Origin and evolution of obligate pollination mutualism in the Phyllanthaceae–*Epicephala* association

コミカンソウ科－ハナホソガ属間における絶対送粉共生系の起源と進化

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摘要

第一章

生物種間の相利共生系は，自然界に普遍的に存在し，生物多様性の起源や維持において極めて大きな役割を果たしている。相利共生系が進化した背景や，それが維持されるためのメカニズムを明らかにすることは，生物多様性の由来を深く理解する上で不可欠だが，そのためには分類学，生態学，あるいは進化学など幅広い方面からの研究が必要である。植物と送粉性種子食者との間の絶対送粉共生系は，古くから相利共生系に関する研究のモデルとなっており，なかでもイチジクとイチジクコバチ，およびユッカとユッカガとの間の共生系は相利共生系のさまざまな生態学的，進化学的特性を明らかにするために役立ってきた。近年新しく発見されたカンコノキ属（コミカンソウ科）とハナホソガ属（ホソガ科）の間の絶対送粉共生系は，イチジクやユッカの系から得られた知見を検証し，新たな視点を付与するためのモデルとしておおいに期待される。またこれらの近縁種には多様な生活史を持つものが多く存在するため，この系は相利共生系がどのような進化的起源を持つかを考える上でも極めて適している。コミカンソウ科－ハナホソガ属の絶対送粉共生系は，近年とりわけ注目を集めている相利共生系の進化研究において多くの重要な知見をもたらすであろう。

第二章

カンコノキ属（コミカンソウ科）とハナホソガ属（ホソガ科）の間の種特異的な絶対送粉共生系は，種多様性が高く，古くから知られるイチジクとイチジクコバチ，ユッカとユッカガの共生系とさまざまな点で類似している。本章では，カンコノキ属－ハナホソガ属共生系における両者の種分化パターンを比較するため，分布域のさまざまな地点から得られた 18 種のカンコノキ属植物と，それらに特異的な 18 種のハナホソガ属昆虫について，分子系統解析を行った。解析には，カンコノキ属においてはリボゾーム DNA の ITS，および ETS 領域，ハナホソガ属においてはミトコンドリア DNA 上のチトクローム酸化酵素サブユニット1遺伝子，および核DNA上のアルギニンキナーゼとタンパク質伸長因子1α領域を用いた。得られた系統樹をもとに，2つの系統樹間の一致度を評価するいくつかの手法を用いて，両者の種分化パターンの類似性を評価した。その結果，カンコノキ属とハナホソガ属の系統樹の間には，ランダムな関係から予測される以上の相関が見られたが，一部の結果は解析に用いた系統樹や手法によって影響を受けた。両者の系統樹の間には完全な一致は見られなかったが，これはハナホソガによる寄主転換が原因となっていると考えられる。観察された植物と送粉者の系統樹の食い違いは，両者の種特異性が複雑な過程に基づいて維持されてきたか，あるいは両者の種間関係が 1 対 1 ではなく，より複雑なものである可能性を示している。

第三章

植物と種子食性送粉者との間の絶対送粉共生系は，種間相互作用の華々しい例であり，多くの場合極めて高い種特異性によって特徴づけられる。こうした共生系において両者の多様化は，密接な相互適応を介した並行的な種分化の繰り返しによって起こったと長い間考えられてきたが，近年の研究はこうした考え方に疑問を投げかける。本章ではカンコノキ属とハナホソガ属の間の絶対送粉共生系における種多様性，および種特異性を分子系統解析により評価した。日本西南部，および台湾における 5 種のカンコノキ属植物から採集したハナホソガについて，ミトコンドリアおよび核 DNA の 3 遺伝子領域を解析したところ，形態的に識別可能な 6 種が含まれていることが分かった。それらは，(1)ウラジロカンコノキおよびカキバカンコノキにそれぞれ特異的な 2 種，(2)キールンカンコノキ上で共存する 2 種，(3)側所的に分布する近縁なカンコノキとヒラミカンコノキを共有する 2 種であった。キールンカンコノキで得られた 2 種のホソガは統計的に姉妹群でないことが示され，いずれかの種が少なくとも過去に 1 度寄主転換を起こしていることが明らかになった。また野外における行動観察から 6 種のホソガはすべて能動的な送粉者であることが分かり，二次的に送粉行動を失うような進化は起こっていなかった。これらの結果はイチジクやユッカの系において近年明らかにされたパターンと同様のものであり，本研究の成果は共生系における種分化や多様化のパターンについてのより一般的な理解に向けて重要な知見を提供している。

第四章

イチジクとイチジクコバチ，およびユッカとユッカガの間に見られるような絶対的な送粉共生系は，近年見つかったカンコノキ属とハナホソガ属の系をはじめとして数例が報告されている。これらの共生系に共通することは，送粉者の幼虫が果実のなかの一部の種子のみを食害するため，植物が種子を残すことができるという点である。本章ではこうした通例にあてはまらない，あらたな絶対送粉共生系の例を報告する。ニューカレドニア産のコミカンソウ属 2 種（コミカンソウ科）における特殊な花は，種特異的に種子に寄生するハナホソガ属の蛾によって能動的に送粉されているが，その幼虫は 1 匹が果実中の 6 つの種子をすべて食い尽くしてしまう。しかし送粉された花の一部にはホソガの

卵が産みつけられないため，果実の一部は食害を免れる。ニューカレドニア産コミカンソウ属には種子食性のハナホソガ属との種特異的な関係が広く見られることから，絶対送粉共生系が一般に見られると考えられる。最後に共生系の進化的安定性や，カンコノキ属で見られる共生系との違いについて考察する。

第五章

本章ではオオシマコバンノキ属（コミカンソウ科）2 種における絶対送粉共生系について報告する。本属は同様にハナホソガ属との共生系が知られているカンコノキ属やニューカレドニア産コミカンソウ属に近縁である。オオシマコバンノキでは夜間，多量の花粉を口吻にたくわえた雌のホソガが花を訪れ，口吻を伸長させて授粉し，直後に花に産卵することが観察された。2 種について野外で採集した雌花を調べた結果，授粉された花はほぼ全てがホソガに産卵されていることが分かり，両種においてハナホソガが唯一の送粉者となっている可能性が強く支持された。一匹のホソガの幼虫は一方の種では果実中の約半分の種子を食害したが，もう一方の種では全ての種子が食害されていた。しかし全く食害を受けない果実も多く見られ，これらの一部には産卵の跡が確認されたことから，ホソガの卵期の死亡が，植物の種子生産が保証されるための重要な要因となっていることが分かった。オオシマコバンノキ属に含まれる植物の多くも同様に特殊な花形態を持っていることから，絶対送粉共生系は属内で広く見られると考えられる。

第六章

コミカンソウ族は全世界に 1200 種以上が知られ，コミカンソウ科においてもっとも大きな族である。これまでの研究からカンコノキ属，オオシマコバンノキ属，コミカンソウ属において，種子寄生的な送粉者であるハナホソガ属（ホソガ科）との絶対的な共生関係が見られることが分かっている。しかし，族内で見られる著しい花形態の多様性は，こうした特殊な送粉様式が必ずしも族内で一般的でないことを物語っている。本章ではコミカンソウ族におけるさまざまな種において花，および果実を採集し，ハナホソガによる種子食害がどれほど系統的に幅広く見られるかを検討した。また野外観察や授粉実験の結果から，それぞれの種について潜在的な送粉者相を推察した。これらの結果から，あらたにコミカンソウ属の 2 つの種群においてハナホソガとの絶対送粉共生系が見られることが分かり，その他の種ではハナホソガ以外の送粉者が存在することが分かった。またヒトツバハギ属の 1 種や，雑草性のコミカンソウ属は昼行性の昆虫によって効果的に送粉されているが，これらは送粉を行わない種子寄生的なハナホソガによって寄生されていることが分かった。これらの結果はコミカンソウ族で見られる花形態の多様性が送粉様式の違いを反映していることを示すと同時に，ハナホソガ属との特殊な共生系の起源の解明に重要な示唆を与えている。

第七章

植物と種子食性昆虫との間の絶対的な共生系は共進化の顕著な例であるが，こうした共生系は自然界で極めて少ない。これまでの研究から，いくつかの生態的要因が共生系の進化のに必要な条件として考えられており，それらを満たす相互作用系が，相利共生系であると考えられている。本章では絶対送粉共生系が知られているコミカンソウ科植物とハナホソガ属昆虫との関係において，その共生系の進化的起源を系統解析に基づき明らかにした。両者の系統推定と，それに基づく祖先的形質復元，および分岐年代推定の結果から，コミカンソウ科において共生系は最も多い場合で 5 回進化したと推定された。ハナホソガの能動的送粉行動は一度起源しており，共生系の進化や維持における革新的適応であったと考えられる。度重なるハナホソガの寄主転換と，新たな共生系における両者の共進化は，ハナホソガ属における適応放散を可能にし，コミカンソウ科植物との相乗的な多様化を促したであろう。

第八章

コミカンソウ科植物は多様な送粉様式や，ハナホソガ属とのさまざまな相互作用をもつことから，共生系や共進化系における生態学的，進化学的な研究にとりわけ適している。両者の間にはさまざまスケールでの非対称な種特異性や種分化のパターンが見られたが，こうした結果は両者の複雑な遺伝的構造や，それが共進化動態におよぼす影響を正しく理解する上で重要である。コミカンソウ科では絶対送粉共生系が独立に何度も起源しているため，共生系の進化を促すと考えられる生態的要因の特定や，共進化系が介在する多様化を解析する上で，極めて重要なモデル系となるであろう。

Summary

CHAPTER 1

Mutualisms are found at all levels of biological organization and often play fundamental roles in the origin and maintenace of biodiversity. Understanding factors that promote evolution and persistence of mutualisms requires studies from various taxonomic, ecological, and evolutionary approaches. Obligate mutualisms between plants and their seed-eating insects have provided important model systems for this purpose, and previous studies in the fig–fig wasp and yucca–yucca moth systems have greatly improved our understandings of various attributes of mutualisms. The recently discovered analogous system between *Glochidion* trees (Phyllanthaceae) and *Epicephala* moths (Gracillariidae) offer promising new opportunities to test and refine these earlier findings. Furthermore, the taxonomic and ecological diversity of related taxa in both groups allows detailed investigation of origin of the complex mutualism. The association between Phyllanthaceae and *Epicephala* holds promise for an ever-growing field of evolutionary biology.

