± 7.7	31.5 ± 19.9	42.4 ± 17.1
Sucrose	0.0	3.5 ± 5.2	3.3 ± 2.9

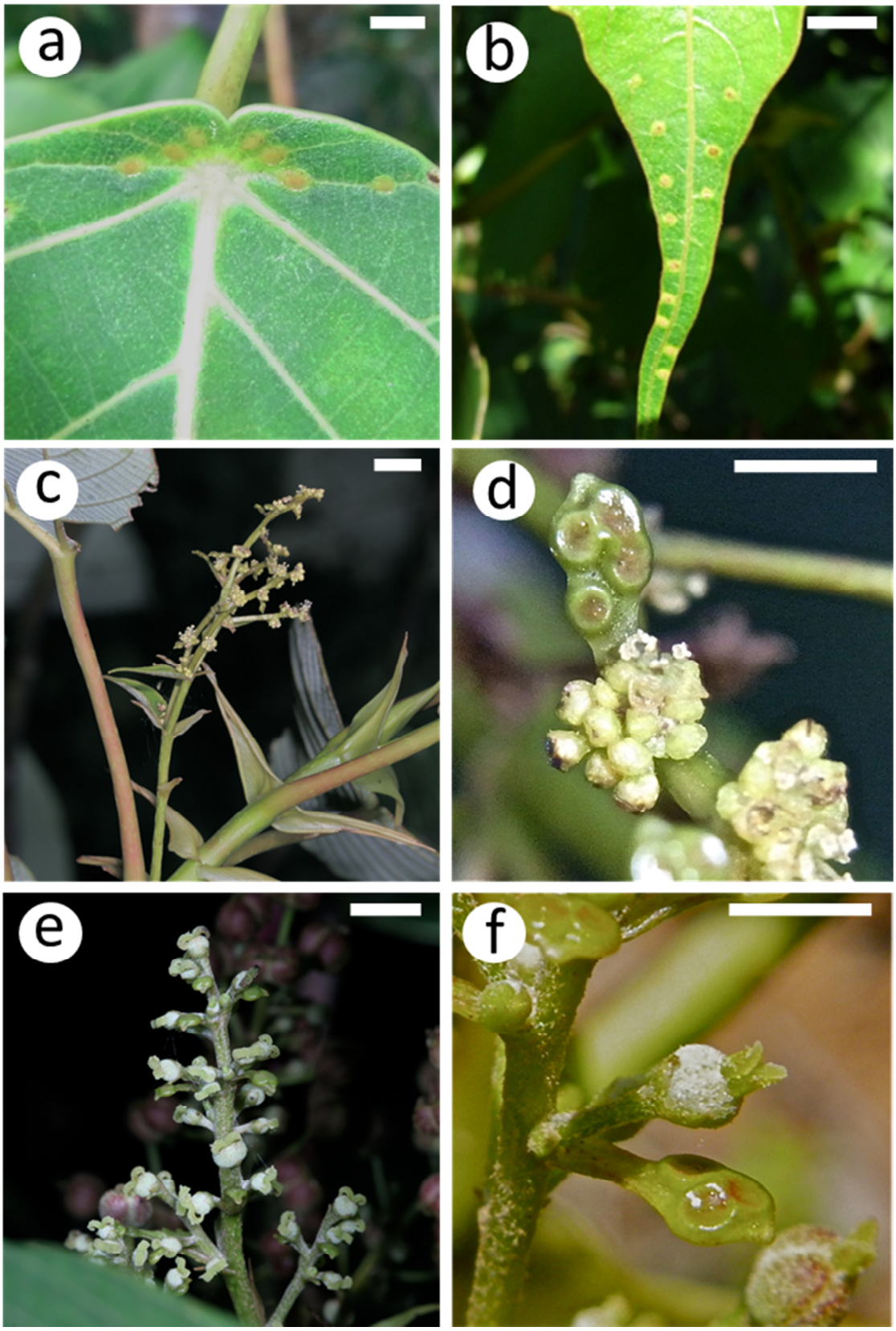
a
b
c
d
e
f

Fig. 2.3.1 Leaves and inflorescences of *Macaranga sinensis*. (a) Extrafloral nectaries near the stalk insertion of leaf lamina; (b) extrafloral nectaries located at the tip of leaf lamina; (c) male inflorescence; (d) male bracteole with disk-shaped glands located at the base of flower clusters; (e) female inflorescence; and (f) female bracteoles with disk-shaped glands at the base of a female flower. Scale bars of (a), (b), (d), and (f) indicate 0.5 cm and those of (c) and (e) denote 1 cm.

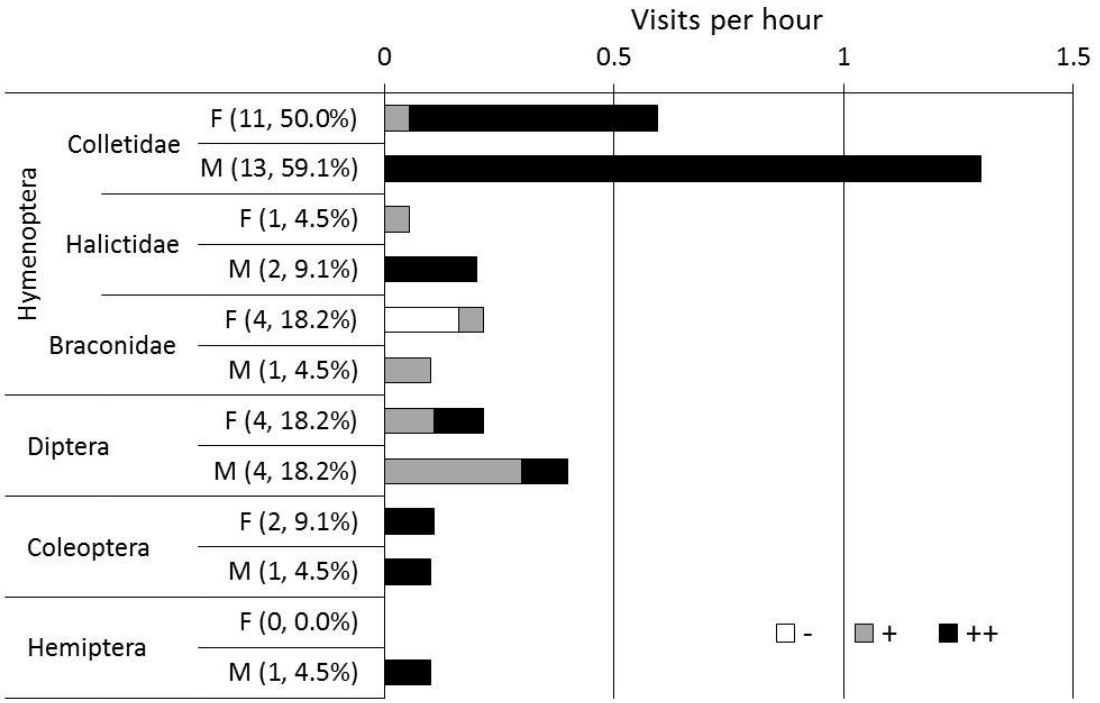

Fig. 2.3.2 Insects captured on female and male flowers of *Macaranga sinensis* per hour. Amount of body pollen is indicated by – (no pollen grains), + (1–10 pollen grains), and ++ (> 10 pollen grains). Actual number of insects collected and the proportion that they represent among the total number of flower visitors are given in the parentheses.

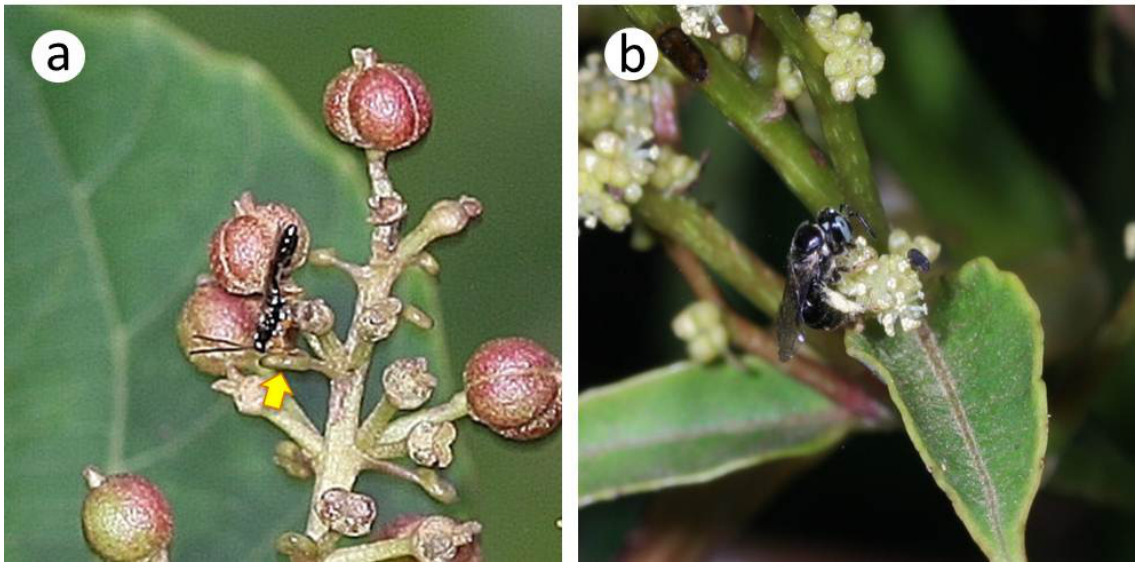

Fig. 2.3.3 Flower visitors. (a) A hymenopteran licking an extrafloral nectary (yellow arrow) on female inflorescences of *Macaranga sinensis*. (b) *Hylaeus* sp. collecting pollen grains.

Chapter 3
Interactions among plants, pollinators and guard ants in ant-plant *Macaranga*

Section 3.1
Density of ant guards on inflorescences and their effects on herbivores and pollinators

Introduction

Many plants have protective mutualistic relationships with ants in which ants are used by plants as deterrents against herbivores (Chapter 1). Although guard ants are useful for plant protection, they have both positive and negative effects on the reproductive organs. Ants may exclude flower- or fruit-damaging herbivores and thereby promote plant reproduction (Willmer & Stone, 1997). Some plant species possess extrafloral nectaries on their inflorescences that attract nectar-harvesting ants; these ants reduce the damage caused by herbivores (Chapter 1). In contrast, ants may inhibit pollination success, mostly because they exclude pollinators from inflorescences (Willmer & Stone, 1997; Altshuler, 1999; Tsuji *et al.*, 2004; Ness, 2006; Willmer *et al.*, 2009). To avoid the reduction in reproductive success caused by ants, many plants have evolved mechanisms that deter ants, including repellent chemicals, slippery waxy shoots, extrafloral nectaries that attract ants away from flowers, and narrow corollas (Chapter 1). The balance of such positive and negative effects may vary depending on a number of factors, such as the aggressiveness of the ants, characteristics of the pollinators, and flower and inflorescence structures.

Some plants that are inhabited by ants in domatia are termed "ant-plants" (Chapter 1). Because the defence of the mutualistic ants is more aggressive than the defence of plants that are facultatively protected by ants (Fiala *et al.* 1989; Itioka 2005), guard ants on ant-plants may strongly deter pollinators and negatively impact plant reproduction. However, relatively little attention has been focused on the effects of ants on the reproductive success of ant-plants. A few studies have reported ant-repellent mechanisms in ant-plant *Acacia* (Fabaceae). *Acacia* plants, which are pollinated mostly by bees (Stone *et al.*, 2003), produce volatile compounds that deter ants from visiting their flowers (Willmer & Stone, 1997; Ghazoul, 2001; Raine *et al.*, 2002; Willmer *et al.*, 2009). Some species of *Acacia* also bear flowers on parts of the plant that are remote from the ant-inhabiting domatia (Raine *et al.*, 2002).

In this section I examine whether mutualistic ants have positive or negative effects on the reproductive organs of ant-plant *Macaranga.* Interestingly, some *Macaranga* species bear food bodies on their inflorescences (Fiala & Maschwitz, 1992a). I first investigated the types and temporal and spatial distributions of food bodies on 11 species of *Macaranga* (eight ant-plants and three non-ant-plants). Second, I determined whether the food bodies increased ant visitation to the inflorescences on three ant-plant species, which possessed different types of food bodies. Third, I examined the effects of mutualistic ants on herbivores and pollinators through ant-exclusion manipulations on one ant-plant species that lacks food bodies on the inflorescences. Based on the results, I discuss the relationships between ants and reproductive functions of ant-plants, as well as interspecific variation in these relationships.

Materials and methods

Study species

I studied 11 species of *Macaranga* whose flowers and fruits could be easily observed at the study sites (Table 3.1.1). Eight were ant-plants inhabited by specialized *Crematogaster* ants (Fiala *et al*., 1999), and three were non-ant-plants protected by facultative mutualistic ants. *Macaranga* is divided into five main clades by molecular phylogenetic analysis: two basal (B1, B2) and three large crown clades (C1, C2, C3) (Kulju *et al*., 2007; Section 2.1) and the ten of the 11 species I studied are in the C1 clade, whereas the remaining species (*M. praestans*) is in the B2 clade. Most of the C1 species studied, other than *M. umbrosa*, are pollinated by thrips *Dolichothrips* spp. breed in the bracteole chambers (Moog *et al*., 2002; Ishida, 2008; Fiala *et al*., 2011). The pollination systems of *M. umbrosa* and *M. praestans* are unknown. In the thrips-pollinated species the pollinator thrips settle on the inflorescences several days before anthesis, and their numbers increase rapidly by recruitment to the inflorescences (Moog *et al*., 2002; Ishida, 2008; Fiala *et al*., 2011).

Study sites

I conducted this study in aseasonal tropical rain forests in Lambir Hills National Park and Long Semiyang, Upper Baram (3°10'N, 115°10'E), Sarawak, Malaysia, from August to December 2009, August to September 2010, and between September and November 2011. See Section 2.2 for site information of Lambir Hills National Park. The daily maximum temperatures in Upper Baram are and ~31°C, and the annual rainfall is ~4000 mm (Samejima *et al*., 2004). The Upper Baram site is covered by secondary forest. All three surveys described below were conducted in Lambir Hills National Park; only details of inflorescence morphology were examined at Upper Baram.

Food rewards for ants on reproductive organs

I made external observations of inflorescences to determine the presence of potential ant attractants and repellents during seven different stages of reproduction listed in Table 3.1.2. I sampled inflorescences and fruits with a long cutter (occasionally supplemented with a canopy crane system).

Visual inspection did not reveal any physical repellents, but did reveal potential attractants, such as food bodies on bracts, receptacles, and pericarps (see Results). Chemical repellents were not tested.

Changes in ant abundance on the inflorescences

To determine whether mutualistic ants were attracted by inflorescence food bodies, I examined changes in ant abundances in relation to the availability of food bodies at different stages of flowering. For this purpose, I counted the number of ants visiting the inflorescences during 5 min observations on different reproductive stages of *M. havilandii*, *M. winkleri*, and *M. trachyphylla* between 0900 and 1300 hours (female inflorescences of *M. trachyphylla* in the flowering stages were unavailable during the fieldwork). Because most *Macaranga* species at the study sites reached >15 m when sufficiently mature to flower, detailed observations were restricted to *M. havilandii*, *M. winkleri*, and *M. trachyphylla*, which have observer-accessible mature inflorescences. Nevertheless, these three species represent the diversity of inflorescence food body types found in *Macaranga*, allowing us to generalize to other congeners. To distinguish between changes in ant activity in the whole colony and activity on the inflorescences, I simultaneously monitored the number of ants on leaves adjacent to the inflorescences. Ant abundance was quantified in stages of high and low food body production during flowering and fruiting. In my classification of the sequence of phenology, these stages were termed Bud, Flower 3, Fruit 2, and Fruit 3 stages (Table 3.1.2). The significance of the differences in ant abundance between Bud and Flower 3 stages and between Fruit 2 and Fruit 3 stages was examined by Wilcoxon rank-sum tests.