CHAPTER 2

Species-specific obligate pollination mutualism between *Glochidion* trees (Phyllanthaceae) and *Epicephala* moths (Gracillariidae) involves a large number of interacting species and resembles the classically known fig–fig wasp and yucca–yucca moth associations. To assess the extent of parallel cladogenesis in *Glochidion–Epicephala* association, I reconstruct phylogenetic relationships of 18 species of *Glochidion* using nuclear ribosomal DNA sequences (internal and external transcribed spacers) and those of the corresponding 18 *Epicephala* species using mitochondrial (the cytochrome oxidase subunit I gene) and nuclear DNA sequences (the arginine kinase and elongation factor-1α genes). Based on the obtained phylogenies, I determine whether *Glochidion* and *Epicephala* have undergone parallel diversification using several different methods for investigating the level of cospeciation between phylogenies. These tests indicate that there is generally a greater degree of correlation between *Glochidion* and *Epicephala* phylogenies than expected in a random association, but the results are sensitive to selection of different phylogenetic hypotheses and analytical methods for evaluating cospeciation. Perfect congruence between phylogenies is not found in this association, which likely resulted from host shift by the moths. The observed significant discrepancy between *Glochidion* and *Epicephala* phylogenies implies that the one-to-one specificity between the plants and moths has been maintained through a complex speciation process, or that there is an underestimated diversity of association between *Glochidion* trees and *Epicephala* moths.

CHAPTER 3

The obligate mutualisms between flowering plants and their seed-parasitic pollinators constitute fascinating examples of interspecific mutualisms, which are often characterized by high levels of species diversity and reciprocal species specificity. The diversification in these mutualisms has been thought to occur through simultaneous speciation of the partners mediated by tight reciprocal adaptation, but recent studies cast doubt on this general view. In this chapter, I examine the diversity and species specificity of *Epicephala* moths (Gracillariidae) that pollinate *Glochidion* trees (Phyllanthaceae) using analysis of mitochondrial and nuclear gene sequences. Phylogenetic analysis of *Epicephala* moths associated with five *Glochidion* species in Japan and Taiwan reveal six genetically isolated species that are also distinguishable by male genital morphology: (1) two species specific to single host species (*G. acuminatum* and *G. zeylanicum*, respectively), (2) two species that coexist on *G. lanceolatum*, and (3) two species that share two closely related, parapatric hosts (*G. obovatum* and *G. rubrum*). Statistical analysis shows that the two species associated with *G. lanceolatum* are not sister species, indicating the colonization of novel *Glochidion* host in at least one lineage. Behavioral observations suggest that all six species possess the actively pollinating habit, thus none of the studied species has become a non-mutualistic 'cheater' that exploits the benefit resulting from pollination by other species. These results parallel recent findings in ecologically similar associations, namely the fig–fig wasp and yucca–yucca moth mutualisms, and contribute to a more general understanding of the factors that determine ecological and evolutionary outcomes in these mutualisms.

CHAPTER 4

A common principle among obligate seed-parasitic pollination mutualisms is that the pollinators consume only a limited amount of the seed crop within a developing fruit (or fig in the case of fig–fig wasp mutualism), thereby ensuring a net benefit to plant reproduction. A novel obligate, seed-parasitic pollination mutualism between two species of New Caledonian *Phyllanthus* (Phyllanthaceae), a close relative of *Glochidion*, and *Epicephala* moths (Gracillariidae) is an exception to this principle. The

highly specialized flowers of *Phyllanthus* are actively and exclusively pollinated by species-specific *Epicephala* moths, whose larvae consume all six ovules of the developing fruit. Some flowers pollinated by the moths remain untouched, and thus a fraction of the fruits is left intact. Additional evidence for a similar association of *Epicephala* moths in other *Phyllanthus* species suggests that this interaction is a coevolved, species-specific pollination mutualism. Implications for the evolutionary stability of the system, as well as differences in mode of interaction with respect to the *Glochidion–Epicephala* mutualism, are discussed.

CHAPTER 5

This chapter reports obligate seed-parasitic pollination mutualisms in *Breynia vitis-idea* and *B. fruticosa* (Phyllanthaceae). The genus *Breynia* is closely related to *Glochidion* and *Gomphidium* (a subgenus of *Phyllanthus*), in which pollination by species-specific, seed-parasitic *Epicephala* moths (Gracillariidae) have been previously reported. At night, female *Epicephala* moths carrying numerous pollen grains on their proboscises visited female flowers of *B. vitis-idea*, actively pollinated flowers, and each subsequently laid an egg. Examination of field-collected flowers indicated that pollinated flowers of *B. vitis-idea* and *B. fruticosa* almost invariably had *Epicephala* eggs, suggesting that these moths are the primary pollinators of the two species. Single *Epicephala* larvae consumed a fraction of seeds within developing fruit in *B. vitis-idea* and all seeds in *B. fruticosa*. However, some of the fruits were left untouched, and many of these had sign of moth oviposition, suggesting that egg/larval mortality of *Epicephala* moths is an important factor assuring seed set in these plants. The overall similarity of the specialized floral structure among *Breynia* species may indicate that this pollination system is fairly widespread within the genus.

CHAPTER 6

With more than 1200 species worldwide, Phyllantheae represents by far the most species-rich tribe within the family Phyllanthaceae. Previous studies have shown that plants of *Glochidion*, *Breynia*, and *Phyllanthus* (subgenus *Gomphidium*) are involved in obligate mutualisms with gracillariid *Epicephala* moths that actively pollinate the flowers as adults and feed on the developing seeds as larvae. However, considerable floral variation within the tribe suggests that this pollination system may not be widespread among the genera and subgenera within the tribe. In this chapter, I examine flowers and fruits of various species within Phyllantheae to determine the taxonomic diversity of plants associated with *Epicephala* moths. I also conduct field observation and pollination experiment to identify potential pollinator fauna for each species. I show that *Phyllanthus* shrubs of section *Anisonema* and an unclassified group of Malagasy endemics are pollinated by seed-parasitic *Epicephala* moths, whereas the rest of the studied species are not involved in the mutualism. Non-pollinating *Epicephala* parasitize the fruits of *Flueggea suffruticosa* and several herbaceous *Phyllanthus*, which are effectively pollinated by diurnal insects. These results show that some of the specialized floral structures found in Phyllantheae are associated with *Epicephala* pollination and provide important insights into the evolution of specialized mutualisms.

CHAPTER 7

Obligate mutualism between plants and seed-parasitic pollinators represents one of the most obvious cases of coevolution, but the origins of such interactions are exceedingly rare in nature. Previous studies have identified several ecological conditions that facilitate evolution of mutualisms, which predict that mutualisms can arise rapidly within lineages with key life history traits. In this chapter, I test this prediction using the association between Phyllanthaceae plants and seed-parasitic *Epicephala* moths that have previously shown to have evolved specialized pollination mutualisms. Phylogenetic reconstruction of the plant and moth lineages, and analyses of ancestral character reconstruction and divergence time estimate suggest that obligate pollination mutualism arose as much as five times within Phyllanthaceae. Active pollination behavior in *Epicephala* originated only once, being an evolutionary key innovation that has facilitated the evolution of the mutualism. Repeated colonization by the moths and subsequent coevolution have provided multiple independent opportunities for adaptive radiation in *Epicephala* and associated reciprocal diversification in Phyllanthaceae plants.

CHAPTER 8

The diversity of pollination systems and ecological associations with *Epicephala* moths found in Phyllanthaceae provides wealth of opportunity for testing various hypotheses on ecological and evolutionary outcomes of mutualisms and coevolutionary interactions. Asymmetries in patterns of specificity and diversification found at various levels in this association provide templates for general understanding of complex genetic structure and their significance in coevolutionary outcome. Independent origins of mutualism found in Phyllanthaceae provide promising opportunities for identifying ecological factors that promote mutualism, and determining the historical role of coevolution in driving reciprocal diversification.

Chapter 1

General introduction

MUTUALISMS AS CENTRAL FOCUS IN BIODIVERSITY STUDIES

Mutualisms are ubiquitous in nature, often ecologically dominant, and provide indispensable services to the origin and maintenace of biodiversity. The origin of eukaryotic life can be traced back to ancient mutualistic symbiosis, and plant cells are the product of symbiotic life with chloroplasts (Dyall et al. 2004). Subsequent success of land plants in terrestrial ecosystems owes to the mutualisms with mychorryzal fungi and nitrogen-fixing bacteria (Blackwell 2000; Redecker et al. 2000), while most flowering plants are dependent on mutualistic pollinators and seed dispersers for successful reproduction (Herrera and Pellmyr 2000). Many of the ants and termites that dominate tropical ecosystems are symbiotic with fungi that they cultivate (Mueller et al. 2001; Aanen et al. 2002), and various plants nourish ants that in turn protect them from herbivores (Heil and McKey 2003; Quek et al. 2004). In marine ecosystems, much of the diversity in coral reefs would simply disappear in the absence of mutualisms between corals and symbiotic algae (Baker 2003), or territorial fish and cultivated algae (Hata and Kato, 2006). Mutualisms are so pervasive on earth that it is often overlooked how central they are to the evolution and maintenance of biodiversity.

Despite their importance to the life on earth, studies of evolutionary and ecological dynamics of mutualisms have attracted very little attention during the past decades (Bronstein 1994). This is in sharp contrast with the great amount of knowledge accumulated on various biological attributes of parasitic and competitive associations. One important reason for this, however, is that understanding of antagonistic interactions has been fundamental to applications in medical or agricultural practices, such as disease control and pest management. It is not until the last couple of decades that researchers have started to focus on investigating various evolutionary questions concerning mutualisms (Herre et al. 1999; Sachs et al. 2004). With the global decline of biodiversity and growing appreciation of its importance to human welfare, we are now quickly learning how mutualisms are pervasive and central to the organization of biodiversity.

FUNDAMENTAL QUESTIONS IN MUTUALISMS

Mutualisms are not associations between altruistic organisms, but best viewed as balanced antagonistic interactions that nonetheless provide net benefits to each other (Axelrod and Hamilton 1981; Bull and Rice; 1991). Consequently, there is a conflict of interest between the partners, and overexploitation of one partner by the other can easily disrupt the balance between the cost and benefit of the mutualism (Sachs et al. 2004). This inherently fragile potential of the mutualism poses a series of fundamental evolutionary questions, and understanding what factors contribute to the evolution and persistence of mutualisms clearly requires various study approaches from taxomonic to ecological and evolutionary point of view (Herre et al. 1999; Pellmyr 2003). For example, identification and characterization of partners in a mutualism provide information about patterns of specificity and diversity of the association that are the basis of any ecological or evolutionary studies. Quantifying the cost and benefit of the mutualism and identifying factors that influence them are primary ecological approaches to understand mechanisms that align conflict of interests (Pellmyr and Huth 1994; Kiers et al. 2003). On the other hand, comparisons across related taxa in a phylogenetic framework can provide important insights into evolutionary outcomes of mutualisms that have persisted for millions of years (Currie et al 2003), or how inherently unstable associations have originated in the first place (Pellmyr and Thompson 1992). All of these approaches provide important insights to various aspects of mutualisms and contribute to a more general understanding of the factors that influence ecological and evolutionary outcomes of these potentially disruptive associations.

OBLIGATE POLLINATION MUTUALISMS AS IMPORTANT MODEL SYSTEMS

Among various types of symbiotic associations, the obligate mutualisms between plants and their seed-parasitic pollinators have provided important model systems for studies of mutualisms. The figs and yuccas have evolved intriguing mutualisms with specific wasps and moths, respectively, that actively pollinate the flowers as adults and, in return, feed on a fraction of the seeds as larvae (Janzen 1979; Weiblen 2002; Pellmyr 2003). These interactions involve fairly large organisms, which make them easier to handle and manipulate experimentally, and are highly species-specific associations, which enable more precise investigations of ecological and evolutionary dynamics of the interactions. Furthermore, the cost and benefit of the mutualism can be easily measured by counting the number of seeds, thus allowing a clear appreciation of the factors that influence ecological consequences of the

interactions. Together, these systems have greatly contributed to the development and testing of a range of theories and ideas concerning various attributes of mutuliasms. For example, phylogenetic studies in the fig–wasp system have revealed the extent to which speciation in figs is tracked by speciation in wasps, or taxonomic and geographic scales to which fig–wasp specificities are structured and maintained (Molbo et al. 2003; Weiblen 2002, 2004). Ecological studies in the yucca–moth system have found possible ecological mechanisms that could stabilize the balance between seed destruction and moth population size (Pellmyr and Huth 1994; Addicott and Bao 1999). Also, comparisons of life history traits in related moth groups have identified trait changes that have been involved during the evolution of the mutualism, and major transition events from mutualism to secondary antagonism (Pellmyr et al. 1996a,b).

PHYLLANTHACEAE–*EPICEPHALA* MUTUALISM: A NOVEL MODEL SYSTEM

Recently, a striking novel example of obligate pollination mutualism was found between seed-parasitic moths of the genus *Epicephala* (Gracillariidae) and their host *Glochidion* trees (Phyllanthaceae; Kato et al. 2003). In this mutualism, the female moth uses her specialized proboscis to actively collect and transport pollen between host flowers. She deposits the pollen in the stigmatic cavity of the female flower and subsequently inserts her long ovipositor to lay an egg in the flower she pollinates. The resultant seeds are the exclusive food for the *Epicephala* larva, but a fraction of the seeds is still viable for plant reproduction. Reciprocal plant specialization to *Epicephala* moth has led to obligate mutual dependence, such that neither of the partners can successfully reproduce in the absence of the other

This newly discovered mutualism provides promosing opportunities to corroborate and refine earlier findings in the fig and yucca systems, as the association is highly species-specific and involves an incredibly diverse lineage (>300 species in *Glochidion*). Despite overall similarity to the fig and yucca systems, there are also major differences, including pollinator taxonomy, proportion of seeds destroyed by each larva, and degrees of pollinator specificity. These differences offer interesting new opportunities for comparative analysis across systems regarding various aspects of mutualism. Furthermore, related taxa of the mutualists potentially include species with divergent life histories, allowing an empirical investigation of the origin and evolutionary consequences of the mutualism. The discovery of this novel association opens up previously unexplored horizon in an increasingly important field of ecology and evolutionary biology.

ORGANIZATION OF THE THESIS

This thesis consists of eight chapters, the first of which is the introduction given here (Chapter 1). In the next two chapters (Chapters 2 and 3), I investigate patterns of historical association between *Glochidion* trees and *Epicephala* moths. I determine the extent to which speciation in the hosts (*Glochidion*) is tracked by speciation in the pollinators (*Epicephala*) in Chapter 2, and assess patterns of specificity and diversity of the association within a small geographic scale in Chapter 3. In the following three chapters (Chapters 4–6), I describe pollination systems and modes of association with *Epicephala* moths in various taxa of Phyllanthaceae to provide basic information upon which to infer the origin of the mutualism. Finally, I interpret this information in a phylogenetic context to study patterns of mutualism evolution and subsequent diversification in the Phyllanthaceae–*Epicephala* association.

Chapter 2

Cospeciation analysis of an obligate pollination mutualism: Have *Glochidion* trees (Phyllanthaceae) and pollinating *Epicephala* moths (Gracillariidae) diversified in parallel?

INTRODUCTION

Obligate pollination mutualism between plants and their seed-parasitic pollinators is perhaps one of the most specialized cases of plant–insect coevolution (Janzen 1979; Wiebes 1979; Pellmyr et al. 1996a; Weiblen 2002; Kato et al. 2003; Pellmyr 2003). Fig–fig wasp and yucca–yucca moth associations are well-known examples, which are diverse and usually species-specific and possess various traits that are thought to have resulted from reciprocal adaptation (Ramírez 1974; Wiebes 1979; Pellmyr 1999, 2003; Weiblen 2002; Cook and Rasplus 2003). These attributes of the interactions have long provided model systems for studies of coevolution as well as evolutionary and ecological aspects of mutualism (reviewed in Herre 1999; Weiblen 2002; Cook and Rasplus 2003; Pellmyr 2003). Recently, a novel example of such interaction was found between trees of the diverse genus *Glochidion* (>300 spp.; Euphorbiaceae) and species-specific, seed-parasitic moths of the genus *Epicephala* (Gracillariidae) (Kato et al. 2003). In this association, the female *Epicephala* moth uses its proboscis to actively collect and transport pollen between *Glochidion* flowers and lays an egg in the style. The *Epicephala* larva feeds solely on the developing seeds of *Glochidion* and destroys a small proportion of the crop. Because *Epicephala* moths are the exclusive pollinators of *Glochidion* trees, neither of the mutualists can successfully reproduce in the absence of the other.

When two interacting lineages have been in intimate association during much or all of their diversification, as in the case of obligate pollination mutualisms or some host–parasite interactions, there is a probability that speciation in one group is paralleled by speciation in the other. This mode of diversification results in a pattern of shared evolutionary history between the two lineages, known as cospeciation (Hafner and Nadler 1988; Hafner et al 1994; Moran and Baumann 1994; Page, 1994; Page and Charleston 1998; Huelsenbeck et al. 2000). Cospeciation can be a non-adaptive process that occurs in the absence of selection. For example, repeated vicariance events followed by shared allopatric speciation can produce a pattern of parallel diversification (Roderick 1997). However, cospeciation can also be reinforced or directly result from adaptive process (Moran and Baumann 1994; Clayton et al. 2003a,b). For example in feather lice and their avian hosts, preening behavior of the host imposes selection on louse body size, which prevents lice from switching between hosts of different sizes (Clayton et al. 2003a). In obligate pollination mutualisms, the pollinators are responsible for the fertilization among conspecific host flowers, and thus some adaptation in the plants to exclude non-legitimate pollinators is likely present. In fact, there are several candidates of reciprocally selected traits that may reinforce plant–pollinator specialization, such as synchronized phenological patterns (Wiebes 1979; Beck and Lord 1988; Patel and Hossaert-McKey 2000), species-specific olfactory signals (Ware et al. 1993; Hossaert-McKey et al. 1994; Song et al. 2001; Grison-Pigé et al 2002, 2003), and reciprocal adaptation between pollinator morphology and floral structure (Ramírez 1974; Herre 1989; Van Noort and Compton 1996; Kato et al. 2003; Weiblen 2004). Thus, knowledge on the degree of cospeciation in obligate pollination mutualisms provides an essential step towards understanding the historical role of coevolution in shaping speciation and diversification in plants and pollinators.

Previous studies using the fig–fig wasp system have indicated a significant level of cospeciation at both lower and higher taxonomic levels (Herre et al. 1996; Weiblen 2000, 2001; Machado et al. 2001; Weiblen and Bush 2002). However, strict congruence of phylogenies has not been found in the fig–fig wasp association. In addition, there are several documented cases in which multiple distantly related fig wasp species associate with a single host, further indicating a lack of strict-sense cospeciation in this association (Wiebes 1979; Compton 1990; Michaloud et al. 1996; Rasplus 1996; Kerdelhue et al. 1999; Lopez-Vaamonde et al. 2002; Molbo et al., 2003). Although analysis of parallel cladogenesis using the yucca–yucca moth system has not been thoroughly conducted, mapping of *Yucca* sections on yucca moth phylogeny indicates that host plant use is relatively conserved at higher taxomonic levels (Pellmyr and Leebens-Mack 1999; Pellmyr, 2003). However, obvious instances of colonization based on existing classification, combined with occurrence of a yucca moth species on multiple yucca hosts indicate that strict-sense cospeciation has not occurred in the yucca–yucca moth system either (Pellmyr et al. 1996b; Pellmyr 1999, 2003; Pellmyr and Balcázar-Lara 2002; Pellmyr and Segraves 2003).