Ant-exclusion experiment

To determine the effects of mutualistic ants on herbivores and pollinators, I conducted an ant-exclusion experiment on *M. havilandii* (Fig. 3.1.1). Although *M. havilandii* does not produce food bodies on the inflorescences, this was the only species for which I was able to obtain accessible inflorescence samples sufficiently large for statistical testing. Experiments were conducted in a paired design because the strength of herbivory may vary by position within the crown even within the same tree; I selected 15 and five pairs of inflorescences on seven male and three female trees, respectively, and chose one inflorescence in each pair for the ant-exclusion treatment and the other for the control. Both inflorescences in each pair were the first and second inflorescences counted from the tip of a target branch, and their

flowering was largely coincident. Before anthesis, I administered adhesive spray (Kinryu spray; SDS Biotech K.K., Tokyo, Japan) to the base of the one or the other inflorescence to repel ants. I resprayed every 2 or 3 days to keep the base sticky. Minute midges were occasionally trapped by the sticky barriers, but I never found herbivores (weevils, leaf beetles, or lepidopteran larvae), pollinator thrips, or ants in the adhesive.

I collected all pairs of male inflorescences at the Flower 3 stage on male trees to determine how ant presence/absence affected pollinator abundance. The numbers of thrips on the inflorescences were counted with the aid of a binocular microscope. I used paired *t*-tests to test for significant differences in numbers of pollinators per branch between treatments and controls. Because I had intended to compare fruit set between inflorescences with and without ants (data not included here), female inflorescences were not collected for pollinator counts.

The intensity of herbivory on male inflorescences was quantified by counting numbers of damaged and intact bracteoles in 12 of the original 15 pairs (three pairs were accidentally lost), and the extent of damage was calculated by dividing the number of damaged bracteoles by the total. Herbivores fed externally on the inflorescences and usually damaged the bracteoles that provide thrips with nectar and breeding sites. I classified each bracteole as damaged when I observed any wounding; in most-damaged cases, more than a quarter of the area was lost to the herbivores. To compare the extent of damage between ant-exclusion and control inflorescences, I used a simple linear regression analysis on arcsine-transformed proportional damage data, where the response variable was the extent of damage in ant-exclusion inflorescences and the explanatory variable was the extent of damage in the control from the same inflorescence pair. Based on the premise that no damage would be observed in ant-exclusion inflorescences when no damage occurred in control inflorescences, the intercepts of the regression lines were set to zero. I determined whether the values of the slopes of the regression lines were significantly different from 1.0.

For female inflorescences, I evaluated the extent of damage at the Flower 3 stage. Because almost all bracteoles had been shed in this stage, I evaluated the damage to flowers (stigmas and ovaries). Herbivores usually grazed the surface of the flower or bored into the ovary. I classified each flower as damaged when more than a quarter of the surface area was grazed or when the whole flower was lost. The extent of damage was calculated by dividing the number of damaged flowers by the total, and this was compared between treatments using the procedure applied to male inflorescences.

Results

Food rewards for ants on plant reproductive organs

I found three types of food bodies on the 11 *Macaranga* species observed (Table 3.1.1). Food bodies on bracts were most frequent (occurring on five species) (Fig. 3.1.2a, b). The food bodies were white or yellowish-white, similar to those on vegetative organs, although smaller. They were produced on the basal parts of bracts several days before the bracts opened. Numbers of these food bodies decreased with time. They became very rare during the Flower 3 stage as ants harvested them, and eventually most bracts bearing them were shed. Food bodies that were consumed were not replaced. During fruiting stages, food bodies were also produced on receptacles (*M. bancana* and *M. trachyphylla*; Fig. 3.1.2c) or on pericarps (*M. havilandii* and *M. umbrosa*; Fig. 3.1.2d). These bodies were similar in appearance to those produced on bracts during the flowering stages. Both types of food bodies were produced during the Fruit 2 stage, and then became very rare by the Fruit 3 stage due to harvesting by ants. In addition to food bodies, red glands were observed on the margins of *M. winkleri* bracteoles, although nectar secretion was invisible to the naked eye. Mutualistic ants sometimes touched the glands with their mandibles.

Changes in ant abundance on inflorescences

I observed increased ant numbers on inflorescences when food bodies were present (Table 3.1.3). More ants visited the inflorescences of *M. winkleri* and *M. trachyphylla* during the Bud stage than during the Flower 3 stage (Table 3.1.3). The number of ant visits to inflorescences of *M. havilandii* (which did not produce food rewards during flowering stages) did not differ between the two stages (Bud and Flower 3) (Table 3.1.3). More ants visited inflorescences of *M. trachyphylla* and *M. havilandii* during the Fruit 2 stage than during the Fruit 3 stage, although the difference was not significant (Table 3.1.3). In contrast, the number of ants visiting inflorescences of *M. winkleri* during fruiting did not differ between these two stages (Table 3.1.3). The number of ants on the leaves did not change significantly in any of the species (Table 3.1.3).

Ant-exclusion experiment

On the male trees of *M. havilandii*, inflorescences from which ants were excluded were significantly more damaged than controls (Fig. 3.1.3a); the slope of the regression line for the relationship between the extent of damage in ant-excluded inflorescences and the extent of damage in the controls was 1.30, which was significantly greater than 1.0 (P = 0.04). Differences between exclusions and controls were large when the extent of damage in the control was large, while the differences were small when controls were damaged only slightly. However, the numbers of pollinator thrips did not differ significantly between the treatments and controls (P = 0.18; Fig. 3.1.3b).

The female inflorescences from which ants were excluded had more damage from herbivores than did controls in four of five pairs (Fig. 3.1.3c). Two ant-excluded

inflorescences received especially serious damage; they were hollowed out deeply into the axis while control inflorescences had no such damage. The difference between treatments and controls was significant; the slope of the regression line was 3.46, which was significantly greater than 1.0 (P = 0.04).

Discussion

I found food bodies on the inflorescences/fruits occurring on seven of eight ant-plant *Macaranga* species (Table 3.1.1). Because ants harvest food bodies, these bodies likely serve as ant attractants. Indeed, observations of inflorescences on three species confirmed that food bodies on reproductive organs significantly increased ant abundances. Because ant abundances on leaves did not change between reproductive stages, these changes were obviously due to redistribution of ant numbers among plant organs, rather than changes in total ant abundances. Although I was unable to examine changes in ant abundances associated with glands on the margins of the bracteoles of *M. winkleri*, they may in fact be nectaries that also provide food rewards. Attraction of mutualistic ants to inflorescences through formation of food bodies has not been reported in ant-plants other than *Macaranga*. Inflorescence food bodies have been reported for *Cordia nodosa* (Boraginaceae) (Solano *et al.*, 2005), but their function is still unclear.

Mutualistic ants on inflorescences may contribute to plant reproduction by protecting flowers from herbivores. In the experiments on *M. havilandii*, ant-free inflorescences were damaged more severely than controls in both male and female trees. During flowering stages, I frequently observed lepidopteran larvae and adults, coleopteran larvae (mostly weevils *Eugryporrhynchus* sp. and leaf beetles), and hemipterans on the inflorescences of the studied *Macaranga* species. They disrupt reproduction of *M. havilandii* by damaging inflorescences, and in some cases, kill whole inflorescences by boring deeply into the inflorescence stems or grazing anthers before pollen is mature (E. Yamasaki, personal observation). On male trees, clear differences were detected in the damage caused by herbivores between ant-excluded and control inflorescences when the damage to controls was extensive; the damage levels were similar when the controls were slightly grazed. This outcome may indicate that ants respond to herbivory during an early stage of grazing and prevent further damage, as shown previously for vegetative parts of *Macaranga* plants (Inui & Itioka, 2007). *M. havilandii*, which I used for the experiment, did not form food bodies during flowering stages, and ant density on flowering inflorescences was no higher than on nonreproductive parts. Species possessing inflorescence food bodies may therefore be more vulnerable to herbivores and need more intense protection from ants than those without food rewards. The strength of ant defence on leaves, which is known to vary greatly among species of *Macaranga*, generally correlates negatively with the strengths of chemical and/or

mechanical defences (Itioka, 2005; Nomura *et al.*, 2011), which may also explain the observed variation in food body production among *Macaranga* species. Food bodies on fruiting inflorescences may similarly contribute to protection by ants, although I was unable to determine the major herbivores during the fruiting stage.

On *M. havilandii*, ants defended inflorescences by excluding herbivores, but they may not have excluded pollinator thrips. In the ant-exclusion experiment on male inflorescences, the abundance of thrips on treated inflorescences did not differ significantly from that on controls. Because *Macaranga*-associated mutualistic ants usually exclude any alien species from their host (Itioka, 2005), the inflorescences of *M. havilandii* are thought to have mechanisms to prevent the elimination of pollinator thrips by ants. One possible mechanism is spatial segregation between ants and pollinators achieved via the architecture of the bracteoles. The thrips usually remain inside the bracteole chambers and feed on nectar from trichome-like nectaries on the adaxial surfaces of bracteoles (Moog *et al.*, 2002; E. Yamasaki, personal observation); ants cannot enter the bracteole chambers because the gaps are too narrow (Fiala *et al.*, 2011; E. Yamasaki, personal observation). Another possible mechanism is that the thrips may repel ants directly through defensive behaviours. Many other thrips belonging to the family Phlaeothripidae, to which the pollinator thrips of *Macaranga* belong, are known to secrete ant-repelling chemicals from their anus (Howard *et al.*, 1983; Suzuki *et al.*, 2004; Tschuch *et al.*, 2005; Tschuch *et al.*, 2008). The second potential mechanism is examined in Section 3.2.

My survey showed that the presence/absence of inflorescence food bodies differed among 11 *Macaranga* species. Inflorescence food rewards were found only on the ant-plant species. Furthermore, the non-ant-plant *M. tanarius*, *M. trichocarpa*, and *M. heynei*, which occurred outside the study sites, do not have inflorescence food bodies (Fiala & Maschwitz, 1992a; A. Kawakita, personal communication). Ant-plant species have close relationships with mutualistic ants, which are more aggressive than the ants associated with non-ant-plant species (Fiala *et al.*, 1989; Itioka, 2005). Thus, ant-plant species can reliably secure strong ant protection for their inflorescences by producing food bodies. In addition, food bodies may have evolved more readily on inflorescences of ant-plant species than on non-ant-plant species because ant-plant species already have food bodies on their vegetative organs. Nevertheless, considerable variation was observed in the presence/absence of food bodies among the ant-plant species. For example, *M. beccariana*, an ant-plant species, did not have any food rewards on its reproductive parts. Factors involved in these variations await investigation in future studies.

Table 3.1.1 Characteristics of 11 *Macaranga* species investigated in this study, with a summary of food body presence/absence on their inflorescences

	Attractants at flowering†		Attractants at fruiting†		Habitat‡	Phenology§	Maximum height¶
	Type	*N* (male, female)	Type	*N*			
Ant-plants							
M. bancana	FB(B)	7/7, 2/2	FB(R)	1/3	P	E	23 m
M. trachyphylla	FB(B)	10/10, 3/3	FB(R)	3/5 (0/2)	P	E	20 m
M. hullettii	FB(B)	1/2 (1/1), –††	–	0/2	P	E	18 m
M. rufescens	FB(B)	0/1, 2/2 (2/2)	–	0/2 (0/2)	P	E	30 m
M. winkleri	FB(B)	7/7, 3/5	–	0/6 (0/1)	P	C	22 m
M. havilandii	–	0/16, 0/9	FB(P)	6/6	P	C	5 m
M. umbrosa	–	–, 0/1	FB(P)	2/2	P	E	15 m
M. beccariana	–	0/4, 0/1	–	0/3 (0/2)	P	E	17 m
Non-ant-plants							
M. gigantea	–	0/3, 0/1	–	0/2 (0/1)	P	E	30 m
M. conifera	–	0/1, 0/1	–	0/1	P	E	24 m
M. praestans	–	–, 0/1	–	0/1	C	E	5 m

† Food bodies on bracts, receptacles, and pericarps are indicated in the two columns headed "Type" as FB(B), FB(R), and FB(P), respectively. The columns headed *N* (male, female) give numbers of individuals with attractants/numbers of individuals observed; numbers in parentheses refer to the Upper Baram area.

‡ Habitat is given as successional stage: P (pioneer) or C (climax), according to Davies (2001) and our observations.

§ Flowering phenology is given as E (episodic) or C (continuous), according to Davies & Ashton (1999) and our observations.

¶ Maximum tree height follows Davies (2001) and Whitmore (2008).

†† Food bodies of inflorescences in *M. hullettii* follow Fiala & Maschwitz (1992a).

Table 3.1.2 Definitions of inflorescence reproductive stages.

Stage	State of inflorescence	Sexual function		Duration
		Male	Female	
Bud	Outer bracts not open	–	–	
Flower 1	Outer bracts open, retained intact	–	+	3–10 days
Flower 2	Outer bracts shed, majority of inner bracts retained Anthers not dehisced (male)	–	+	3–6 days
Flower 3	Majority of inner bracts (male and female of *M. praestans*) or bracteoles (female other than *M. praestans*) shed Anthers dehiscing (male)	+	+	3–6 days
Fruit 1	All bracteoles shed	–	–	5–7 days
Fruit 2	Fruits increase in size	–	–	10–20 days
Fruit 3	Fruits have attained a mature size.	–	–	10–12 days

Table 3.1.3 Abundances of food bodies and numbers of ant visits to inflorescences and leaves at different reproductive stages

		Inflorescences					Leaves		
		Food bodies		Ant visit			Ant visit		
		Bud	Flower 3	Bud	Flower 3	*P*	Bud	Flower 3	*P*
M. winkleri	♀	+++	+	1.55	0.33	0.007*	0.09	0.00	0.28
	(*N* = 1)			(*n* = 11)	(*n* = 15)		(*n* = 11)	(*n* = 15)	
	♂	+++	+	32.20	11.75	0.05*	13.67	8.50	0.25
	(*N* = 1)			(*n* = 15)	(*n* = 4)		(*n* = 15)	(*n* = 4)	
M. trachyphylla	♂	+++	+	5.75	0.67	0.02*	1.38	1.00	0.74
	(*N* = 1)			(*n* = 8)	(*n* = 3)		(*n* = 8)	(*n* = 3)	
M. havilandii	♀	–	–	0.36	0.50	0.59	2.29	2.75	0.39
	(*N* = 3)			(*n* = 14)	(*n* = 12)		(*n* = 14)	(*n* = 12)	
	♂	–	–	0.53	0.00	0.27	5.47	1.40	0.09
	(*N* = 3)			(*n* = 15)	(*n* = 6)		(*n* = 15)	(*n* = 6)	
		Inflorescences					Leaves		
		Food bodies		Ant visit			Ant visit		
		Fruit 2	Fruit 3	Fruit 2	Fruit 3	*P*	Fruit 2	Fruit 3	*P*
M. winkleri	(*N* = 1)	–	–	0.17	0.00	0.77	0.17	0.00	0.77
				(*n* = 6)	(*n* = 2)		(*n* = 6)	(*n* = 2)	
M. trachyphylla	(*N* = 1)	+++	+	14.60	5.00	0.09	6.80	3.00	0.30
				(*n* = 20)	(*n* = 2)		(*n* = 20)	(*n* = 2)	
M. havilandii	(*N* = 3)	+++	+	9.57	5.67	0.06	3.21	2.11	0.22
				(*n* = 28)	(*n* = 18)		(*n* = 28)	(*n* = 18)	

Abundances of food bodies are indicated by +++ (present), + (very few), and – (absent). *N* and *n* are the number of trees and inflorescences, respectively. *P* refers to the significance of the Wilcoxon rank-sum tests for significant differences between inflorescences or leaves of Bud and Flower 3 stages and between those of Fruit 2 and Fruit 3 stages.