While previous studies using the fig–fig wasp and yucca–yucca moth systems provided insights into

macroevolutionary patterns in these specialized interactions, the mutualism between *Glochidion* and *Epicephala* offers a novel opportunity to corroborate and refine these earlier observations. This system is particularly suited for such analysis, as the association is highly species-specific and extremely diverse (Kato et al., 2003). The genus *Glochidion* comprises more than 300 species distributed in tropical Asia, Australia, and Polynesia (Govaerts et al. 2000; Hoffmann and McPherson 2003) with multiple species commonly occurring in sympatry. *Epicephala* moths associated with *Glochidion* plants are currently all undescribed, but individual moths associated with different *Glochidion* hosts can potentially be distinguished morphologically (Kato et al. 2003). In principle, they differ in the genitalic structure of the males, while there are also slight differences among the female genitalic characters (Kato et al. 2003). In this study, I provide the first analysis of the level of cospeciation between *Glochidion* trees and *Epicephala* moths using molecular phylogenetic analysis of 18 species of *Glochidion* and the corresponding 18 species of *Epicephala*. Phylogenetic analyses are based on nucleotide sequences of the internal and external transcribed spacer regions of the nuclear ribosomal DNA (ITS and ETS) for *Glochidion* trees, and those of the mitochondrial cytochrome oxidase subunit I gene (COI) and nuclear arginine kinase (ArgK) and elongation factor-1α (EF-1α) genes for *Epicephala* moths.

Apart from plant–pollinator interactions, studies of cospeciation have focused primarily on host–parasite associations, such as those between insect ectoparasites and their avian/mammalian hosts (Hafner and Nadler 1998; Page 1990, 1996; Hafner et al. 1994; Clayton et al. 2003a), bacterial symbionts and their invertebrate hosts (Moran and Baumann 1994; Peek et al. 1998; Clark et al. 2000; Shoemaker et al. 2002), and herbivorous insects and their host plants (Farrell and Mitter 1990, 1998; Farrell et al. 1992; Funk et al. 1995; Roderick 1997; Becerra 1997). These studies have shown that phylogenetic congruence is imperfect or absent for most of the interactions, but a few rare cases are known in which two phylogenies are consistent with the hypothesis of strict cocladogenesis (Peek et al. 1998; Clark et al. 2000; Lo et al. 2003; Degnan et al. 2004). Recent growing interest in this area has lead to the development of various analytical tools for testing the hypothesis of cospeciation, such as Brooks' parsimony analysis (Brooks 1981), reconciliation analysis (TreeMap; Page 1994), event-based method (TreeFitter; Ronquist 1995), maximum-likelihood method (Huelsenbeck et al. 1997), Jungles analysis (Charleston 1998), and distance-based method (ParaFit; Legendre et al. 2002). These methods consider various assumptions for inferring the optimal evolutionary scenario for host–parasite associations and use different null hypotheses to test the significance of cospeciation. I therefore use several of these tests to determine whether *Glochidion* trees and *Epicephala* moths have undergone parallel diversification and propose possible interpretations of the observed pattern.

MATERIALS AND METHODS

SAMPLING

A list of species sampled and their locality information are given in Table 1. I sampled 18 *Glochidion* species collected from various localities within the distribution of the genus (Table 1). Although there is little information concerning intrageneric taxonomy of *Glochidion* (Webster 1994; Govaerts et al. 2000), my sampling includes representatives of the three morphological groups previously identified by Levin (1986) and thus cover a wide range of morphological variation found within this large genus. Representatives of *Breynia* and *Sauropus* were sampled as outgroups, which likely represent sister groups of *Glochidion* (Webster 1994).

For *Epicephala*, I sampled a single individual from each of the 18 morphologically delimited species collected from their respective *Glochidion* hosts. Kato et al. (2003) showed that *Epicephala* moths associated with different *Glochidion* species can also be distinguished genetically, based on sequence variation of the COI gene among *Epicephala* moths collected at various localities in Japan and Taiwan. Therefore, the use of a single individual to represent each *Epicephala* species in this study is fairly reasonable. Description of these moth species will be provided elsewhere, as the results of the current analysis would not be affected by nomenclature. For outgroups, two *Epicephala* moths associated with *Breynia* and *Phyllanthus*, the close relatives of *Glochidion*, were used.

MOLECULAR METHODS

I extracted plant genomic DNA from field collected, silica-gel dried leaves either using Plant Tissue Kit (Qiagen, Valencia, CA) or following a standard CTAB protocol. We PCR-amplified the fragments spanning the entire ITS-1, 5.8S rDNA, and ITS-2 regions using primers ITS-5 and ITS-4 (White et al. 1990) and the entire intergenic spacer region between 28S and 18S rDNA including ETS using primers 28S-IGS and 18S-IGS (Baldwin and Markos 1998). PCR conditions for amplifying the fragment containing the ITS region were as follows: initial denaturation step at 94°C for 5 min, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C, and final extension at 72°C for 7 min. Those for amplifying ETS were: initial denaturation at 94°C for

Table 1. A list of species analyzed in the study with information on their collection locality. Because all species of *Epicephala* are currently undescribed, the species names of their host plants are provided in parentheses. *Glochidion* sp. is an undescribed species occurring at Pindai, New Caledonia.

Host plant	Associated moth	Collection locality
Glochidion acuminatum	*Epicephala* sp. (*acuminatum*)	Yakkachi, Amami Island, Japan
G. benthamianum	*E.* sp. (*benthamianum*)	Mt. Molly, Queensland, Australia
G. caledonicum	*E.* sp. (*caledonicum*)	Hienghéne, New Caledonia
G. collinum	*E.* sp. (*collinum*)	Mt. Victoria, Fiji
G. concolor	*E.* sp. (*concolor*)	Namosi, Fiji
G. cordatum	*E.* sp. (*cordatum*)	Namosi, Fiji
G. ferdinandii	*E.* sp. (*ferdinandii*)	Mt. Lewis, Queensland, Australia
G. harveyanum	*E.* sp. (*harveyanum*)	Mt. Windsor, Queensland, Australia
G. lanceisepalum	*E.* sp. (*lanceisepalum*)	Lambir, Borneo Island, Malaysia
G. lanceolatum	*E.* sp. (*lanceolatum*)	Banna, Ishigaki Island, Japan
G. obovatum	*E.* sp. (*obovatum*)	Cape Toi, Miyazaki, Japan
G. phillipicum	*E.* sp. (*phillipicum*)	Nanjin, Taiwan
G. pungens	*E.* sp. (*pungens*)	Mt. Lewis, Queensland, Australia
G. rubrum	*E.* sp. (*rubrum*)	Banna, Ishigaki Island, Japan
G. seemanii	*E.* sp. (*seemanii*)	Navai, Fiji
G. velutinum	*E.* sp. (*velutinum*)	Mt. Popa, Myanmar
G. zeylanicum	*E.* sp. (*zeylanicum*)	Henoko, Okinawa Island, Japan
G. sp.	*E.* sp. (sp.)	Pindai, New Caledonia
Breynia distica	*E.* sp. (*Breynia distica*)	Pindai, New Caledonia
Sauropus granulosus		Vientiane, Laos
	E. sp. (*Phyllanthus koumacensis*)	Koumac, New Caledonia

Table 2. A list of primers originally designed for the present study. ETS primers are located in this order (top to bottom) from the 5′ to 3′-end of the ETS region.

Locus	Primer name	Sequence
ETS	ETS-F2	5′-GGGAAATGGCAAGCAAAATGG-3′
	ETS-F1	5′-GCYTTTCTCGGTGTATTCG-3′
	ETS-R2	5′-CATCGCACTAAGACCCACC-3′
	ETS-R1	5′-TAGGCAACAACAATTCTTAAG-3′
ArgK	ArgK-F4	5′-ATTTAGACTCTGGTGTTGG-3′
	ArgK-R4	5′-ATGCCGTCGTACATCTCCTT-3′
EF-1α	ef1af2	5′-CCCATTTCKGGCTGGCAYGGAGA-3′
	ef1ar2	5′-GATTTACCRGWACGACGRTC-3′

5 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 68°C, and 3 min at 72°C, with a final extension at 72°C for 7 min. All PCR amplifications were aided by Ex Taq DNA polymerase (TaKaRa, Otsu, Japan) and carried out on GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). PCR products were purified using QiaQuick PCR Purification Kit (Qiagen). Sequencing was performed on both strands using the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and electrophorased on an ABI 3100 sequencer (Perkin-Elmer). The primers as used in amplification were used for sequencing the ITS regions. For ETS, we used 18S-E (Baldwin and Markos 1998) in combination with additional sequencing primers listed in Table 2.

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Genomic DNA of *Epicephala* moths was extracted either from larvae or adults reared from fruits of their respective hosts using DNeasy Tissue Kit (Qiagen). I PCR-amplified the COI region using primers described in Kato et al. (2003) and the ArgK and EF-1α regions using primers given in Table 2. PCR conditions for both regions were identical to those for ITS, except that annealing temperature was 40°C, 50°C, and 60°C for COI, ArgK, and EF-1α, respectively. The products were purified and sequenced as described above using the amplification primers and additional internal sequencing primers provided in Kato et al. (2003) for the COI region.

PHYLOGENETIC ANALYSIS

Alignment of COI, ArgK, and EF-1α was straightforward and required no gaps. Sequences of the ITS and ETS regions varied in length among species and were aligned using Clustal X (Thompson et al. 1997) with manual correction of obvious misalignments. Because 5′-end of the outgroup ETS sequences were highly dissimilar with respect to

those of the ingroup, I excluded nearly half of the ETS region we obtained for the outgroup and coded as missing data in the analysis. Gaps within the ITS and ETS regions were also treated as missing data throughout the analysis. The obtained sequences have been deposited in the GenBank database under accession numbers AY525678–AY525757 and AY538751–AY538770.

All phylogenetic analyses were done using PAUP* version 4.0b10 (Swofford 2002), unless otherwise mentioned. Because the ITS region contained only 11 parsimony-informative characters within the ingroup and did not provide sufficient resolution to phylogenetic estimates, ITS and ETS were analyzed simultaneously throughout the analysis. The COI, ArgK, and EF-1α regions were analyzed both separately and simultaneously. Concordance of phylogenetic signals among data partitions (ITS-1, ITS-2, and ETS for *Glochidion* and COI, ArgK, and EF-1α for *Epicephala*) was assessed using the incongruence length difference test (ILD test; Farris et al. 1994). The test was performed using 1000 replications after removing constant and uninformative characters, as suggested by Cunningham (1997) and Lee (2001), which indicated no significant heterogeneity among data partitions ($P > 0.4$ for all pairwise comparisons). Recent studies have illustrated problems with the ILD test in assessing incongruence among data partitions (Dolphin et al., 2000; Barker and Lutzoni, 2002; Darlu and Lecointre, 2002), and thus I did not use this test as a criterion for data combinability. However, tree topologies obtained in the separate analyses were highly concordant among data partitions (see Results), thus supporting the rationale for performing simultaneous analyses.

I obtained most-parsimonious (MP) trees by heuristic searches with 100 random addition analyses and tree bisection-reconnection (TBR) branch-swapping. Heuristic searches were run without collapsing branches with zero length in order to obtain fully resolved trees, which is required in subsequent tests of cospeciation. Robustness of the MP trees was validated with bootstrap analysis with 1000 replications and decay indices (Bremer 1994). Command file for calculating decay indices were produced using TreeRot.v2 (Sorenson 1999).

I also performed maximum-likelihood (ML) analysis by heuristic searches with 10 random addition analyses and TBR branch-swapping. To obtain fully resolved trees, branches with effectively zero length were not collapsed in the searches. The program Modeltest 3.0 (Posada and Crandall 1998) was used to select appropriate models of base substitution and to estimate model parameters. Nodal support was assessed using bootstrap analysis with 100 replications.

COSPECIATION TESTS

To assess whether species of *Glochidion* and *Epicephala* have undergone parallel diversification, I used reconciliation analysis, as implemented in the programs TreeMap 1.1 (Page 1994) and TreeFitter 1.0 (Ronquist 1995). TreeMap uses a simple model to find optimal reconstructions of the history of the association by maximizing cospeciation events and minimizing host shifts (Page 1994). In situations where host shifts are likely to be common, however, this methodology is not guaranteed to find optimal solutions (Page and Charleston 1998). A more recent version of this program, TreeMap 2.0b (Charleston and Page 2001) implements the Jungles analysis (Charleston 1998), which considers all potentially optimal solutions and offers a more appropriate means of dealing with host shifts. However, this program is currently limited in size and complexity of data sets that can be computed in allowable time and memory. Thus, in this study I used the program TreeMap 1.1 with the heuristic search option to reconcile plant and moth trees. On the other hand, TreeFitter uses different algorithm and optimality criterion for reconciling the two phylogenies. Costs are assigned to the four types of cophylogenetic events (cospeciation, duplication, sorting, and host shift; for detailed terminology, see Page [1994]; Page and Charlton [1998]), and optimal solutions are found by minimizing the global cost of the reconstruction. Because TreeFitter is computationally simpler than TreeMap, reconstructions involving many host shifts can be recovered. The disadvantage of this method is that the placement of each cophylogenetic event on the phylogeny can not be output.

In both TreeMap and TreeFitter, one can test the null hypothesis that the two phylogenies are randomly related by comparing the scores of optimal reconstructions (number of cospeciation events for TreeMap and global cost for TreeFitter) with those of randomly obtained phylogenies through permutational procedure. Because these programs require fully resolved trees, I tested all combinations of the obtained tree topologies between *Glochidion* and *Epicephala* to account for phylogenetic uncertainty. TreeFitter also allows assignment of different costs to the four types of events, so I varied these costs to assess its effect on the test results. All tests were performed based on 999 permutations.

In addition to TreeMap and TreeFitter, I also used the method ParaFit (Legendre et al. 2002), which, rather than tree topologies, uses matrices of patristic distances (summed branch lengths along a phylogenetic tree) or phylogenetic distances calculated directly from sequence data. Whereas TreeMap and TreeFitter requires fully resolved trees and thus are sensitive to selection of different phylogenetic hypotheses, Parafit is less likely to

provide different results among several optimal phylogenies. In this test, distance matrices of the two groups are transformed to principal coordinates (Gower 1966), and the trace statistic is calculated by taking plant–pollinator associations into account. Null hypothesis that the two groups are randomly associated is tested through permutational procedure; plant–pollinator relationships are permuted to obtain a null distribution of the test statistic against which the observed value is tested. This method also allows one to test whether each plant–pollinator association contributes significantly to the global fit of the two phylogenies. This is done by calculating trace statistics with and without a given plant–pollinator link, and testing the difference between the two statistics by permutation. Numerical simulations indicated that these tests have correct rate of type-I error under various error conditions (Legendre et al. 2002). In this study, I used patristic distances calculated from the ML trees of *Glochidion* and *Epicephala*. The ML trees obtained from both separate and simultaneous analyses of *Epicephala* were used in the test. Principal coordinates were calculated using the DistPCoA software (Legendre and Anderson 1998), and the test was performed by 999 permutations using the program ParaFit (Legendre 2001).

If *Glochidion* and *Epicephala* phylogenies show significantly higher correlation than expected by chance, the observed topological incongruence may simply be explained by systematic error (e.g., limited number of informative characters or inadequate taxon sampling), rather than actual biological processes such as host shifting. Therefore, I tested the hypothesis that the same tree topology underlies *Glochidion* and *Epicephala* data sets using the likelihood-based Shimodaira-Hasegawa test (SH-test; Shimodaira and Hasegawa 1999; Goldman et al. 2000). In this test, the likelihood score of the best topology for a given data set is compared to the scores of alternative topologies obtained from other data sets. All unique topologies obtained in the MP and ML searches of both separate and simultaneous analyses were used in the test (branches with effectively zero length were collapsed).

In addition, I accounted for the possibility of introgressive hybridization and/or incomplete lineage sorting of ancestral polymorphisms within the *Epicephala* lineage, which potentially causes incongruence between *Glochidion* and *Epicephala* phylogenies (Herre et al. 1996; Machado et al. 2001; Demastes et al. 2003). If such stochastic processes are involved in the *Epicephala* moth evolution, different loci are expected to undergo different evolutionary history due to recombination and/or different modes of inheritance. Therefore, topological congruence among different loci within *Epicephala* can be used to assess whether or not phylogenetic estimates were affected by genetic introgression and/or lineage sorting. For this purpose, I performed the SH-test among COI, ArgK, and EF-1α to determine the level of phylogenetic conflict among loci. Each data set was tested against all unique topologies obtained in the MP and ML analyses of the alternative data sets (branches with effectively zero length were collapsed).

RESULTS

The complete ITS-1 and ITS-2 regions had 222 and 219 aligned nucleotide sites, respectively. These regions had very little sequence variation among the ingroup; of the combined 441 nucleotide sites, only 11 were parsimony-informative for the ingroup, and pairwise sequence difference among the ingroupwas 1.5 ± 0.7% (mean ± SD; range, 0–3.1%). For ETS, I obtained 1492 aligned nucleotide sites, of which 46 were parsimony-informative for the ingroup. Sequence divergence among the ingroup was comparable to that of the ITS region (pairwise sequence difference, 1.6 ± 0.5%; range, 0.2–2.8%). For the outgroup taxa, only the 3′-end half (721 aligned nucleotide sites) of the ETS region was included in the analysis due to alignment ambiguity; 5′-end half of the region was coded as missing data.

Simultaneous parsimony analysis of the ITS and ETS regions resulted in a single shortest tree (Fig. 1). This tree was identical to the single MP tree obtained by ETS alone, and all 11 informative characters among the ingroup within the ITS region mapped to the ETS phylogeny without homoplasy, indicating that phylogenetic signals between ITS and ETS are highly concordant. Simultaneous likelihood analysis also resulted in a tree that is identical to the MP topology (Fig. 1).

The data matrices of COI, ArgK, and EF-1α consisted of 1325, 767, and 496 bp, respectively, of which 206, 63, and 32 were parsimony-informative for the ingroup. Pairwise sequence difference among the ingroup for COI, ArgK, and EF-1α was 7.0 ± 1.4% (0.2–9.7%), 3.7 ± 1.1% (0–6.0%), and 2.6 ± 0.9% (0–4.5%), respectively. Parsimony analysis of individual data sets produced 9, 15, and 18 shortest trees, respectively. However, MP trees derived from the EF-1α data set contained branches with zero length, and if these branches were not collapsed, the number of MP trees increased to 378. Likelihood analysis of individual data sets produced trees that are either identical to one or a subset of the MP trees (branches with effectively zero length were not collapsed). Thus, for the following TreeFitter and TreeMap analyses, we considered the fully resolved MP topologies as the *Epicephala* phylogeny.

Simultaneous parsimony analysis of the combined data set resulted in a single MP tree, and