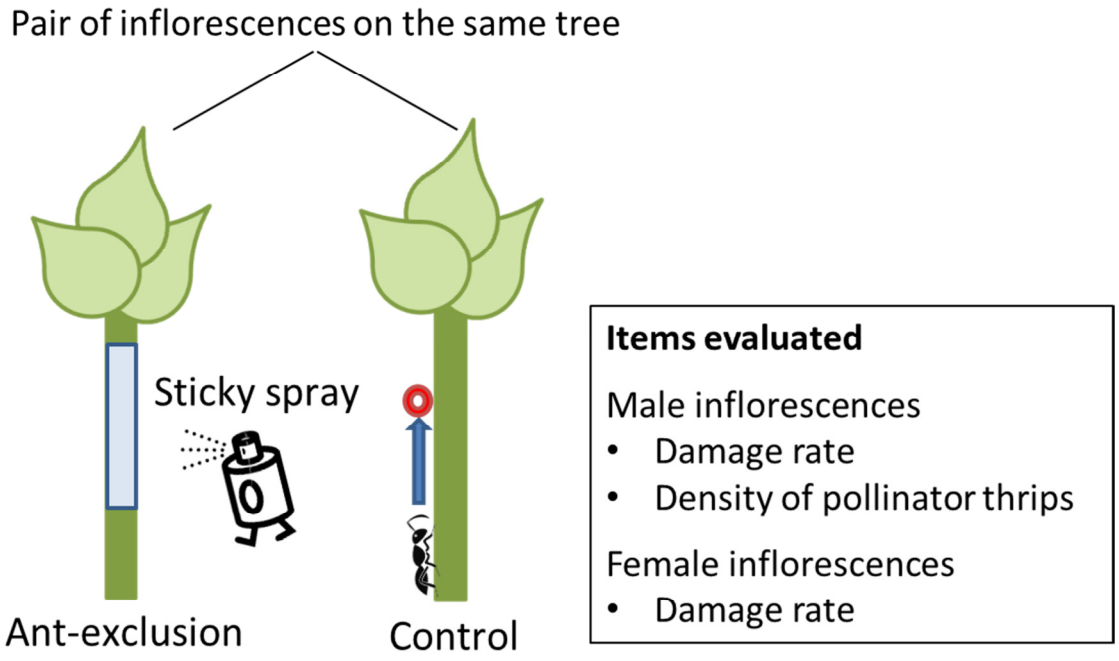

Fig. 3.1.1 Methods of ant-excluding experiment.

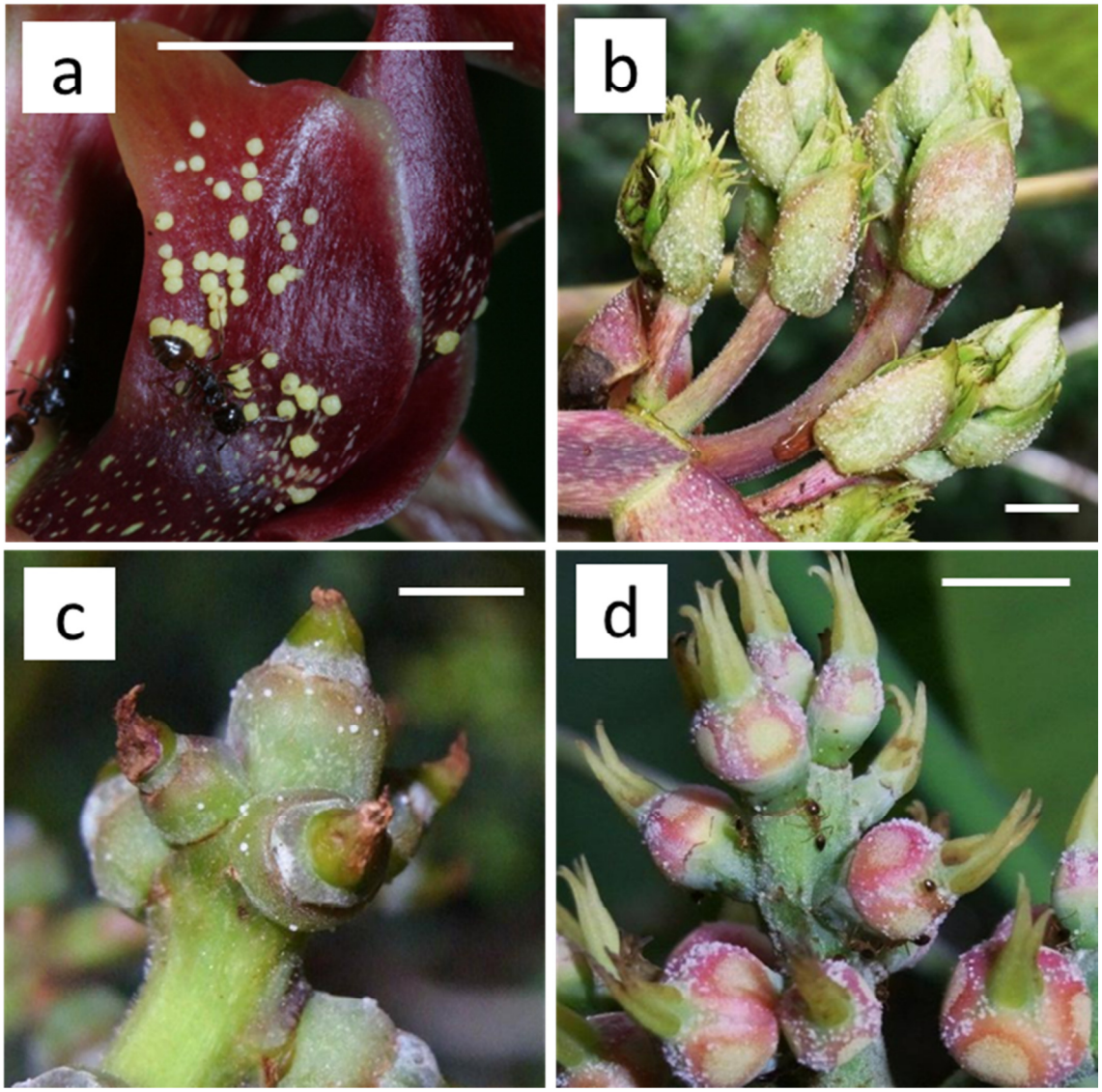

Fig. 3.1.2 Food bodies on inflorescences. (a) Food bodies on the bracts of male *Macaranga winkleri*. (b) Food bodies on the bracts of male *M. trachyphylla*. (c) Food bodies on the receptacles of *M. trachyphylla*. (d) Food bodies on the pericarps of *M. havilandii.* Scale bars are 1 cm.

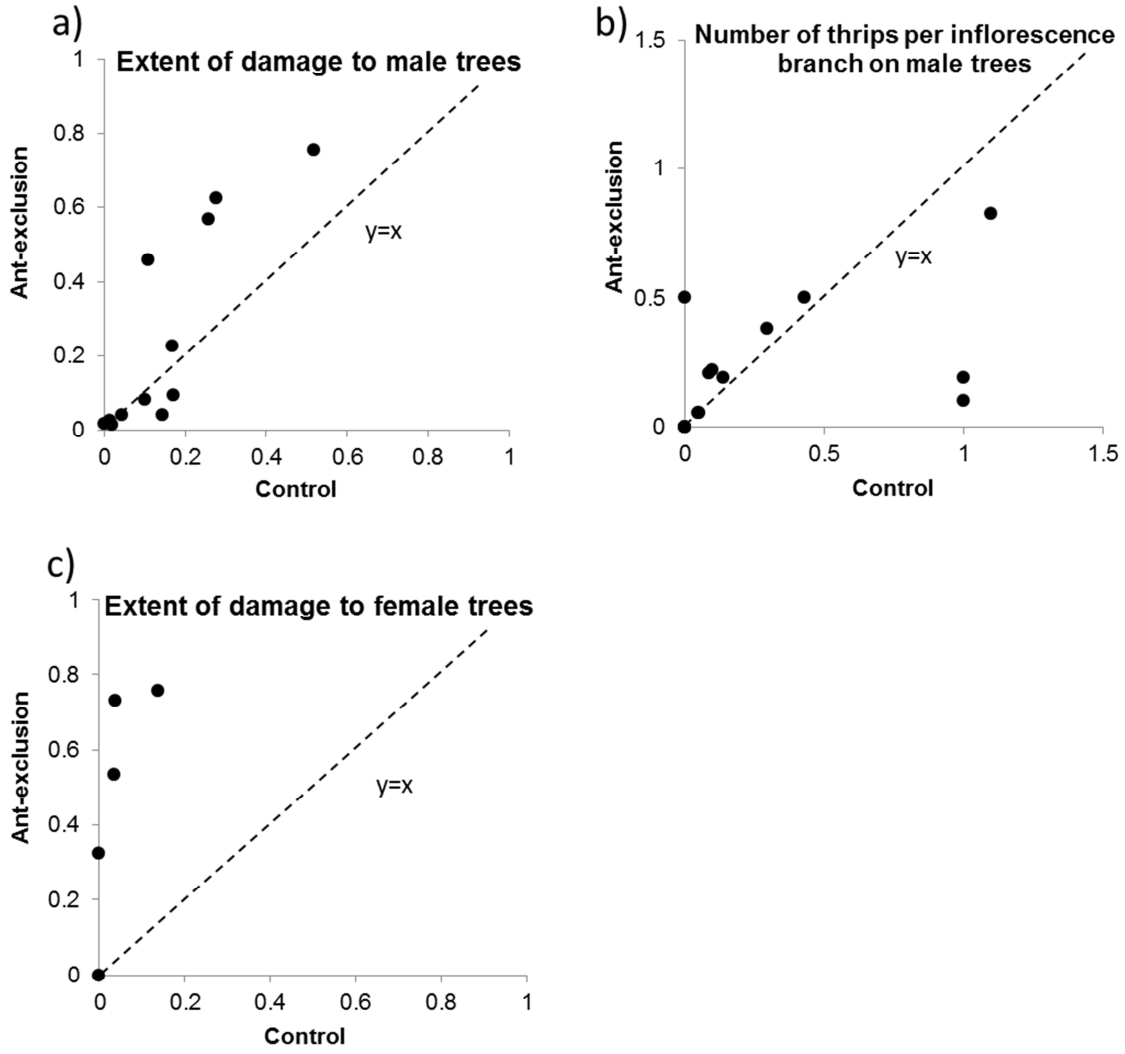

Fig. 3.1.3 Responses to treatments (ant exclusion vs. control on *M. havilandii*) measured as (i) extent of damage attributable to herbivores and (ii) number of pollinator thrips on inflorescences. (a) Extent of damage on male trees (b) Number of thrips per inflorescence branch on male trees (c) Extent of damage on female trees. Symbols above the diagonal ($y = x$) indicate cases where ants protected inflorescences from herbivores (a, c) or ants excluded pollinators (b).

Section 3.2
Anal secretions of pollinator thrips of *Macaranga winkleri* repel guard ants

Introduction

Many plants have protective mutualistic relationships with ants (Chapter 1). Although ants are useful for plant defence, they may also negatively affect reproduction of the plant because they exclude its pollinators as well as its grazers. Therefore plants have evolved diverse strategies for deterring ants from their flowers, thereby circumventing this problem (Chapter 1). However, only a small body of literature exists on the strategies by which ant-plants avoid negative effects of ants on pollination, which is surprising because defence by ants is much stronger in ant-plants than in plants that attract ant guards facultatively. The potentially negative effects of ant symbionts on pollination are also more severe in ant-plants. Rare studies report that *Acacia* species that are ant-plants secrete ant-repellent volatiles from their flowers (Willmer & Stone, 1997; Raine *et al.*, 2002; Willmer *et al.*, 2009) and locate reproductive parts far from nests and foods for ants (Raine *et al.*, 2002), thereby reducing conflicts between ants and pollinators.

All of the ant-plant *Macaranga* species for which pollination has been investigated are pollinated by one of two undescribed species of thrips in the genus *Dolichothrips* (*Dolichothrips* sp. 1 and *Dolichothrips* sp. 2: Phlaeothripidae) (Moog *et al.*, 2002; Fiala *et al.*, 2011) that preferentially feed and breed on the inflorescences of *Macaranga*. They are diminutive insects reaching about 2 mm in length and have limited flight capability (Lewis, 1973), attributes that in isolation would make them easily killed and excluded by ants. However, the earlier ant-exclusion experiment on *M. havilandii* demonstrated that ants do not exclude pollinator thrips while protecting inflorescences against herbivory (Yamasaki *et al.*, 2013a; Section 3.1).

In the present study, I examined the ways in which pollinator thrips avoid or reduce ant attacks. *M. winkleri* was chosen for the study because the species of ant it hosts is among the most aggressive of ant guards in relationships with *Macaranga* (Itioka *et al.*, 2000; Itioka, 2005). First, to determine how ants respond to pollinator thrips and other insects, I conducted bioassays using individual test insects (pollinator thrips, congeneric ants from *Macaranga* species other than *M. winkleri*, herbivores and generalist flower visitors). After observing pollinator thrips secreting droplets from their anuses when encountering ants, I analysed the constituents of these secretions. Finally, to determine whether ant guards are chemically repelled by the secretions of pollinator thrips, I

conducted chemical bioassays using the whole anal secretion and its main chemical constituent.

Materials and methods

Study species

Macaranga winkleri is a dioecious pioneer tree species distributed throughout the island of Borneo (Whitmore, 2008). Among its congeners, this tree has one of the strongest ant defences (expressed by the aggressiveness and biomass of symbiotic ants (Itioka *et al.*, 2000; Itioka, 2005)). *M. winkleri* flowers continually year-round at the study site (Davies & Ashton, 1999), where they are pollinated by *Dolichothrips* sp. 1 (Fiala *et al.*, 2011). Male and female inflorescences of *M. winkleri* are panicles 20–35 cm and 10–26 cm in length, respectively; each panicle has many small flowers lacking petals (Whitmore, 2008). Single greenish bracteoles subtend 6–15 and 2–5 male and female flowers, respectively. A red-coloured bract subtends a series of these flowers and bracteoles. The most abundant flower visitors other than *Dolichothrips* sp.1 were weevils (*Eugryporrhynchus* sp.: Curculionidae); leaf beetles and hemipterans have also been observed (E. Yamasaki, unpublished data).

Study site

The study was conducted in an aseasonal tropical rain forest in Lambir Hills National Park from September 2011 to June 2013 (See Section 2.2 for detail information of the site). The site was covered by a primary mixed dipterocarp forest, with *M. winkleri* growing in gaps, along the edges of the forest and on riverbanks.