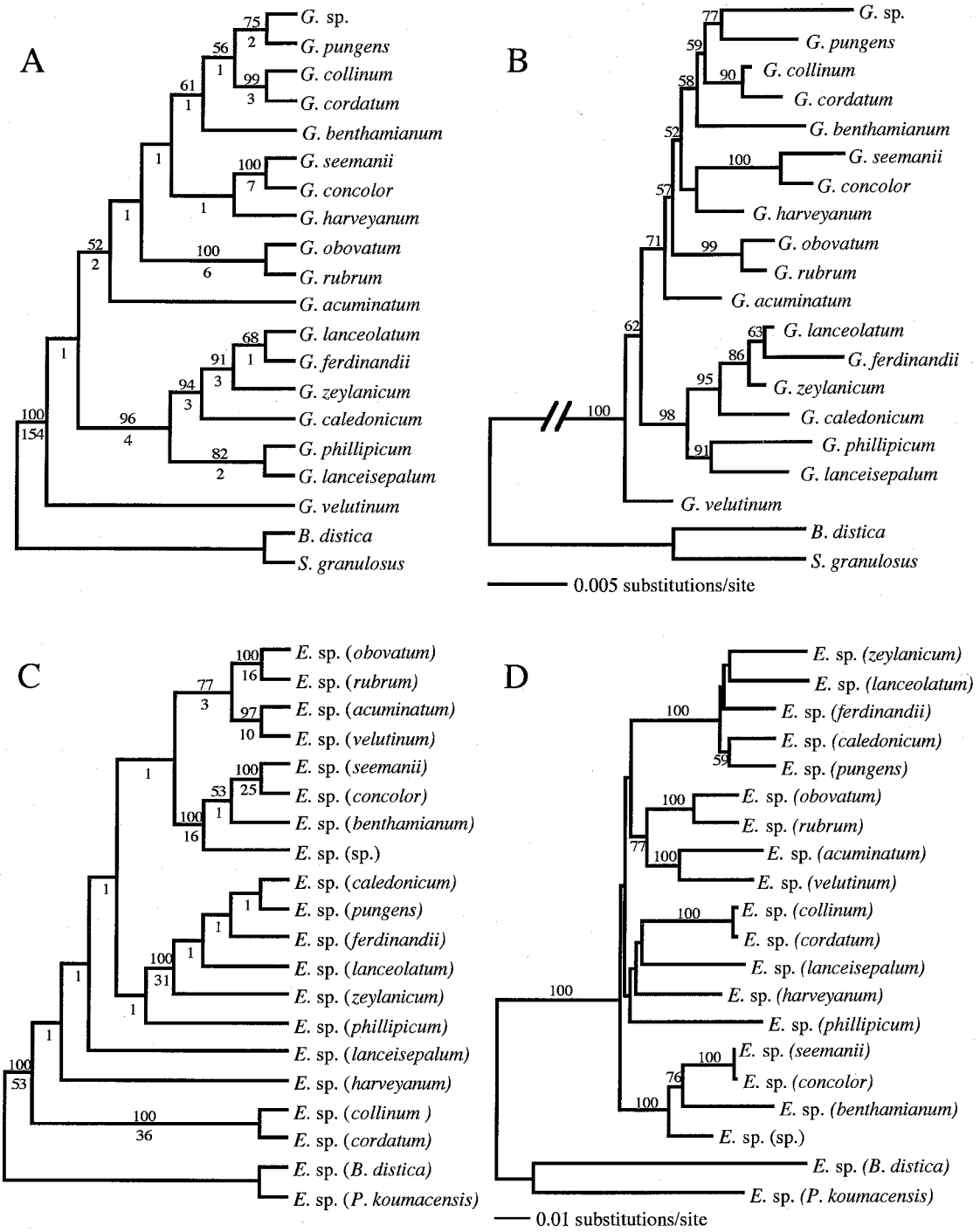


Fig. 1. Maximum-parsimony and maximum-likelihood trees for the 18 species of *Glochidion* and *Epicephala* analyzed in the study. *Glochidion* phylogenies (A, B) were estimated using the combined ITS and ETS sequence data; those of *Epicephala* (C, D) were based on the combined data set of COI, ArgK, and EF-1α sequences. (A) The single most-parsimonious tree of 372 steps (consistency index excluding uninformative characters [CI] = 0.91, retention index [RI] = 0.93). (B) Maximum-likelihood phylogeny estimated using HKY + Γ substitution model (-ln likelihood = 4776.0229; empirical base frequencies with rate heterogeneity; transition/transversion ratio = 1.7532; gamma shape parameter = 0.7701). (C) The single most-parsimonious tree of 1286 steps (CI = 0.50; RI = 0.56). (D) Maximum-likelihood phylogeny estimated using GTR + Γ + I substitution model (-ln likelihood = 10138.0841; empirical base frequencies with rate heterogeneity; gamma shape parameter = 0.7580; proportion of invariable sites = 0.5315; transformation parameters [A-C] = 1.9845, [A-G] = 5.7716, [A-T] = 6.0535, [C-G] = 2.0840, [C-T] = 19.0841, [G-T] = 1.0000). Numbers above and below branches are bootstrap values and decay indices, respectively. Because *Epicephala* moths analyzed in this study are all undescribed, the species names of their respective hosts are provided in parentheses.

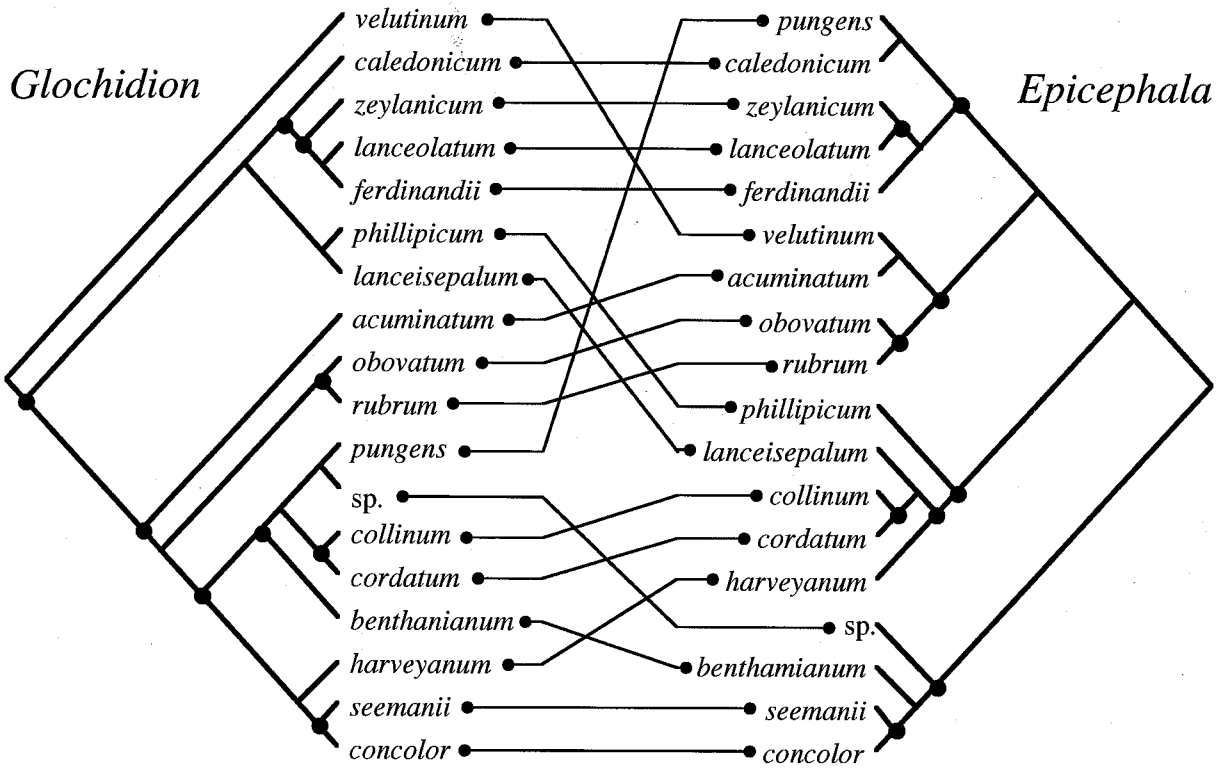


Fig. 2. Phylogenetic trees for *Glochidion* (left) and *Epicephala* (right) with information on plant–moth associations. The *Glochidion* tree is the maximum-likelihood topology inferred from the combined ITS + ETS sequences; the *Epicephala* tree is the maximum-likelihood tree based on the combined COI, ArgK, and EF-1α sequences. Cospeciating nodes as inferred from the TreeMap analysis are indicated with circles. Because *Epicephala* moths analyzed in this study are all undescribed, only the species names of their respective hosts are given. Species of *Glochidion* are also designated by their species names.

the likelihood analysis produced a topology that is different from the MP topology (Fig. 1). Because the MP and ML topologies obtained in the simultaneous analysis were different from either of the trees derived from separate data sets, these two topologies were also used in the following tests of cospeciation.

COSPECIATION TESTS

The TreeFitter and TreeMap analyses were performed using the single MP/ML tree derived from the combined ITS + ETS data set as the *Glochidion* phylogeny and all unique topologies obtained in separate and simultaneous analyses of COI, ArgK, and EF-1α as the *Epicephala* phylogeny. Figure 2 shows the comparison between the *Glochidion* phylogeny and simultaneous ML topology of *Epicephala* with information on plant–pollinator relationships. Although *Glochidion* and *Epicephala* phylogenies are not strictly identical, there is a tendency that associated plant and pollinator occupy a similar position on the cladogram. Indeed, the result of TreeFitter analysis, using default cost settings, indicated that there is a better overall fit between *Glochidion* and *Epicephala* phylogenies than expected by chance alone, although 16 % of the trees inferred by EF-1α did not show significantly better fit (Table 3). I also tested whether these results are sensitive to cost settings by varying the costs of sorting and host shifting, the two events that reduce the overall fit between phylogenies. Because the obtained results were essentially similar among most comparisons, only the results obtained using the simultaneous *Epicephala* ML and MP topologies are presented here. When sorting and switching costs were varied among 0.5, 1, and 2 (maximum of four-fold difference), overall fit was still significant, suggesting that these results are not sensitive to cost settings (Table 4). Using the ML topology for *Epicephala*, the overall fit disappeared when cospeciation events were made almost impossible by assigning a very high cost (100), although this was not observed when the three other events were prevented (Table 3). This suggests that the significant phylogenetic correlation observed between the *Glochidion* tree and *Epicephala* ML tree is best explained by underlying cospeciation. When the MP topology was used, correlation became non-significant when either cospeciation or host shift was prevented, indicating a mixed pattern of cospeciation and host shift between the *Glochidion* tree and *Epicephala* MP tree.

The number of cospeciation events on the reconciled *Glochidion–Epicephala* tree inferred by TreeMap varied between 6 and 10, depending on the

Table 3. The results of the TreeFitter and TreeMap analyses. The single tree derived from the combined ITS and ETS sequences of *Glochidion* were tested against all unique *Epicephala* topologies derived from each of COI, ArgK, and EF-1α as well as the combined data. The number of trees with non-random association followed by the total number of trees examined is given for each set of trees. For the TreeMap analysis, the inferred number of cospeciation events is also given.

	TreeFitter		TreeMap		
Tree	Non-random (%)	Range of *P*-value	Non-random (%)	Range of *P*-value	Cospeciations
COI	9/9 (100)	0.001–0.009	2/9 (22)	0.015–0.255	7–9
ArgK	15/15 (100)	0.001–0.007	8/15 (53)	0.001–0.085	8–10
EF-1α	318/378 (84)	0.001–0.094	16/378 (4)	0.010–0.524	6–9
Combined (parsimony)	1/1 (100)	0.003	0/1 (0)	0.220	7
Combined (likelihood)	1/1 (100)	0.005	1/1 (100)	0.019	9

Table 4. The results of the TreeFitter analysis under various cost settings. The cost setting at the top is the default. The test was performed between the single optimal topology derived from the combined ITS and ETS sequences of *Glochidion* and each of the likelihood and parsimony topologies derived from the combined COI, ArgK, and EF-1α sequences of *Epicephala*. Probabilities are based on 999 permutations. The asterisks represent significance by 5%.

COST SETTINGS				*P*-VALUES	
Cospeciation	Duplication	Sorting	Host shift	*Epicephala* ML	*Epicephala* MP
0	0	1	2	0.006*	0.004*
0	0	0.5	2	0.003*	0.002*
0	0	1	1	0.008*	0.004*
0	0	2	0.5	0.009*	0.003*
0	0	2	1	0.004*	0.001*
100	0	1	2	1.000	1.000
0	100	1	2	0.001*	0.003*
0	0	100	2	0.005*	0.004*
0	0	1	100	0.010*	0.068

Table 5. The result of ParaFit analysis conducted using patristic distances of the maximum-likelihood tree derived from the combined ITS and ETS sequences of *Glochidion* and that obtained from the combined COI, ArgK, and EF-1α sequences of *Epicephala*. Probabilities are based on 999 permutations. The null hypothesis of the global test is that the association between *Glochidion* plants and *Epicephala* moths are random on the phylogeny. In the test of individual links, the null hypothesis states that a given plant–moth association is established at random. Because all *Epicephala* moths are undescribed, species names of their respective host plants are given. Asterisks represent significance by 5%.

Plant	Moth	*P*-values
Global fit		0.005*
Individual links		
G. acuminatum	*E.* sp. *(acuminatum)*	0.112
G. benthamianum	*E.* sp. *(benthamianum)*	0.577
G. caledonicum	*E.* sp. *(caledonicum)*	0.018*
G. collinum	*E.* sp. *(collinum)*	0.019*
G. concolor	*E.* sp. *(concolor)*	0.055
G. cordatum	*E.* sp. *(cordatum)*	0.046*
G. ferdinandii	*E.* sp. *(ferdinandii)*	0.012*
G. harveyanum	*E.* sp. *(harveyanum)*	0.025*
G. lanceisepalum	*E.* sp. *(lanceisepalum)*	0.984
G. lanceolatum	*E.* sp. *(lanceolatum)*	0.003*
G. obovatum	*E.* sp. *(obovatum)*	0.118
G. phillipicum	*E.* sp. *(phillipicum)*	0.189
G. pungens	*E.* sp. *(pungens)*	0.996
G. rubrum	*E.* sp. *(rubrum)*	0.090
G. seemanii	*E.* sp. *(seemanii)*	0.080
G. sp.	*E.* sp. (sp.)	0.191
G. velutinum	*E.* sp. *(velutinum)*	0.320
G. zeylanicum	*E.* sp. *(zeylanicum)*	0.005*

tree used as the *Epicephala* phylogeny (Table 3). The number of cospeciations was higher than expected by chance in only 22, 53, and 4% of the trees derived from COI, ArgK, and EF-1α, respectively (Table 3). Using trees obtained from the combined data set, the number of nodes shared with *Glochidion* phylogeny was greater than expected by chance in the ML topology but not in the MP topology (Table 3).

Because TreeFitter and TreeMap uses fully resolved trees and thus are sensitive to the selection of different optimal trees, I also performed the ParaFit analysis, which takes tree structure into account, as complementary to the above two analyses. The global test using ParaFit corroborated results obtained by TreeFitter that there is a significant correlation between *Glochidion* and *Epicephala* trees, either when trees derived from individual loci or the combined data were used as the *Epicephala* phylogeny ($P = 0.002$ for COI, $P = 0.009$ for ArgK, $P = 0.019$ for EF-1α, and $P = 0.005$ for the combined data). However, the test of individual links indicated that not all plant–moth associations contribute to the global fit between the two phylogenies. Table 5 shows the test results of individual plant–pollinator links. Because the results were similar among *Epicephala* phylogenies derived from individual loci and the combined data, only the results obtained using the simultaneous *Epicephala* ML tree is presented. The results show that some associations, such as those between *G. benthamianum*, *G. pungens*, and *G. lanceisepalum* and their respective *Epicephala* pollinators do not contribute to the overall phylogenetic structure (Table 5), which can also be inspected visually by comparison of the *Glochidion–Epicephala* phylogenies (Fig. 2). These results indicate that while *Glochidion* and *Epicephala* phylogenies are consistent with a global cospeciation pattern, there are also cases of apparent mismatch between the two trees.

Since the results of TreeFitter and Parafit indicated a significantly better fit between *Glochidion* and *Epicephala* phylogenies than expected by chance, I tested the hypothesis that the same topology underlies plant and pollinator phylogenies, but different trees are obtained due to systematic error. The results of SH-test indicated that there is a significant disagreement in the most likely topology supported by *Glochidion* and *Epicephala* data sets (Table 6), indicating that the observed incongruence between *Glochidion–Epicephala* phylogenies can not be explained by sampling error. We also examined whether introgressive hybridization or incomplete

Table 6. Ln likelihoods of *Glochidion* and *Epicephala* trees under alternative data sets. Significance levels of differences between likelihoods were tested using the Shimodaira-Hasegawa test. Significant results indicate that the score of the best tree for a given data set is significantly higher than the scores of the optimal trees based on alternative data sets. Results were essentially identical when either parsimony or likelihood topologies were tested; only the results obtained using likelihood topologies are presented. Combined, the combined COI, ArgK, and EF-1α data set; Delta, difference between the likelihood of trees; *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, — not significant.

Data set	Tree	ln(L)	Delta	*P*-values
ITS+ETS	ITS+ETS	-4776.023		
	COI	-4990.144	214.121	***
	ArgK	-5019.020	242.997	***
	EF-1α	-5065.531	289.508	***
	Combined	-4987.503	211.480	***
COI	ITS+ETS	-6123.876	198.240	***
	COI	-5925.635		
	ArgK	-5943.389	17.654	—
	EF-1α	-5994.402	68.766	**
	Combined	-5934.736	9.101	—
ArgK	ITS+ETS	-2668.136	138.366	***
	COI	-2553.716	23.946	—
	ArgK	-2529.770		
	EF-1α	-2565.434	35.664	—
	Combined	-2536.350	6.58	—
EF-1α	ITS+ETS	-1448.858	68.196	***
	COI	-1390.066	9.403	—
	ArgK	-1390.346	9.683	—
	EF-1α	-1380.662		
	Combined	-1388.891	8.229	—
Combined	ITS+ETS	-10526.186	388.102	***
	COI	-10234.149	96.065	*
	ArgK	-10146.835	8.751	—
	EF-1α	-10147.726	9.642	—
	Combined	-10138.084		

lineage sorting of ancestral polymorphisms within *Epicephala* account for the discrepancies between plant–moth phylogenies by assessing topological congruence among the COI, ArgK, and EF-1α trees. The results of the SH-test indicated that there is no significant incongruence among topologies supported by different loci, although the optimal topologies based on EF-1α was not supported by the COI data set (Table 6). These results indicate that the assumption of shared evolutionary history for the three loci is not rejected, and thus stochastic processes are unlikely responsible for most of the observed incongruence between phylogenies of *Glochidion* and *Epicephala*.

DISCUSSION

PHYLOGENETIC ANALYSES AND COSPECIATION TESTS

Simultaneous analyses of ITS and ETS for *Glochidion* and COI, ArgK, and EF-1α for *Epicephala* recovered several well-supported groupings, which were mostly apical on the phylogeny (Fig. 1). However, higher-level relationships were left poorly resolved in both the plant and moth phylogenies (Fig. 1). Particularly, statistical support for the majority of higher nodes in the *Epicephala* phylogeny was very low, which was also the case in separate analyses of individual loci. Because none of the loci analyzed in this study were significantly affected by multiple substitutions (A. Kawakita, unpublished data), the observed poor phylogenetic resolution may indicate that the genetic loci used in this study are of limited use for higher-level phylogenetics in *Glochidion* and *Epicephala*, which is possibly due to rapid initial diversification of these lineages.

Despite uncertainty in phylogenetic estimation, the results of TreeFitter and ParaFit demonstrated that there is a greater level of correlation between *Glochidion* and *Epicephala* trees than expected in a random association. These results were generally unaffected by selection of different optimal trees or cost structures (Tables 3, 4), indicating that the

association between *Glochidion* and *Epicephala* phylogenies is significantly structured. However, the TreeMap results were sensitive to selection of different optimal trees. Using TreeMap, 22, 53, and 4% of the topologies derived from COI, ArgK, and EF-1α, respectively, were inferred to have greater number of nodes shared with *Glochidion* phylogeny than expected by chance, but the remaining trees were not significantly different from random trees (Table 3). Likewise, the *Epicephala* ML tree inferred from the simultaneous analysis showed significant evidence for cospeciation, but this did not hold for the MP tree. These results indicate that while our cospeciation analysis generally suggested a significant correlation between *Glochidion* and *Epicephala* phylogenies, the results were influenced by uncertainty in phylogenetic estimation, at least under the criterion used in the TreeMap analysis.

In addition to uncertainty in phylogenetic estimation, the method of evaluating cospeciation also affected the results. The two topology-based tests performed in this study, TreeMap and TreeFitter, produced more or less conflicting results, although the topologies tested were identical in the two tests. Whereas TreeFitter suggested that nearly all the *Epicephala* trees are consistent with the hypothesis of cospeciation, only 7% of the topologies tested in TreeMap had a greater number of nodes shared with *Glochidon* phylogeny than expected by chance (Table 3). One possible explanation for this apparently different outcomes is that the TreeMap program used in this study is not guaranteed to find optimal reconstructions, particularly in situations where host shifts are likely to be common, as in our data sets (Ronquist 1995; Charleston 1998; Page and Charleston 1998; Percy et al. 2004). On the other hand, TreeFitter uses a different algorithm that deals with host shifts more appropriately, thus optimal reconstructions involving any number of host shifts can be recovered. Therefore, it is possible that TreeMap underestimated the degree of cospeciation in this association, leading to the observed conflict in the TreeMap and TreeFitter results. In any case, the evidence for cospeciation between *Glochidion* and *Epicephala* found in this study should be interpreted with caution, because of uncertainty in phylogenetic estimation and sensitivity of the results to different analytical methods.