Ant behavioural experiment

I compared behaviours of ants against different insects in the following experimental design (Fig. 3.2.1). Ant workers (50–100) were collected from each *M. winkleri* tree and kept for a few hours before experimentation in a circular plastic cup (6 cm diameter, 4 cm tall) lined on the bottom with burnt plaster. A piece of host stem halved lengthways was introduced into each cup to habituate the ants to their new circumstances. Each ant colony in a cup was kept for less than 2 days and used in a maximum of four trials using different test insects. I introduced one of the following groups of test insects into the cup: pollinator thrips (N = 24); congener ants from *M. trachyphylla* or *M. beccariana* (N = 14); weevils (*Eugryporrhynchus* sp.), which are common herbivores on *Macaranga* inflorescences (N = 20); and stingless bees (*Trigona erythrogastra*), which are common generalist pollinators at the study site but do not pollinate *Macaranga* species (N = 14). In each trial, an individual test insect was carefully placed near the plant stem segment using forceps. The behaviours

of the first 10 ants that touched the test insect with their antennae were classified into three categories (escape, antennal drumming, and caution/attack; Table 3.2.1). The frequencies of "escape" and "caution/attack" behavioural categories were compared among treatments using Tukey tests. I stopped the trials when the test insects died during the observation period.

Chemical analysis of thrips anal secretions

Anal droplets from pollinator thrips were collected and analysed by gas chromatography (GC)-mass spectrometry. First, I stimulated the thrips (observed by stereomicroscopy) with fine soft brushes. When the insects raised their abdomens and secreted droplets, I captured the droplets on pieces of glass microfibre filter (Whatman, Little Chalfont, Buckinghamshire, UK) previously rinsed in n-hexane. Each pollinator thrips was stimulated 3–5 times, until secretion stopped. After absorbing droplets from 25–30 thrips, the thrips secretions were dissolved by soaking each filter in ~1 mL of n-hexane held in a glass vial. Each solution was carefully concentrated to a volume of 200–400 μL under N_2 gas flow; 500 ng of 10-undecenoic acid was added as an internal standard. Chemical compounds were analysed with a GC-17A gas chromatograph and a QP5050A mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a DB-WAX glass capillary column (inner diameter, 0.25 mm; length, 30 m; film thickness, 0.25 μm; Agilent Technologies, Ipswich, UK) using helium as the carrier gas. The injector was operated in splitless mode for 0.75 min. Oven temperature was programmed as follows: 50°C for 1 min, raised at a rate of 20°C min^{-1} to 150°C, and then to 240°C at a rate of 5°C min^{-1}. Peaks detected were identified by comparison with mass spectra in the NIST 11 database. Identifications of compounds detected were verified by comparing GC retention times and mass spectra to those of authentic standards. The relative amount of each compound was calculated by comparing its peak area with that of 10-undecenoic acid, the internal standard; the individual equivalent of each compound was obtained by dividing the relative amount by the number of thrips individuals.

Ant responses to chemicals

I conducted chemical bioassays to determine whether pollinator thrips chemically deter ant guards of *M. winkleri* by secreting repellent anal droplets. I prepared Teflon® (Dupont, Wilmington, DE, USA) rods (diameter, 1.5 mm; length, 5 mm) by rinsing them in n-hexane, followed by application to each of one of the following test chemicals: (1) hexane only (control), (2) hexane crude extract of pollinator thrips, (3) 200 ng of decanoic acid (the main constituent of anal droplets secreted by pollinator thrips) dissolved in n-hexane and (4) hexane crude extract of congener ants from other *Macaranga* species. Hexane crude extracts of congener ants were used to test whether the ants distinguished the chemicals on

the Teflon® rods and exhibited appropriate behaviours. For (2) and (4), each of 15 pollinator thrips and five congener ant workers collected from *M. trachyphylla* or *M. beccariana* were soaked in n-hexane for 5 min, respectively. In preliminary chemical analyses, I demonstrated that constituents of anal droplets and the hexane crude extracts of the pollinator thrips were almost identical. After application of the test chemicals, the hexane was evaporated from the Teflon® rods, each of which was then inserted into a cup containing ants held under conditions identical to those in the behavioural experiments. Ant responses were recorded using the same categories used in the ant behavioural experiments. The frequencies of "escape" and "caution/attack" behaviours in response to each chemical were compared to controls using Dunnett's multiple comparison tests.

Results

Ant behavioural experiment

All trials with ants from other *Macaranga* species were discontinued after 2.07 ± 1.98 (mean ± SD) ants had touched the test ants because the ants bit one another and struggled until both were dead. Caution/attack ant behaviours were observed in the tests with the stingless bees, weevils and pollinator thrips; significant differences were observed among treatments (thrips vs. weevils, $t = 5.36$, $P < 0.001$; thrips vs. stingless bees, $t = 20.10$, $P < 0.001$; weevils vs. stingless bees, $t = 14.74$, $P < 0.001$) (Fig. 3.2.2). Stingless bees always shifted locations when touched by the ants, but weevils often held their ground. Ants escaped from pollinator thrips more frequently than from weevils ($t = 7.84$, $P < 0.001$) or stingless bees ($t = 7.56$, $P < 0.001$) (Fig. 3.2.2). Ant escape behaviour often occurred when the thrips raised their abdomens; among a total of 84 escape behaviours, 57 occurred after thrips raised their abdomens. I often saw thrips secreting yellow droplets from their anuses when encountering ants (Fig. 3.2.3).

Chemical analysis of thrips anal secretions

Several fatty acids were detected in the anal droplets (Table 3.2.2). Among them, n-decanoic acid was the main constituent, accounting for 75% of total weight (65.56 ± 39.74 ng per individual).

Ant responses to chemicals

Frequencies of caution/attack behaviours in response to crude extracts of pollinator thrips and n-decanoic acid did not differ significantly from frequencies in response to the control ($t = 0.30$ and 0.58, $P = 0.88$ and 0.98); this behaviour occurred more frequently in responses to crude extracts of congener ants than in responses to the controls ($t = 8.12$, $P < 0.001$) (Fig. 3.2.4). Escape behaviour occurred more often in responses to crude extracts of

pollinator thrips and n-decanoic acid than in responses to the controls (t = 6.15 and 4.41, P < 0.001 and 0.001). No significant differences were observed in the proportions of escape behaviours in responses to crude extracts of congener ants and responses to the controls (t = 0.61, P = 0.86) (Fig. 3.2.4).

Discussion

The extremely aggressive ant guards of *Macaranga winkleri* had different responses to different insect groups. They aggressively attacked congeneric ants from other *Macaranga* hosts. The intensity of this interaction was so strong that I found it necessary to discontinue bioassays as soon as the congener ants made contact. Stingless bees, which were generalist flower visitors at the study site, were also strongly attacked by the guard ants. Inflorescence-feeding weevils provoked less aggressive interactions by ants. Weevils may avoid ant attacks to some extent by staying still during ant encounters; this strategy may be effective because the ants react so strongly to movement. Moreover, ant bites were rarely fatal for weevils, which have particularly hard exoskeletons. The weevils appear to be specialists on *Macaranga* because they have been found on the inflorescences of this genus and nowhere else. This habitat specificity may relate to the ability of weevils to endure ant aggression, an ability that may represent an evolutionary predisposition. Compared to congeneric ants, stingless bees and weevils, pollinator thrips received ant attacks only rarely, and the thrips rather often repelled ant guards. The experimental results on ant–pollinator thrips relationships were consistent with those of ant-excluding experiments conducted with other *Macaranga* species, which report that ants exclude herbivores but not pollinator thrips (Yamasaki *et al*., 2013a). Ant deterrence by pollinator thrips was probably related to thrips anal secretions. When individuals of *Dolichothrips* sp. 1 encountered the ants, they often raised their abdomens and secreted anal droplets from which the ants fled.

The ants may be chemically repelled by constituents of thrips anal secretions. Although crude extracts of congener ants were vigorously attacked by ant guards of *M. winkleri*, pollinator thrips secretions and their main constituent, n-decanoic acid, were rarely attacked by the ants; indeed, they functioned as ant repellents. My preliminary survey also confirmed that n-decanoic acid is a main constituent of anal secretions in both *Dolichothrips* sp. 1 and *Dolichothrips* sp. 2 collected from several other *Macaranga* species (E. Yamasaki, unpublished data). n-Decanoic acid is common in nature (Torto *et al*., 1996; Jürgens *et al*., 2006; Pino *et al*., 2010); it has been detected in defensive and venomous secretions of diverse arthropods (Steidle & Dettner, 1995; Salles *et al*., 2006) and in the anal droplets of some species of the family Phlaeothripidae, including pollinator thrips of the ant-plant *Macaranga* (Suzuki *et al*., 2004).

Ants generally have negative effects on pollination processes and plants have evolved diverse ways of blocking ant access to their flowers. In most of the blocking mechanisms, plants modify their morphologies (by developing slippery waxy inflorescence shoots, narrow corollas and ant-distracting extrafloral nectaries, among other means) and/or the plants produce repellent secretions (e.g. ant-repelling volatiles, toxic nectar) (Chapter 1). To my knowledge, the present study is the first report to demonstrate that pollinator insects repel ants in mutualistic relationships with the host plant.

Importantly, all the observed ant-plant *Macaranga* species had the Enclosing type inflorescences (Section 2.1) and many of them are pollinated by thrips *Dolichothrips* spp. (Moog *et al.*, 2002; Fiala *et al.*, 2011) that secrete ant-repelling droplets from their anuses. While aggressive ant guards are patrolling on the plants, the pollinator thrips can stay on the inflorescences of ant-plants without being excluded by ant guards because they secrete ant-repellents and maybe flower-enclosing bracteoles protect the pollinators. Not only ant-plant species but also the related species that are facultatively protected by ants are pollinated by *Dolichothrips* spp. Fig. 3.2.5 shows the most parsimonious phylogenetic tree from Davies *et al.* (2001) containing many species in sect. *Pachystemon*, *Pruinosae* and *Winklerianae* and Bicolor group, which include ant-plants, and some species in other infrageneric groups, based on DNA sequence data of ITS region and 81 morphological trait data. Information about main flower visitors and whether ant-plant or non-ant-plant is mapped on the tree. Considered based on a principle of parsimony, *Dolichothrips* pollination may have occurred only once, at the base of *M. winkleri* clade (Fig. 3.2.5). On the other hand, the parsimonious reconstruction of ancestral morphologies in Davies *et al.* (2001) indicates that ant-plant and non-ant-plant states have changed in total five times, and ant-plant species have occurred between two to four times in the *Dolichothrips*-pollinated clade (Fig. 3.2.5). Development of a pollination system resistant to ant attacks may have predisposed the evolution of ant-plants in the genus *Macaranga*.

Table 3.2.1 Definitions of behavioural categories recognised in ants encountering test insects/chemicals in bioassays.

Category	Definition
Escape	Ants touching insect with antennae for < 2 s, subsequently fleeing while increasing walking speed or switching direction.
Antennal drumming	Ants touching insect with antennae for < 2 s, subsequently moving away without changing speed or direction or ants touching insect with antennae for > 2 s, then moving away.
Caution/Attack	Ants touching insect with antennae, then opening their mandibles or biting the test insects/chemicals.

Table 3.2.2 Relative amounts of constituents in anal droplets secreted by *Dolichothrips* sp. 1 (means ± SD per individual).

Compounds	Individual equivalent (ng)
Heptanoic acid	0.20 ± 0.44
Octanoic acid	1.08 ± 0.37
Nonanoic acid	0.33 ± 0.58
n-Decanoic acid	65.56 ± 39.74
**m/z*: 41, 55, 69, 43, 68, 67, 54, 84, 81, 71	6.37 ± 8.15
9-Decenoic acid	13.91 ± 10.23

* Ion fragments of an unidentified fatty acid are provided in rank order of intensity.
See "*Chemical analysis of thrips anal secretions*" in the Materials and Methods for definitions of "relative amount" and "individual equivalent".

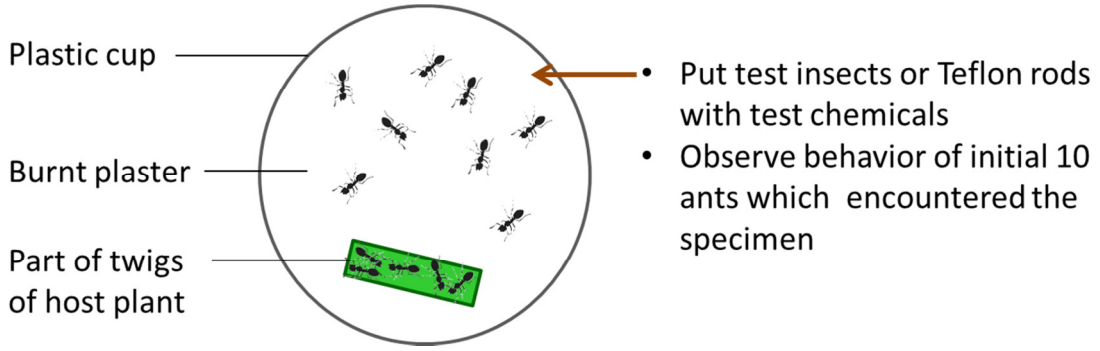

Fig. 3.2.1 Methods of "ant behavioural experiment" and "ant responses to chemicals".

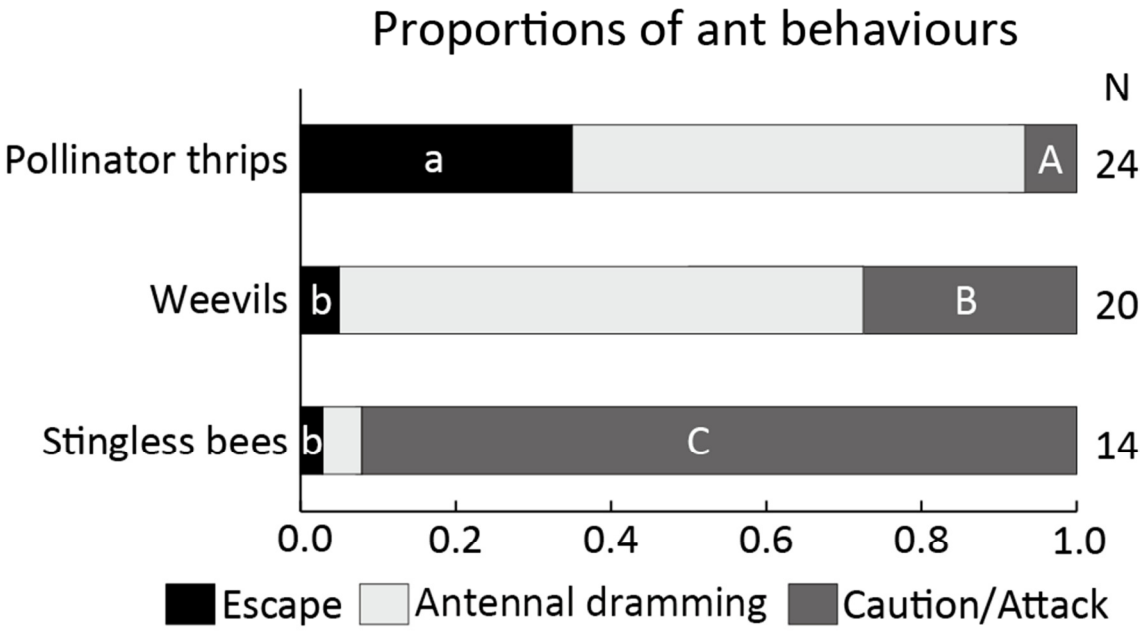

Fig. 3.2.2 Proportions of different behavioural categories in ants responding to pollinator thrips, inflorescence-feeding weevils and stingless bees, generalist flower visitors. Different lower- and uppercase letters indicate significant differences in "escape" and "caution/attack" behaviours, respectively (Tukey tests, $P < 0.05$). See Table 3.2.1 for definitions of behavioural categories.

Fig. 3.2.3 Pollinator thrips secreting yellowish droplets from their anuses when encountering ants.

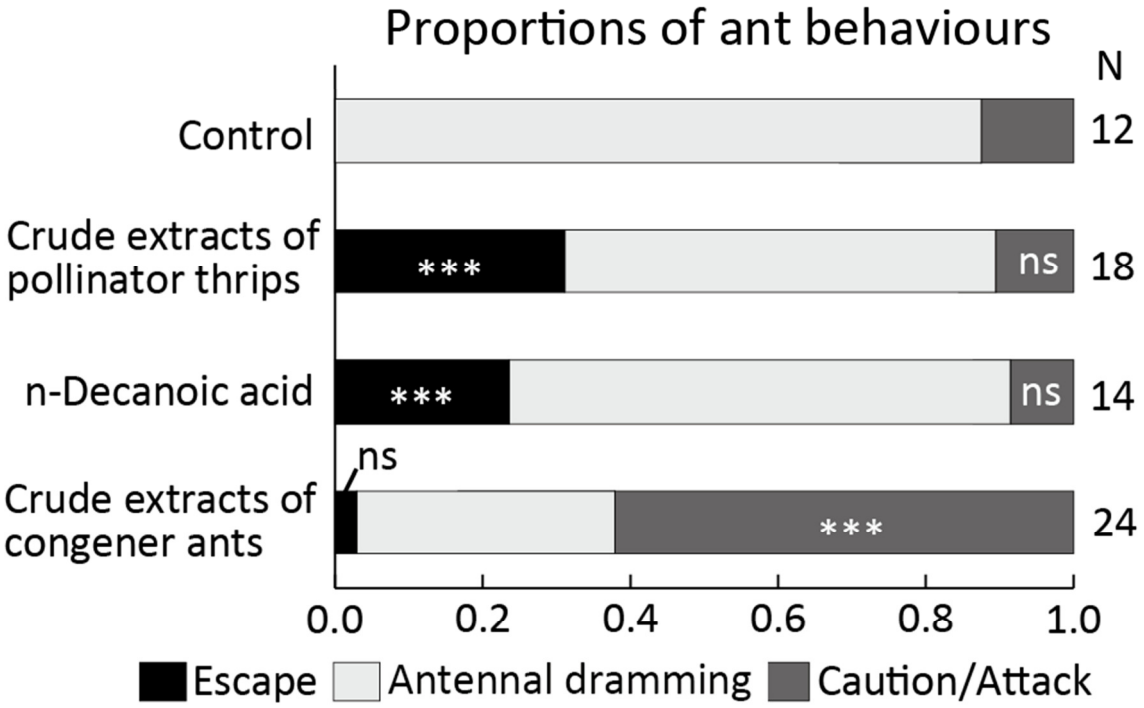

Fig. 3.2.4 Proportions of different behavioural categories in ants responding to different chemicals. Significances of differences in the proportions of "escape" and "caution/attack" behaviours between each test chemical and controls are indicated by *** ($P < 0.01$) and ns ($P > 0.05$) (Dunnett's multiple comparison tests).

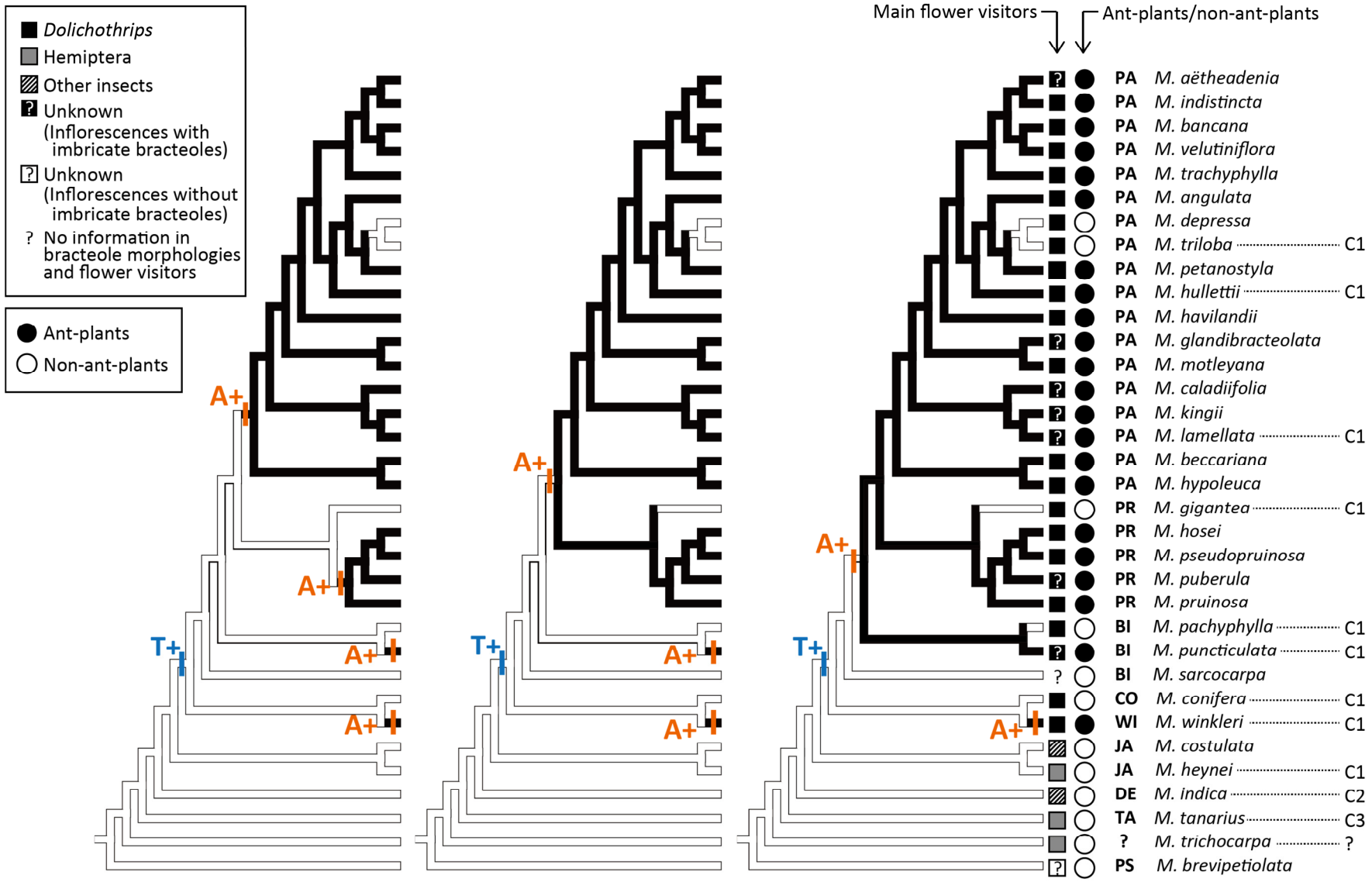
Dolichothrips
Hemiptera
Other insects
Unknown (Inflorescences with imbricate bracteoles)
Unknown (Inflorescences without imbricate bracteoles)
No information in bracteole morphologies and flower visitors
Ant-plants
Non-ant-plants
Main flower visitors
Ant-plants/non-ant-plants
A+
T+
PA M. aëtheadenia
PA M. indistincta
PA M. bancana
PA M. velutiniflora
PA M. trachyphylla
PA M. angulata
PA M. depressa
PA M. triloba C1
PA M. petanostyla
PA M. hullettii C1
PA M. havilandii
PA M. glandibracteolata
PA M. motleyana
PA M. caladiifolia
PA M. kingii
PA M. lamellata C1
PA M. beccariana
PA M. hypoleuca
PR M. gigantea C1
PR M. hosei
PR M. pseudopruinosa
PR M. puberula
PR M. pruinosa
BI M. pachyphylla C1
BI M. puncticulata C1
BI M. sarcocarpa
CO M. conifera C1
WI M. winkleri C1
JA M. costulata
JA M. heynei C1
DE M. indica C2
TA M. tanarius C3
? M. trichocarpa ?
PS M. brevipetiolata

Fig. 3.2.5 Most parsimonious phylogenetic trees around ant-plant *Macaranga* species based on DNA data of ITS region and 81 morphological trait data (Davies *et al.*, 2001). The three trees show same topologies and three evolutionary patterns of ant-plants obtained by most parsimonious reconstruction method. T+ and A+ indicate gaining of thrips pollination and ant-plants based on most parsimonious reconstruction method, respectively. Information of main flower visitors and whether ant-plant or non-ant-plant is shown by squares and circles, respectively. Infrageneric groups are indicated by two capital letters; PA = sect. *Pachystemon*, PR = sect. *Pruinosa*, WI = sect. *Winklerianae*, PS = sect. *Pseudorottlera*, BI = Bicolor group, CO = Conifera group, JA = Javanica group, DE = Denticulata group, TA = Tanarius group (Whitmore, 2008). Species included in phylogeny obtained in Section 2.1 are indicated on the right side with the group recognized in the section (C1, C2, C3 or ?).

Chapter 4
General Discussion

In the genus *Macaranga*, pollination systems and the strength of biological defence have changed repeatedly, and their evolution has not occurred independently. Among the three inflorescence morphology types, the Inconspicuous type is considered to be ancestral, while the Discoid-gland and Enclosing types evolved afterward, although which of these two types evolved first is unknown. Each of the three inflorescence types did not converge upon a monophyletic group and may have originated independently multiple times. On the other hand, the evolution of ant-plants has occurred two to four times in only one of the crown clades (Blattner *et al.*, 2001; Davies *et al.*, 2001; Bänfer *et al.*, 2004).

In species exhibiting Discoid-gland-type inflorescences, extrafloral nectaries on leaves, which attract ant guards, may have been diverted to inflorescences and served as rewards for pollinators (Section 2.3). Because *Macaranga* species throughout the genus exhibit protective mutualisms with ants and possess extrafloral nectaries on leaves, this type of evolution may have occurred easily. While attracting pollinator insects, disk-shaped nectaries on inflorescences may also attract guard ants that can deter pollinators (Yamasaki *et al.*, 2013). This conflict between pollination and protective mutualisms with ants might be resolved by plant adaptations such as ant-repelling chemicals. Alternatively, the conflict may be negligible because the frequency of encountering ants and pollinators is low or because the advantage of protection of floral organs by ants exceeds any disadvantage of pollination interference by ants. Whether a conflict between pollination and protection by ants exists in species with disk-shaped glands, and if it exists, how the conflict is resolved, are subjects for future studies.

Multiple evolution of the Enclosing type of inflorescence may be related to conflicts between pollination and protection by ants. Species with inflorescences of this type may be pollinated by small insects, such as thrips and hemipterans (Moog *et al.*, 2002; Ishida *et al.*, 2009; Fiala *et al.*, 2011), which can crawl into the bracteole chambers to visit flowers. Because the bracteole can function to physically separate ants and pollinators on the inflorescence, interference with pollination by ants may have been a selective force for the evolution of flower-covering bracteoles that eliminate ants from flowers. To explore this possibility, the functions of bracteoles should be examined in the Enclosing-type species of various clades.

Ant-plants have evolved multiple times in the clade of Enclosing types pollinated by *Dolichothrips* spp., but evolution has not occurred outside of the clade. Acquisition of *Dolichothrips* pollination, which is highly resistant to ant attacks, may have facilitated the evolution and maintenance of active ant defence in *Macaranga* (Sections 3.1, 3.2). In turn,

the pollination system might also be maintained by strong defence by ants, as pollinator shifts from *Dolichothrips* to other animals is not likely to occur under strong defence by ants.

In the evolutionary history of the genus outlined above, two evolutionary events by which plant traits involved in protective mutualisms with ants might have affected the evolution of pollination and vice versa can be recognised. The first event is that disk-shaped glands on bracteoles for pollinators originated by "exaptation", in which existing traits achieve new functions from pre-existing extrafloral nectaries that attract ant guards on leaves (Section 2.3; Yamasaki *et al.*, 2013). Such exaptation may also have occurred in *Acacia* (Fabaceae) (Knox *et al.*, 1985), *Euphorbia* (Euphorbiaceae) (Traveset & Sáez, 1997) and related genera of *Triadica*, *Neoshirakia*, *Excoecaria* and *Homalanthus* (Euphorbiaceae) (E. Yamasaki, unpublished data). The glands serve as extrafloral nectaries next to flowers used for pollination. As in the case of *Macaranga*, these species or their relatives also exhibit protective mutualisms with ants and possess extrafloral nectaries on leaves or petioles (Janzen, 1966; Boughton, 1981; Blüthgen & Reifenrath, 2003; So, 2004; Carrillo *et al.*, 2012). Exaptation is thought to have played an important role in various evolutionary processes in a variety of organisms (Gould & Vrba, 1982). Previous studies have reported evolution by exaptation of plant traits involved in chemical or physical defence to pollination and vice versa (Armbruster *et al.*, 1997; Wragg & Johnson, 2011). Further studies could reveal additional cases in which exaptation has played an important role in the evolution of pollination systems and defence.

The second example of an evolutionary relationship between protective mutualisms with ants and pollination is that pollination by *Dolichothrips*, which is resistant to ant attacks, might have reduced pollination interference by ants and enabled the evolution of strong defence by ants (Chapter 3). In this case, a change in the pollination system resolved a conflict between pollination and defence and released defensive traits from evolutionary constraints. Most ant-plants potentially face the conflict that as the intensity of protection provided by ants increases, the more likely pollinators will be excluded by the ants. Three-way interactions among ant-plants, pollinators and guard ants have only been investigated in *Macaranga* and *Acacia*. Ant-plant *Acacia* species pollinated by generalist insects place flowers far from ant nests and food sources, and they deter ant guards from their flowers using ant-repelling volatile compounds (Willmer & Stone, 1997; Raine *et al.*, 2002; Willmer *et al.*, 2009). Mechanisms for avoiding ant guard interference with pollination may vary among plant groups depending on plant characteristics such as pollinator identity, flower/inflorescence characteristics, strength of defence by ants and allocation of resources to ant guards.

To date, different preadaptations have been proposed as essential for the evolution of ant-plants; these include hollow organs or stems allowing ant inhabitation (sometimes with the aid of excavation by ants), the absence of resin secretion from wounds that prevent ants from gnawing entrance holes on plant organs, queen-attracting volatiles emitted without grazing and year-round food production (Fiala & Maschwitz, 1992b; Davidson & McKey, 1993; Brouat & McKey, 2000; Blattner *et al.*, 2001; Davies *et al.*, 2001). In addition to these prerequisites, the present findings indicate that a mechanism for avoiding ant attacks on pollinators may also strongly influence the evolution of ant-plants. While the previously suggested prerequisites are necessary for ants to settle in the plants at all times, the results of this dissertation have suggested a prerequisite that is necessary for ensuring host plant reproduction with the existence of guard ants. To sustain this hypothesis, the effects of ants on pollination and strategies to avoid ant interference with pollination should be investigated in many ant-plant and non-ant-plant species other than *Macaranga* and *Acacia*. Melastomataceous ant-plants, such as *Tococa*, *Maieta* and *Clidemia*, may be ideal groups for such studies, as multiple origins of ant-plant species have been suggested in the family (Vasconcelos, 1991; Michelangeli, 2000). *Tococa* and *Clidemia* are pollinated by various bees (Renner, 1989; Michelangeli, 2000), and they may possess mechanisms to segregate pollinator bees from ant guards, as in the ant-plant *Acacia*.

To the best of my knowledge, evolutionary relationships have primarily been reported between plant traits involved in pollination and defence (Knox *et al.*, 1985; Armbruster, 1997; Traveset & Sáez, 1997; Wragg & Johnson, 2011). Plants exhibit various relationships with animals other than those involving pollination and herbivory; these include seed dispersal, cultivation mutualisms and myrmecotrophic mutualisms in which plants absorb nutrients from ant nests in their bodies (Fenner, 2000; Hata & Kato, 2002; Rico-Gray & Oliveira, 2007). The evolution of pollination and defence systems may be more strongly related to each other due to their ubiquity and to the higher diversity of these interactions compared to other interactions. Various insects, birds, mammals and other animals act as pollinators and/or herbivores, whereas a limited number of animal taxa are involved in other interactions. Corresponding with the diversity of herbivore and pollinator interactions, plants have evolved various strategies for pollination and for defence against herbivores, whereas plant strategies for adapting to animals in other interaction are not as diverse. Therefore, among the combinations of pollination and defence strategies, some may be susceptible to conflicts between pollination and defence, or plant traits involved in pollination or defence may be diverted to other uses.

Among the defence strategies of plants, protective mutualisms with ants may be more prone to interact with pollination than with physical or chemical defences. First, plant traits involved in physical (e.g. tough leaves or trichomes) or chemical (secondary

metabolites such as tannins or phenols) defences are rarely used to attract and reward pollinators. On the other hand, among the plant traits involved in protective mutualisms with ants, extrafloral nectaries, the most common reward for ant guards, can potentially be used as a reward for pollinators. Second, because physical or chemical defences are usually expressed locally in organs damaged by herbivores, pollinators are rarely affected by the defence systems. In contrast, because ant guards usually indiscriminately attack plant visitors, pollinators can also be attacked by the ants. While only a small percentage of angiosperm species potentially have protective mutualisms with ants (Marazzi *et al.*, 2013; Weber & Keeler, 2013), the flowering plant species that do are principal components in tropical forests (Koptur, 1992; Heil & McKey, 2003). The evolutionary relationships between pollination and protective mutualisms are therefore crucial for studying the evolution of plants that sustain tropical forests.

Appendix

Appendix 1 Inflorescence/floral traits assessed on herbarium specimens. Inflorescence morphology types of Enclosing, Discoid-gland and "absent" are shown as E, D and A, respectively. *N* indicates numbers of specimens observed (numbers of male and female specimens are shown separately). Presence/absence of disk-shaped glands on the middle part of the bracteole surfaces are indicated by "+" and "–". Data not available are indicated by NA.

Species	Bracteole type	*N*		Disk-shaped glands	Length of bracteoles (mm)	Width of bracteoles (mm)	Internode between two bracteoles (mm)	Length of stigma (mm)
		♂	♀					
M. albescens	A	3	3	−	1.28	0.55	2.78	2.63
M. alchorneoides	A	3	0	−	1.14	1.12	2.76	NA
M. aleuritoides	D	3	3	+	1.19	0.39	2.00	6.43
M. alnifolia	D	2	2	+	1.43	0.37	3.58	4.37
M. angustifolia	D	3	2	+	4.19	2.18	5.23	0.34
M. auriculata	A	2	1	−	1.27	1.07	1.75	6.73
M. barteri	E	2	2	−	4.65	3.35	1.38	0.53
M. bicolor	E	4	0	−	2.33	2.17	0.99	NA
M. bifoveata	D	3	3	+	1.90	1.66	3.51	2.92
M. brachytricha	NA	0	0	NA	NA	NA	NA	NA
M. clavata	E	3	3	−	2.94	2.88	1.81	1.60
M. conifera	E	3	0	−	1.41	1.77	0.80	NA
M. densiflora	A	3	0	−	1.67	0.96	1.83	1.30
M. denticulata	D	5	4	+	1.01	1.03	NA	0.69
M. diepenhorstii	E	3	1	−	2.84	2.70	1.01	0.43
M. domatiosa	A	3	3	−	1.43	0.22	3.41	7.33

Appendix 1 Continued.

Species	Bracteole type	N		Disk-shaped glands	Length of bracteoles (mm)	Width of bracteoles (mm)	Internode between two bracteoles (mm)	Length of stigma (mm)
		♂	♀					
M. ducis	A	2	0	−	2.43	1.27	1.88	NA
M. echinocarpa	NA	0	0	NA	NA	NA	NA	NA
M. gabunica	E	1	1	−	2.33	2.53	2.13	0.47
M. gigantea	E	3	0	−	3.40	3.43	1.91	NA
M. grallata	A	1	1	−	0.00	0.00	4.00	3.47
M. grandifolia	E	3	1	−	4.21	3.34	2.15	0.30
M. heterophylla	E	2	0	−	7.86	4.73	2.20	NA
M. heynei	E	4	0	−	5.69	4.39	1.97	NA
M. hispida	D	5	3	+	2.61	1.10	2.57	7.60
M. hullettii	E	3	0	−	3.61	2.69	2.07	NA
M. hurifolia	E	2	0	−	3.05	2.72	1.13	NA
M. inamoena	A	2	1	−	0.00	0.00	4.73	8.00
M. indica	D	5	2	+	3.19	1.33	7.30	2.03
M. induta	NA	0	2	−	NA	NA	NA	3.33
M. involucrata	D	2	2	+	0.97	0.60	2.20	2.77
M. klaineana	A	2	0	−	1.53	1.53	1.73	NA
M. lamellata	E	0	0	−	NA	NA	NA	NA
M. lowii var. *kostermansii*	A	3	2	−	1.39	0.72	1.56	6.57
M. lowii var. *lowii*	A	2	0	−	1.17	0.93	1.93	NA
M. mauritiana	A	2	0	−	2.15	2.20	3.55	NA

Appendix 1 Continued.

Species	Bracteole type	N ♂	N ♀	Disk-shaped glands	Length of bracteoles (mm)	Width of bracteoles (mm)	Internode between two bracteoles (mm)	Length of stigma (mm)
M. monandra	E	3	0	−	4.71	3.42	1.22	NA
M. novo-guineensis	E	3	0	−	1.99	1.54	1.03	NA
M. oblongifolia	A	1	0	−	2.30	2.37	1.25	NA
M. obovata	D	2	1	+	1.85	0.80	1.75	3.60
M. pachyphylla	E	5	1	−	2.46	2.36	0.85	1.03
M. pearsonii	E	3	0	−	2.54	2.16	1.72	NA
M. poggei	E	2	0	−	2.30	2.37	1.25	NA
M. puncticulata	E	4	1	−	3.58	2.70	1.53	1.37
M. praestans	A	3	4	−	1.68	0.73	3.08	24.24
M. quadriglandulosa	E	3	3	−	2.72	2.42	1.23	3.40
M. repando-dentata	NA	0	3	NA	NA	NA	NA	38.11
M. rhizinoides	A	3	3	−	1.12	0.82	3.37	1.78
M. saccifera	E	1	0	−	2.97	3.17	1.13	NA
M. schweinfurthii	E	2	0	−	2.53	3.17	1.58	NA
M. siamensis	E	4	2	−	3.79	3.14	2.58	1.20
M. sinensis	D	3	3	+	3.17	1.09	3.46	0.68
M. subdentata	A	2	1	−	1.07	1.35	3.38	3.67
M. tanarius	E	3	3	−	6.00	4.97	2.98	3.83
M. tessellata	D	2	1	+	1.38	0.90	2.02	4.40
M. trichocarpa	E	4	3	−	4.28	2.63	1.51	0.88

Appendix 1 Continued.

Species	Bracteole type	N ♂	N ♀	Disk-shaped glands	Length of bracteoles (mm)	Width of bracteoles (mm)	Internode between two bracteoles (mm)	Length of stigma (mm)
M. triloba	E	4	1	−	2.62	2.42	1.29	0.30
M. umbrosa	E	0	0	−	NA	NA	NA	NA
M. winkleri	E	4	3	−	2.83	2.70	2.08	0.26

Appendix 2 Existence of disk-shaped glands on leaves and inflorescences (bract, bracteole or bract+bracteole) described in the taxonomic literature of *Macaranga* (Davies, 2001; Whitmore, 2008). Since the information in the literature is usually based on herbarium specimens, it is possible that glands on leaves and/or inflorescences have been overlooked in some species. Nevertheless, the information is concordant with authors' observations of live plants in 16 of 17 species (marked with asterisks), suggesting that the possibility of error is likely to be small. The presence of disk-shaped glands on leaves and bracts of *Macaranga denticulata*, which was not mentioned in Whitmore (2008), is based on our own observation. We considered the term "glands" in the literature as disk-shaped glands, while more specific terms "granular glands," "conical glands," and "gland-tipped" were not considered disk-shaped.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
African species	*M. kilimandscharica*	+	bract
	M. angolensis	+	
	M. assas	+	
	M. barteri	+	
	M. beillei	+	
	M. capensis	+	
	M. conglomerata	+	
	M. heterophylla	+	
	M. heudelotii	+	
	M. mellifera	+	
	M. occidentalis	+	
	M. paxii	+	
	M. poggei	+	
	M. schweinfurthii	+	
	M. spinosa	+	
	M. vermoesenii	+	
	M. gabunica		
	M. hurifolia		
	M. klaineana		
	M. longipetiolata		
	M. magnistipulosa		
	M. monandra		
	M. pierreana		
	M. saccifera		
	M. staudtii		
Angustifolia	*M. angustifolia*	+	bracteole

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. sandsii	+	bracteole
	M. allorobinsonii	+	
	M. crassistipulosa	+	
	M. faiketo	+	
	M. hartleyana	+	
	M. inermis	+	
	M. myriantha	+	
	M. pleioneura	+	
	M. pleopstemon	+	
	M. polyadenia	+	
	M. villosula	+	
	M. lanceolata		
Bicolor	*M. parabicolor*	+	bracteole
	M. pachyphylla	+	
	M. puncticulata	+	
	M. sarcocarpa	+	
	M. bicolor		
	M. congestiflora		
Bruneoflococca	*M. amentifera*	+	bracteole
	M. coggygria	+	bracteole
	M. hystrichogyne	+	bracteole
	M. stellimontium	+	bracteole
	M. stenophylla	+	bracteole
	M. sterrophylla	+	bracteole
	M. uxoris	+	bracteole
	M. carrii	+	
	M. hengkyana	+	
	M. palustris	+	
	M. rorokae	+	
	M. versteeghii	+	
	M. intonsa		bracteole
	M. albescens		
	M. brunneofloccosa		
	M. clemensiae		
	M. induta		
	M. melanosticta		
	M. trichanthera		
Coniferae	**M. recurvata*	+	
	M. amissa		
	**M. conifera*		

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. didymocarpa		
	M. diepenhorstii		
Coriacea	*M. coriacea*	+	
	M. alchorneoides		
	M. corymbosa		
	M. lutescens		
	M. vedeliana		
	M. vieillardii		
Denticulata	*M. indica*	+	bracteole
	**M. denticulata*	+	bract
	M. pustulata	+	
	M. neodenticulata		
	M. peltata		
	M. rhizinoides		
Dioica	*M. astrolabica*	+	bracteole
	M. bifoveata	+	bracteole
	M. decipens	+	bracteole
	M. densiflora	+	bracteole
	M. galorei	+	bracteole
	M. involucrata	+	bracteole
	M. neobritannica	+	bracteole
	M. punctata	+	bracteole
	M. serratifolia	+	bracteole
	M. similis	+	bracteole
	M. strigosa	+	bracteole
	M. subpeltata	+	bracteole
	M. warburgiana	+	bracteole
	M. acerifolia	+	
	M. carolinensis	+	
	M. dallachyana	+	
	M. dioica	+	
	M. ducis	+	
	M. inamoena	+	
	M. lugubris	+	
	M. novoguineensis	+	
	M. rufibarbis	+	
	M. hoffmannii		
	M. louisiadum		
Gracilis	*M. advena*	+	bracteole
	M. misimae	+	bracteole

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. domatiosa	+	
	M. gracilis	+	
	M. kostermansii	+	
	M. lumiensis	+	
	M. suleensis	+	
Javanica	**M. costulata*	+	bracteole
	M. cumingii	+	bracteole
	M. endertii	+	bracteole
	M. heynei	+	bracteole
	M. laciniata	+	bracteole
	**M. sinensis*	+	bracteole
	M. spathicalix	+	bracteole
	M. sumatrana	+	bracteole
	M. kinabaluensis	+	bract + bracteole
	M. javanica	+	
	M. sylvatica	+	
	M. loheri		
	M. waturandangii		
Longistipulata	*M. aleuritoides*	+	bracteole
	M. balabacensis	+	bracteole
	M. caudata	+	bracteole
	M. chrysotricha	+	bracteole
	M. eymae	+	bracteole
	M. fallacina	+	bracteole
	M. hispida	+	bracteole
	M. tessellata	+	bracteole
	M. thomasii	+	bracteole
	M. papuana	+	bract
	M. belensis	+	
	M. pleytei	+	
	M. reiteriana	+	
	M. barkeriana		bracteole
	M. cucullata		bracteole
	M. longistipulata		bracteole
	M. racemohispida		
	M. salicifolia		
	M. suwo		
Mappa	*M. seemannii*	+	bracteole
	M. amplifolia	+	
	M. choiseuliana	+	

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. grandifolia	+	
	M. grayana	+	
	M. johannium	+	
	M. leytensis	+	
	M. magna	+	
	M. magnifolia	+	
	M. mappa	+	
	M. noblei	+	
	M. raivavaeensis	+	
	M. caesariata		bracteole
	M. megacarpa		bracteole
	M. stipulosa		bracteole
	M. yakasii		bracteole
	M. fragrans		
	M. marikoensis		
	M. ovatifolia		
	M. thompsonii		
	M. whitomorei		
Mauritiana	*M. mauritiana*		bract
Oblongifolia	*M. boutonioides*	+	
	M. cuspidata	+	
	M. grallata	+	
	M. macropoda	+	
	M. oblongifolia	+	
	M. obovata	+	
	M. sphaerophylla	+	
	M. alnifolia		
	M. echinocarpa		
	M. ferruginea		
sect. *Pachystemon*	*M. angulata*	+	
	M. caladiifolia	+	
	M. calcicola	+	
	M. depressa	+	
	**M. hullettii*	+	
	M. kingii	+	
	M. petanostyla	+	
	M. rostrata	+	
	**M. trachyphylla*	+	
	**M. umbrosa*	+	
	M. velutiniflora	+	

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. aetheadenia		
	M. ashtonii		
	**M. bancana*		
	**M. beccariana*		
	M. constricta		
	M. glandibracteolata		
	M. griffithiana		
	**M. havilandii*		
	M. hypoleuca		
	M. indistincta		
	M. lamellata		
	M. motleyana		
	M. triloba		
	M. velutina		
sect. *Pruinosae*	*M. siamensis*	+	bracteole
	M. nicobarica	+	
	**M. gigantea*		
	M. hosei		
	M. pearsonii		
	M. pentaloba		
	M. pruinosa		
	M. puberula		
	**M. rufescens*		
Pseudorottlera	*M. anceps*	+	
	M. andamanica	+	
	M. brevipetiolata	+	
	M. chlorolepis	+	
	M. digyna	+	
	M. gamblei	+	
	M. glaberrima	+	
	M. lowii	+	
	**M. praestans*	+	
	M. rarispina	+	
	M. strigosissima	+	
	M. subdentata	+	
	M. baccaureifolia		
	M. pepysiana		
	M. setosa		
Tanarius	*M. salomonensis*	+	bracteole
	M. darbyshirei	+	

Appendix 2 Continued.

Infrageneric group	Species	Disk-shaped glands on leaves	Disk-shaped glands on inflorescences
	M. harveyana	+	
	M. quadriglandulosa	+	
	M. brachytricha		
	M. clavata		
	M. herculis		
	M. lineata		
	M. minahassae		
	M. nusatenggarensis		
	M. pilosula		
	**M. tanarius*		
	M. tentaculate		
	M. tsonane		
sect. *Winklerianae*	**M. winkleri*		
	M. winkleriella		
Group uncertain	*M. cassandrae*	+	bracteole
	M. graeffeana	+	bracteole
	M. aenigmatica	+	
	M. celebica	+	
	M. henryi	+	
	M. kurzii	+	
	M. sampsonii	+	
	M. taitensis	+	
	M. thorelii	+	
	**M. trichocarpa*	+	
	M. membranacea		bracteole
	M. vitiensis		bracteole
	M. attenuata		
	M. auctoris		
	M. huahineensis		
	M. stonei		
	M. venosa		

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Literature cited

Agarwal VM, Rastogi N. 2008. Role of floral repellents in the regulation of flower visits of extrafloral nectary-visiting ants in an Indian crop plant. *Ecological Entomology* **33**: 59–65.

Albrecht M, Duell P, Obrist MK, Kleijn D, Schmid B. 2009. Effective long-distance pollen dispersal in *Centaurea jacea*. *PloS One* **4**: e6751.

Altshuler DL. 1999. Novel interactions of non-pollinating ants with pollinators and fruit consumers in a tropical forest. *Oecologia* **119**: 600–606.

Armbruster WS. 1984. Partitioning and sharing of pollinators by 4 sympatric species of *Dalechampia* (Euphorbiaceae) in Panama. *Annals of the Missouri Botanical Garden* **71**: 1–16.

Armbruster WS. 1997. Exaptations link evolution of plant-herbivore and plant-pollinator interactions: a phylogenetic inquiry. *Ecology* **78**: 1661–1672.

Armbruster WS, Lee J, Baldwin BG. 2009. Macroevolutionary patterns of defense and pollination in *Dalechampia* vines: adaptation, exaptation, and evolutionary novelty. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 18085–18090.

Bänfer G, Fiala B, Weising K. 2004. AFLP analysis of phylogenetic relationships among myrmecophytic species of *Macaranga* (Euphorbiaceae) and their allies. *Plant Systematics and Evolution* **249**: 213–231.

Beardsley PM, Yen A, Olmstead RG. 2003. AFLP phylogeny of *Mimulus* section *Erythranthe* and the evolution of hummingbird pollination. *Evolution* **57**: 1397–1410.

Beattie AJ. 1985. *The evolutionary ecology of ant–plant mutualisms*. Cambridge, UK: Cambridge University Press.

Bernardello G. 2007. A systematic survey of floral nectaries. In: NicolsonSW, Nepi M, Pacini E, ed. *Nectaries and nectar*. Dordrecht, The Netherlands: Springer, 19–128.

Blattner FR, Weising K, Bänfer G, Maschwitz U, Fiala B. 2001. Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). *Molecular Phylogenetics and Evolution* **19**: 331–344.

Blüthgen N, Gottsberger G, Fiedler K. 2004. Sugar and amino acid composition of ant-attended nectar and honeydew sources from an Australian rainforest. *Austral Ecology* **29**: 418–429.

Blüthgen N, Reifenrath K. 2003. Extrafloral nectaries in an Australian rainforest: structure and distribution. *Australian Journal of Botany* **51**: 515–527.

Boughton VH. 1981. Extrafloral nectaries of some Australian phyllodineous acacias. *Australian Journal of Botany* **29**: 653–664.

Brouat C, McKey D. 2000. Origin of caulinary ant domatia and timing of their onset in plant ontogeny: evolution of a key trait in horizontally transmitted ant-plant symbioses. *Biological Journal of the Linnean Society* **71**: 801–819.

Carrillo J, Wang Y, Ding J, Siemann E. 2012. Induction of extrafloral nectar depends on herbivore type in invasive and native Chinese tallow seedlings. *Basic and Applied Ecology* **13**: 449–457.

Chamberlain SA, Holland JN. 2008. Density-mediated, context-dependent consumer-resource interactions between ants and extrafloral nectar plants. *Ecology* **89**: 1364–1374.

Corlett RT. 2004. Flower visitors and pollination in the Oriental (Indomalayan) Region. *Biological Reviews of the Cambridge Philosophical Society* **79**: 497–532.

Culley TM, Weller SG, Sakai AK. 2002. The evolution of wind pollination in angiosperms. *Trends in Ecology & Evolution* **17**: 361–369.

Davidson DW, McKey D. 1993. The evolutionary ecology of symbiotic ant-plant relationships. *Journal of Hymenoptera Research* **2**: 13–83.

Davies SJ. 2001. Systematics of *Macaranga* sect. *Pachystemon* and *Pruinosae* (Euphorbiaceae). *Harvard Papers in Botany* **6**: 371–448.

Davies SJ, Ashton PS. 1999. Phenology and fecundity in 11 sympatic pioneer species of *Macaranga* (Euphorbiaceae) in Borneo. *American Journal of Botany* **86**: 1786–1795.

Davies SJ, Lum SKY, Chan R, Wang LK. 2001. Evolution of myrmecophytism in western Malesian *Macaranga* (Euphorbiaceae). *Evolution* **55**: 1542–1559.

De Jong TJ, Batenburg JC, Klinkhamer PGL. 2005. Distance-dependent pollen limitation of seed set in some insect-pollinated dioecious plants. *Acta Oecologica* **28**: 331–335.

Dejean A, Corbara B, Leroy C, Delabie JHC, Rossi V, Céréghino R. 2011. Inherited biotic protection in a neotropical pioneer plant. *PloS one* **6**: e18071.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Dressler RL. 1982. Biology of the orchid bees (Euglossini). *Annual Review of Ecology and Systematics* **13**: 373–394.

Dutton EM, Frederickson ME. 2012. Why ant pollination is rare: new evidence and implications of the antibiotic hypothesis. *Arthropod–Plant Interactions* **6**: 561–569.

Fægri K, van der Pijl L. 1979. *The principles of pollination ecology*. Oxford, UK: Pergamon Press.

Falcão PF, de A. Melo-de-Pinna GF, Leal IR, Almeida-Cortez JS. 2003. Morphology and anatomy of extrafloral nectaries in *Solanum stramonifolium* (Solanaceae). *Canadian Journal of Botany* **81**: 859–864.

Fenner M. 2000. *Seeds: the ecology of regeneration in plant communities*. Wallingford, Oxon: CABI Publishing.

Fiala B, Grunsky H, Maschwitz U, Linsenmair KE. 1994. Diversity of ant-plant interactions: protective efficacy in *Macaranga* species with different degrees of ant association. *Oecologia* **97**: 186–192.

Fiala B, Jakob A, Maschwitz U, Linsenmair KE. 1999. Diversity, evolutionary specialization and geographic distribution of a mutualistic ant–plant complex: *Macaranga* and *Crematogaster* in South East Asia. *Biological Journal of the Linnean Society* **66**: 305–331.

Fiala B, Maschwitz U. 1991. Extrafloral nectaries in the genus *Macaranga* (Euphorbiaceae) in Malaysia: comparative studies of their possible significance as

predispositions for myrmecophytism. *Biological Journal of the Linnean Society* **44**: 287–305.

Fiala B Maschwitz U. 1992a. Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). *Botanical Journal of the Linnean Society* **110**: 61–75.

Fiala B, Maschwitz U. 1992b. Domatia as most important adaptations in the evolution of myrmecophytes in the paleotropical tree genus *Macaranga* (Euphorbiaceae). *Plant Systematics and Evolution* **180**: 53–64.

Fiala B, Maschwitz U, Pong TY, Helbig AJ. 1989. Studies of a South East Asian ant-plant association: protection of *Macaranga* trees by *Crematogaster borneensis*. *Oecologia* **79**: 463–470.

Fiala B, Meyer U, Hashim R, Maschwitz U. 2011. Pollination systems in pioneer trees of the genus *Macaranga* (Euphorbiaceae) in Malaysian rainforests. *Biological Journal of the Linnean Society* **103**: 935–953.

Galen C, Sherry RA, Carroll AB. 1999. Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. *Oecologia* **118**: 461–470.

Galen C. 2005. Catching ants with honey: an experimental test of distraction and satiation as alternative modes of escape from flower-damaging ants. *Oecologia* **144**: 80–87.

Galen C, Cuba J. 2001. Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution; International Journal of Organic Evolution* **55**: 1963–1971.

Galen C, Sherry RA, Carroll AB. 1999. Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. *Oecologia* **118**: 461–470.

Gaume L, Zacharias M, Borges RM. 2005. Ant-plant conflicts and a novel case of castration parasitism in a myrmecophyte. *Evolutionary Ecology Research* **7**:435–452.

Ghazoul J. 2001. Can floral repellents pre-empt potential ant-plant conflicts? *Ecology Letters* **4**: 295–299.

Gómez JM, Zamora R. 1996. Wind pollination in high-mountain populations of *Hormathophylla spinosa* (Cruciferae). *American Journal of Botany* **83**: 580–585.

Goodwillie C. 1999. Wind pollination and reproductive assurance in *Linanthus parviflorus* (Polemoniaceae), a self-incompatible annual. *American Journal of Botany* **86**: 948–954.

Gould SJ, Vrba ES. 1982. Exaptation—a missing term in thescience form. *Paleobiology* **8**: 4–15.

Guariguata MR, Ostertag R. 2001. Neotropical secondary forest succession: changes in structural and functional characteristics. *Forest Ecology and Management* **148**: 185–206.

Guerrant EO, Fiedler PL. 1981 Flower defenses against nectar-pilferage by ants. *Biotropica* **13**(suppl.), 25–33.

Harley R. 1991. The greasy pole syndrome. In: Huxley CR and Cutler DF, eds. *Ant-plant interactions*. Oxford, UK: Oxford University Press. 430–433.

Hata H, M Kato. 2002. Weeding by the herbivorous damselfish *Stegastes nigricans* in monocultural algae farms. *Marine Ecology Progress Series*. **237**: 227–231.

Heil M, Fiala B, Kaiser W, Linsenmair KE. 1998. Chemical contents of *Macaranga* food bodies: adaptations to their role in ant attraction and nutrition. *Functional Ecology* **12**: 117–122.

Heil M, Fiala B, Maschwitz U, Linsenmair KE. 2001. On benefits of indirect defence: short- and long-term studies of antiherbivore protection via mutualistic ants. *Oecologia* **126**: 395–403.

Heil M, McKey D. 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. *Annual Review of Ecology, Evolution, and Systematics* **34**: 425–453.

Hernández-Cumplido J, Benrey B, Heil M. 2010. Attraction of flower visitors to plants that express indirect defence can minimize ecological costs of ant-pollinator conflicts. *Journal of Tropical Ecology* **26**: 555–557.

Hesse E, Pannell JR. 2011. Density-dependent pollen limitation and reproductive assurance in a wind-pollinated herb with contrasting sexual systems. *Journal of Ecology* **99**: 1531–1539.

Holland JN, Chamberlain S a., Miller TEX. 2011. Consequences of ants and extrafloral nectar for a pollinating seed-consuming mutualism: ant satiation, floral distraction or plant defense? *Oikos* **120**: 381–388.

Howard DF, Blum MS, Fales HM. 1983. Defense in thrips: forbidding fruitiness of a lactone. *Science* **220**: 335–336.

Horikawa Y. 1972. *Atlas of the Japanese flora: an introduction to plant sociology of East Asia*. Tokyo: Gakken [In Japanese]

Inui Y, Itioka T. 2007. Species-specific leaf volatile compounds of obligate *Macaranga* myrmecophytes and host-specific aggressiveness of symbiotic *Crematogaster* ants. *Journal of Chemical Ecology* **33**: 2054–2063.

Ishida C. 2008. Pollination system of *Macaranga* (Euphorbiaceae); plants providing breeding sites as reward for pollination. Master's thesis, Kyoto University, Japan [in Japanese].

Ishida C, Kono M, Sakai S. 2009. A new pollination system: brood-site pollination by flower bugs in *Macaranga* (Euphorbiaceae). *Annals of Botany* **103**: 39–44.

Itioka T. 2005. Diversity of anti-herbivore defences in *Macaranga*. In: Roubik DW, Sakai S, Hamid AA, eds. *Pollination Ecology and the Rain Forest: Sarawak Studies*. New York: Springer, 158–171.

Itioka T, Inoue T, Kaliang H, Kato M, Nagamitsu T, Momose K, Sakai S, Yumoto T, Mohamad SU, Hamid AA, Yamane S. 2001. Six-year population fluctuation of the giant honey bee *Apis dorsata* (Hymenoptera: Apidae) in a tropical lowland dipterocarp forest in Sarawak. *Annals of the Entomological Society of America* **94**: 545–549.

Itioka T, Nomura M, Inui Y, Itino T, Inoue T. 2000. Difference in intensity of ant defense among three species of *Macaranga* myrmecophytes in a Southeast Asian Dipterocarp forest. *Biotropica* **32**: 318–326.

Janzen DH. 1966. Coevolution of mutualism between ants and acacias in Central America. *Evolution* **20**: 249–275.

Janzen DH. 1977. Why don't ants visit flowers? *Biotropica* **9**: 252.

Junker RR, Blüthgen N. 2008. Floral scents repel potentially nectar-thieving ants. *Evolutionary Ecology Research* **10**: 295–308.

Jürgens A, Dötterl S, Meve U. 2006. The chemical nature of fetid floral odours in stapeliads (Apocynaceae–Asclepiadoideae–Ceropegieae). *New Phytologist* **172**, 452–468.

Karrenberg S, Kollmann J, Edwards PJ. 2002. Pollen vectors and inflorescence morphology in four species of *Salix*. *Plant Systematics and Evolution* **235**: 181–188.

Kato M, Takimura A, Kawakita A. 2003. An obligate pollination mutualism and reciprocal diversification in the tree genus *Glochidion* (Euphorbiaceae). *Proceedings of the National Academy of Sciences of the United States of America* **100**: 5264–5267.

Knox RB, Kenrick J, Bernhardt P, Marginson R, Beresford G, Baker I, Baker HG. 1985. Extrafloral nectaries as adaptations for bird pollination in *Acacia terminalis*. *American Journal of Botany* **72**: 1185–1196.

Koptur S. 1992. Extrafloral nectary-mediated interactions between insects and plants. In: Bernays E, ed. *Insect-plant interactions*. Boca Raton, Florida: CRC Press, 81–129.

Kulju KKM, Sierra SEC, Draisma SGA, Samuel R, van Welzen PC. 2007. Molecular phylogeny of *Macaranga*, *Mallotus*, and related genera (Euphorbiaceae s.s.): insight from plastid and nuclear DNA sequence data. *American Journal of Botany* **94**: 1726–1743.

Lee JY, Baum SF, Oh SH, Jiang CZ, Chen JC, Bowman JL. 2005. Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. *Development* **132**: 5021–5032.

Levin DA, Kerster HW. 1974. Gene flow in seed plants. *Evolutionary Biology* **7**: 139–220.

Lewis T. 1973. *Thrips: their biology, ecology and economic importance*. London, UK: Academic Press.

Listabarth C. 1993. Insect-induced wind pollination of the palm *Chamaedorea pinnatifrons* and pollination in the related *Wendlandiella* sp. *Biodiversity and Conservation* **2**: 39–50.

Lock JM, Hall JB. 1982. Floral biology of *Mallotus oppositifolius* (Euphorbiaceae). *Biotropica* **14**: 153–155.

Mackay DA, Whalen MA. 1991. Some associations between ants and euphorbs in tropical Australia. In: Huxley C, Cutler DF, ed. *Ant–plant interactions*. Oxford: Oxford Univ. Press, 238–249.

Maddison WP, Maddison DR. 2010. Mesquite: a modular system for evolutionary analysis. Version 2.73. http://mesquiteproject.org.

Mangla Y, Tandon R. 2011. Insects facilitate wind pollination in pollen-limited *Crateva adansonii* (Capparaceae). *Australian Journal of Botany* **59**: 61–69.

Marazzi B, Bronstein JL, Koptur S. 2013. The diversity, ecology and evolution of extrafloral nectaries: current perspectives and future challenges. *Annals of Botany* **111**: 1243–1250.

Martins DJ. 2009. Pollination and facultative ant-association in the African leopard orchid *Ansellia africana*. *Journal of East African Natural History* **98**: 67–77.

Michelangeli FA. 2000. A cladistic analysis of the genus *Tococa* (Melastomataceae) based on morphological data. *Systematic Botany* **25**: 211–234.

Momose K, Yumoto T, Nagamitsu T, Kato M, Nagamasu H, Sakai S, Harrison RD, Itioka T, Hamid AA, Inoue T. 1998. Pollination biology in a lowland dipterpcarp forest in Sarawak, Malaysia. I. Characteristics of the plant-pollinator community in a lowland dipterocarp forest. *American Journal of Botany* **85**: 1477–1501.

Moog U, Fiala B, Federle W, Maschwitz U. 2002. Thrips pollination of the dioecious ant plant *Macaranga hullettii* (Euphorbiaceae) in Southeast Asia. *American Journal of Botany* **89**: 50–59.

Ness JH. 2006. A mutualism's indirect costs: the most aggressive plant bodyguards also deter pollinators. *Oikos* **113**: 506–514.

Nomura M, Hatada A, Itioka T. 2011. Correlation between the leaf turnover rate and anti-herbivore defence strategy (balance between ant and non-ant defences) amongst ten species of *Macaranga* (Euphorbiaceae). *Plant Ecol*ogy **212**: 143–155.

Nowicke JW, Takahashi M. 2002. Pollen morphology, exine structure and systematics of Acalyphoideae (Euphorbiaceae), Part 4, Tribes Acalypheae *pro parte* (*Erythrococca*, *Claoxylon*, *Claoxylopsis*, *Mareya*, *Mareyopsis*, *Discoclaoxylon*, *Micrococca*, *Amyrea*,

Lobanilia, *Mallotus*, *Deuteromallotus*, *Cordemoya*, *Cococceras*, *Trewia*, *Neotrewia*, *Rockinghamia*, *Octospermum*, *Acalypha*, *Lasiococca*, *Spathiostemon*, *Homonoia*), Plukenetieae (*Haematostemon*, *Astrococcus*, *Angostyles*, *Romanoa*, *Eleutherostigma*, *Plukenetia*, *Vigia*, *Cnesmone*, *Megistostigma*, *Sphaerostylis*, *Tragiella*, *Platygyna*, *Tragia*, *Acidoton*, *Pachystylidium*, *Dalechampia*), Omphaleae (*Omphalea*), and discussion and summary of the complete subfamily. *Review of Palaeobotany and Palynology* **121**: 231–336.

Okuyama U, Kawakita A. 2013. DNA extraction protocol from plants (altered CTAB method). In: Kawakita A, Okuyama U, eds. *Biology of Species Interactions*. Tokyo: Bun-ichi Sogo Shuppan Co., 273–278. [In Japanese]

Oliveira PS, Rico-Gray V, Castillo-Guevara CD-C and C. 1999. Interaction between ants, extrafloral nectaries and insect herbivores in Neotropical coastal sand dunes: herbivore deterrence by visiting ants increases fruit set in *Opuntia stricta* (Cactaceae). *Functional Ecology* **13**: 623–631.

Ollerton J, Winfree R, Tarrant S. 2011. How many flowering plants are pollinated by animals? *Oikos* **120**: 321–326.

Pacala SW. 1996. Forest models defined by field measurements: estimation, error analysis and dynamics. *Ecological Monographs* **66**: 1–43.

Peakall R, Handel SN, Beattie AJ. 1991. The evidence for, and importance of, ant pollination. In: Huxley C, Cutler DF ed. *Ant-plant interactions*. Oxford: Oxford Univ. Press. 421–433.

Peeters L, Totland O. 1999. Wind to insect pollination ratios and floral traits in five alpine *Salix* species. *Canadian Journal of Botany* **77**: 556–563.

Pellmyr O. 2003. Yuccas, yucca moths, and coevolution: A review. *Annals of the Missouri Botanical Garden* **90**: 35–55.

Pino JA, Márquez E, Quijano CE, Castro D. 2010. Volatile compounds in noni (*Morinda citrifolia* L.) at two ripening stages. *Ciência e Tecnologia de Alimentos* **30**: 183–187.

Proctor M, Yeo P, Lack A. 1996. *The natural history of pollination*. London: Harper Collins.

Prŷs-Jones OE, Willmer PG. 1992. The biology of alkaline nectar in the purple toothwort (*Lathraea clandestina*): ground level defences. *Biological Journal of the Linnean Society* **45**: 373–388.

Quek S-P, Davies SJ, Itino T, Pierce NE. 2004. Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of *Macaranga* (Euphorbiaceae). *Evolution* **58**: 554–570.

R Development Core Team. 2010. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. Vienna, Austria. URL http://www.R-project.org.

R Development Core Team. 2013. *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Raine NE, Willmer P, Stone GN. 2002. Spatial structuring and floral avoidance behavior prevent ant-pollinator conflict in a Mexican ant-acacia. *Ecology* **83**: 3086–3096.

Renner SS. 1989. A survey of reproductive biology in Neotropical Melastomataceae and Memecylaceae. *Annals of the Missouri Botanical Garden* **76**: 469–518.

Rico-Gray V, Oliveira PS. 2007. *The Ecology and Evolution of Ant–Plant Interactions*. Chicago: The University of Chicago Press.

Roubik DW, Sakai S, Karim AAH. 2005. *Pollination Ecology and the Rain Forest*. Springer: New York.

Sakai S, HarrisonRD,MomoseK, KurajiK,NagamasuH, Yasunari T,Chong L, Nakashizuka T. 2006. Irregular droughts trigger mass flowering in aseasonal tropical forests in Asia. *American Journal of Botany* **93**: 1134–1139.

Salles HC, Volsi ECFR, Marques MR, Souza BM, dos Santos LD, Tormena CF, Mendes MA, Palma MS. 2006. The venomous secrets of the web droplets from the viscid spiral of the orb-weaver spider *Nephila clavipes* (Araneae, Tetragnatidae). *Chemistry & Biodiversity* **3**, 727–741.

Samejima H, Marzuki M, Nagamitsu T, Nakasizuka T. 2004. The effects of human disturbance on a stingless bee community in a tropical rainforest. *Biological Conservation* **120**: 577–587.

Schoonhoven LM, Jermy T, van Loon JJA. 1998. *Insect–plant biology*. London: Chapman & Hall.

Schmid VS, Schmid S, Steiner J, Zillikens A. 2010. High diversity of ants foraging on extrafloral nectar of bromeliads in the Atlantic rainforest of southern Brazil. *Studies on Neotropical Fauna and Environment* **45**: 39–53.

Schulke B, Waser NM. 2001. Long-distance pollinator flights and pollen dispersal between populations of *Delphinium nuttallianum*. *Oecologia* **127**: 239–245.

Sierra SEC, Aparicio M, Gebraad MJH, Kulju KKM, van Welzen PC. 2007. The morphological range in *Mallotus* (Euphorbiaceae) and a taxonomic revision of its section *Rottleropsis* (including axenfeldia) in Malesia, Thailand and Africa. *Blumea* **52**: 21–113.

Sierra SEC, Aparicio M, Kulju KKM, Fišer Ž, van Welzen PC, van der Ham RWJM. 2006. Re-shaping *Mallotus* [Part 1]: Expanded circumscription and revision of the genus *Cordemoya* (Euphorbiaceae). *Blumea - Biodiversity, Evolution and Biogeography of Plants* **51**: 519–540.

Sierra SEC, Kulju KKM, Fišer Ž, Aparicio M, van Welzen PC. 2010. The phylogeny of *Mallotus* s.str. (Euphorbiaceae) inferred from DNA sequence and morphological data. *Taxon* **59**: 101–116.

Sierra SEC, van Welzen PC. 2005. A taxonomic revision of *Mallotus* section *Mallotus* (Euphorbiaceae) in Malesia. *Blumea - Biodiversity, Evolution and Biogeography of Plants* **50**: 249–274.

Slik JWF. 2005. Assessing tropical lowland forest disturbance using plant morphological and ecological attributes. *Forest Ecology and Management* **205**: 241–250.

So ML. 2004. The occurrence of extrafloral nectaries in Hong Kong plants. *Botanical Bulletin of Academia Sinica* **45**: 237–245.

Solano PJ, Belin-Depoux M, Dejean A. 2005. Formation and structure of food bodies in *Cordia nodosa* (Boraginaceae). *Comptes Rendus Biologies* **328**: 642–647.

Steidle JLM, Dettner K. 1995. The chemistry of the abdominal gland secretion of six species of the rove beetle genus *Bledius*. *Biochemical Systematics and Ecology* **23**, 757–765.

Steiner KE, Whitehead VB. 1991. Oil flowers and oil bees: further evidence for pollinator adaptation. *Evolution* **45**: 1493–1501.

Stellman P. 1984. The significance of biotic pollination in a nominally anemophilous plant: *Plantago lanceolata*. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen Series C-biological and Medical Sciences*. **87**: 95–119.

Steven JC, Waller DM. 2007. Isolation affects reproductive success in low-density but not high-density populations of two wind-pollinated *Thalictrum* species. *Plant Ecology* **190**: 131–141.

Stone GN, Raine NE, Prescott M, Willmer PG. 2003. Pollination ecology of acacias (Fabaceae, Mimosoideae). *Australian Systematic Botany* **16**: 103–188.

Subedi A, Chaudhary RP, Achterberg C V., Heijerman T, Lens F, Van Dooren TJM, Gravendeel B. 2011. Pollination and protection against herbivory of Nepalese Coelogyninae (Orchidaceae). *American Journal of Botany* **98**: 1095–1103.

Suzuki T, Haga K, Tsutsumi T, Matsuyama S. 2004. Analysis of anal secretions from phlaeothripine thrips. *Journal of Chemical Ecology* **30:** 409–423.

Tamura S, Kudo G. 2000. Wind pollination and insect pollination of two temperate willow species, *Salix miyabeana* and *Salix sachalinensis*. *Plant Ecology* **147**: 185–192.

Thien LB, Azuma H, Kawano S. 2000. New perspectives on the pollination biology of basal angiosperms. *International Journal of Plant Sciences* **161**: S225–S235.

Torto B, Njagi PGN, Hassanali A, Amiani H. 1996. Aggregation pheromone system of nymphal gregarious desert locust, *Schistocerca gregaria* (forskål [*sic*]). *Journal of Chemical Ecology* **22**, 2273–2281.

Totland Ø, Sottocornola M. 2001. Pollen limitation of reproductive success in two sympatric alpine willows (Salicaceae) with contrasting pollination strategies. *American Journal of Botany* **88**: 1011–1015.

Traveset A, Sáez E. 1997. Pollination of *Euphorbia dendroides* by lizards and insects: Spatio-temporal variation in patterns of flower visitation. *Oecologia* **111**: 241–248.

Tschuch G, Lindemann P, Moritz G. 2008. An unexpected mixture of substances in the defensive secretions of the tubuliferan thrips, *Callococcithrips fuscipennis* (Moulton). *Journal of Chemical Ecology* **34**: 742–747.

Tschuch G, Lindemann P, Niesen A, Csuk R, Moritz G. 2005. A novel long-chained acetate in the defensive secretion of thrips. *Journal of Chemical Ecology* **31**: 1555–1565.

Tsuji K, Hasyim A, Nakamura H, Nakamura K. 2004. Asian weaver ants, *Oecophylla smaragdina*, and their repelling of pollinators. *Ecological Research* **19**: 669–673.

Van Welzen PC, Sierra SEC. 2006. The *Mallotus wrayi* complex (Euphorbiaceae). *Blumea* **51**: 373–388.

Vandepitte K, Roldán-Ruiz I, Honnay O. 2009. Reproductive consequences of mate quantity versus mate diversity in a wind-pollinated plant. *Acta Oecologica* **35**: 548–553.

Vasconcelos HL. 1991. Mutualism between *Maieta guianensis* Aubl., a myrmecophytic melastome, and one of its ant inhabitants: ant protection against insect herbivores. *Oecologia* **87**: 295–298.

Vesprini JL, Galetto L, Bernardello G. 2003. The beneficial effect of ants on the reproductive success of *Dyckia floribunda* (Bromeliaceae), an extrafloral nectary plant. *Canadian Journal of Botany* **81**: 24–27.

Vikas, Tandon R. 2011. Reproductive biology of *Azadirachta indica* (Meliaceae), a medicinal tree species from arid zones. *Plant Species Biology* **26**: 116–123.

Wagner D, Kay A. 2002. Do extrafloral nectaries distract ants from visiting flowers ? An experimental test of an overlooked hypothesis. *Evolutionary Ecology Research* **4**: 293–305.

Warren SD, Harper KT, Booth GM. 1988. Elevational distribution of insect pollinators. *American Midland Naturalist* **120**: 325–330.

Weber MG, Keeler KH. 2013. The phylogenetic distribution of extrafloral nectaries in plants. *Annals of Botany* **111**: 1251–1261.

Weiblen GD. 2002. How to be a fig wasp. *Annual Review of Entomology* **47**: 299–330.

Whalen MA, Mackay DA. 1988. Patterns of ant and herbivore activity on five understory euphorbiaceous saplings in submontane Papua New Guinea. *Biotropica* **20**: 294–300.

Whitmore TC. 2008. *The genus* Macaranga – *a prodromus*. Kew: Kew Publishing.

Willmer PG, Nuttman C V., Raine NE, Stone GN, Pattrick JG, Henson K, Stillman P, McIlroy L, Potts SG, Knudsen JT. 2009. Floral volatiles controlling ant behaviour. *Functional Ecology* **23**: 888–900.

Willmer PG, Stone GN. 1997. How aggressive ant-guards assist seed-set in *Acacia* flowers. *Nature* **388**: 165–167.

Wragg PD, Johnson SD. 2011. Transition from wind pollination to insect pollination in sedges: experimental evidence and functional traits. *The New Phytologist* **191**: 1128–1140.

Yamasaki E, Inui Y, Sakai S. 2013a. Production of food bodies on the reproductive organs of myrmecophytic *Macaranga* species (Euphorbiaceae): effects on interactions with herbivores and pollinators. *Plant Species Biology* in press.

Yamasaki E, Kawakita A, Sakai S. 2013b. Modified leaves with disk-shaped nectaries of *Macaranga sinensis* (Euphorbiaceae) provide reward for pollinators. *American Journal of Botany* **100**: 628–632.

Yamasaki E, Sakai S. 2013. Wind and insect pollination (ambophily) of *Mallotus* spp. (Euphorbiaceae) in tropical and temperate forests. *Australian Journal of Botany* **61**: 60–66.