Given that the *Glochidion* and *Epicephala* phylogenies did not show prefect congruence, I further explored potential sources of incongruence between *Glochidion* and *Epicephala* phylogenies. Phylogenetic conflict could be due to host shifts, but this can also arise from systematic error or stochastic processes such as genetic introgression and/or incomplete lineage sorting of ancestral polymorphisms. The results of SH-test showed that *Glochidion* and *Epicephala* data sets do not share the same underlying evolutionary history, indicating that systematic error can not explain the conflict between plant and moth phylogenies. In fact, there are several apparent cases of incongruence between *Glochidion* and *Epicephala* phylogenies (Fig. 2). For example, the *Epicephala* moth pollinating *G. pungens* is nested within a clade including pollinators of *G. lanceolatum*, *G. caledonicum*, *G. zeylanicum*, and *G. ferdinandii* with very strong support, while its host is grouped with species distantly related to these four *Glochidion* species (Figs. 1, 2). Similarly, the *Epicephala* species pollinating *G. benthamianum* groups with moths associated with *G. seemanii*, *G. concolor*, and *G.* sp., but its host, *G. benthamianum*, does not occupy the corresponding position on the host phylogeny (Figs. 1, 2). These associations that deviate from the global cospeciation pattern were also identified in the tests of individual links in the ParaFit analysis (Table 5).

The possibility that stochastic processes produced incongruent plant–moth phylogenies was indirectly tested on the basis of congruence among multiple, unlinked loci within the *Epicephala* moth lineage. The results of SH-test among COI, ArgK, and EF-1α showed that the three loci are generally consistent with a shared evolutionary history (Table 6). Although trees derived from EF-1α were not supported by the COI data set (Table 6), general congruence between two recombining nuclear loci as well as between nuclear and cytoplasmically inherited loci indicates that genetic introgression or lineage sorting among *Epicephala* species do not explain most, if not all, of the phylogenetic incongruence between plants and pollinators. Therefore, I consider that the observed incongruence between *Glochidion* and *Epicephala* phylogenies is most likely attributed to host shifts by the moths. It is possible that introgression and/or lineage sorting are occurring within the *Glochidion* lineage, resulting in an underestimation of the degree of cospeciation in this association. This possibility could not be tested in this study, because ITS and ETS are closely linked and are assumed to share the same evolutionary history (Baldwin and Markos 1998; Bena et al. 1998). Phylogenetic analysis using independent loci in *Glochidion* is difficult due to a lack of genetic variation within many of the commonly analyzed loci, such as intergenic regions of the chloroplast genome that are often considered fast-evolving (A. Takimura and A. Kawakita, unpublished data).

COSPECIATION BETWEEN *GLOCHIDION* AND *EPICEPHALA*

Previous studies addressing parallel diversification in plant–herbivore interactions often did not find cospeciation of the interacting lineages

(Futuyma and McCafferty 1990; Farrell et al. 1992; Funk et al. 1995; Becerra 1997; Mardulyn et al. 1997; Smith and Bush 1997; Nyman et al. 2000; Scheffer and Wiegmann 2000; Ronquist and Liljeblad 2001; Bucheli et al. 2002; Cook et al. 2002; Lopez-Vaamonde et al. 2003; but see Farrell and Mitter 1990, 1998; Roderick 1997). Rather, host shifts were prevalent, and dramatic shifts among distantly related plant taxa were commonly observed. Therefore, the overall cospeciation pattern found in this study, together with that in the fig–fig wasp system (Herre et al. 1996; Weiblen 2000, 2001; Machadao et al. 2001; Weiblen and Bush 2002), represents a special case in which plants and associated insects diversified in parallel. Unfortunately, analysis of phylogenetic congruence does not identify processes that underlie the pattern of cospeciation. For example, cospeciation may arise through a number of processes including shared allopatric speciation, coevolution, and adaptation by only one group in response to the other (Roderick 1997; Clayton et al. 2003a,b). Thus, it is possible that plants and pollinators cospeciate as the result of shared vicariance events and that adaptive evolution may not be important in driving the overall cospeciation pattern.

However, several observations suggest that reciprocal selection may reinforce cospeciation in obligate pollination mutualisms. For example, Weiblen and Bush (2002) demonstrated that the degree of cospeciation between *Sycomorus* figs and *Ceratosolen* pollinators is greater than that observed between the same set of host figs and non-pollinating, gall-inducing fig wasps of the genus *Apocryptophagus*. They attributed this difference to the extent of reproductive requirements by which pollinating fig wasps are constrained, such as pollen compatibility and/or reciprocal adaptation between fig wasp morphology and narrow ostiolar entrance of the host fig (Ramírez 1974; Herre 1989; van Noort and Compton 1996). Similar constraints may also have been important in preventing host shifts by *Epicephala* moths. In *Glochidion*, the structure of the style exhibits great interspecific variation and is the principal species-diagnostic characteristic within the genus, which is in marked contrast with morphological uniformity of the male flowers (Airy Shaw 1978; Chakrabarty and Gangopadhyay 1995; Kato et al. 2003). Because *Epicephala* moths pollinate *Glochidion* flowers and oviposit in the styles using diverse and specific methods (Kato et al. 2003), this structural difference may reinforce host plant specialization and have played an important role in shaping the overall cospeciation pattern between *Glochidion* and *Epicephala*.

The relative importance of reciprocal adaptation in driving parallel diversification can potentially be assessed by the amount of pollinator and non-pollinator cospeciation with their host plants. For example, *Glochidion* plants are associated with leaf-mining *Diphtheroptila* moths that belong to the subfamily Gracillariinae together with *Epicephala* (Meyrick 1916; M. Kato, personal observations), and thus these moths are good candidates for such analysis. However, this may in turn reveal similar levels of congruence with host phylogeny in pollinators and herbivores, either because cospeciation does not involve an adaptive component or the two groups show similar degrees of adaptation in response to their hosts. In fact in the fig–fig wasp system, the phylogeny of non-pollinating fig wasps is often concordant to that of the pollinators, which is attributed to possible shared use of specific olfactory signals in host recognition (Machado et al. 1996; Lopez-Vaamonde 2001). Thus, a complete understanding of the historical role of coevolution in driving cospeciation requires both robust phylogenetic framework and identification of reciprocally selected traits that actually function to reinforce cospeciation.

HOST SHIFTS BY *EPICEPHALA* MOTHS

The intimate association between *Glochidion* and *Epicephala* is perhaps one of the most extreme cases of species-specific, plant–insect interaction known (Kato et al. 2003). However, if this one-to-one rule had been maintained throughout the history of their diversification, how could the pollinators have shifted to novel hosts? If a host shift occurs successfully without violating this rule, the pollinator colonizing a new host must drive the original pollinator extinct, or the pollinator must be primarily absent on the new host. The former scenario assumes that host shift does not result in stable coexistence of two pollinator species on a single host. Although some theories suggest that the presence of multiple symbionts produces an unstable situation for a mutualism (Bull and Rice 1991; Maynard Smith and Szathmary 1995; Herre 1999; Herre et al. 1999), recent documented cases of species-specificity breakdown in figs indicate that two fig wasp species commonly reproduce and pollin ate in a single host fig (Molbo et al. 2003). This situation is assumed to have lasted for at least a few million years, indicating that long-term coexistence on a shared host can occur (Molbo et al. 2003). Furthermore, common observation of multiple pollinator yucca moth species on a single yucca host indicates that coexistence of multiple pollinator species on a shared host do not necessarily lead to exclusion of others by a single species (Addicott 1996; Pellmyr et al. 1996b; Addicott and Bao 1999; Pellmyr 1999). Thus, we consider that the observed apparent host shift by *Epicephala* moths did not result solely from the colonization/exclusion process.

The second scenario assuming the primary absence of pollinator does not appear to be a plausible condition in obligate pollination mutualisms, as neither the plant nor pollinator can successfully reproduce without the other. However, this process may exert where the plant colonizes a region without its original pollinator, followed by colonization of the isolated plant population by an unassociated pollinator species. For example, independent colonization of oceanic islands (e.g., French Polynesia) by unrelated plant and pollinator could lead to formation of novel associations involving host shift. Also, some *Glochidion* species have very wide distribution ranges (e.g., *G. acuminatum* occurs from Japan to India and *G. phillipicum* from Taiwan to Australia), providing a possibility that multiple, distantly related *Epicephala* species pollinate a single *Glochidion* species allopatrically across its geographic range. In plant–herbivore interactions, there are well-documented examples in which local herbivores colonize and specialize to recently introduced host plants (Feder et al. 1988, 1994, 1997; McPheron et al. 1988; Carroll and Boyd 1992; Carroll et al. 1997, 1998; Filchak et al. 1999; Groman and Pellmyr 2000), supporting the plausibility of this process in organization of novel associations.

Alternative to the above two scenarios, the assumed one-to-one specificity between plants and pollinators may be routinely violated in the *Glochidion–Epicephala* association. Recently, Molbo et al. (2003) showed that in the fig–fig wasp system, a single host species often harbor two or more pollinator species and suggested a widespread cryptic diversity of pollinator fig wasps in this association. Importantly, the wasp species co-occurring on a single host are not necessarily sister species, and thus repeated host shifts were needed to explain the observed pattern of association (Molbo et al. 2003; also see Wiebes 1979; Compton 1990; Michaloud et al. 1996; Rasplus 1996; Kerdelhue et al. 1999; Lopez-Vaamonde et al. 2002). This situation is analogous to that in the yucca–yucca moth association in which multiple distantly-related pollinator species are commonly observed on a single yucca host (Addicott 1996; Pellmyr et al. 1996b; Addicott and Bao 1999; Pellmyr 1999). Furthermore, the breakdown of one-to-one specificity is paralleled in several other obligately mutualistic interactions in which reassessment of species diversity has lead to the discovery of unexpected complexity of associations between the participants, including those between reef-building corals and dinoflagellates (Rowan and Knowlton 1995; Rowan et al. 1997; Rowan 1998), fungus-growing ants and their fungi (Chapela et al. 1994; Mueller et al. 1998), and myrmecophytic plants and their mutualistic ants (Fiala et al. 1999; Ward 1999; Feldhaar et al. 2003; Quek et al. 2004). Importantly, in many of these interactions, the lack of one-to-one specificity has been frequently invoked as explanations for the previously documented poor concordance between phylogenies of the two groups (Rowan and Knowlton 1995; Pellmyr et al 1996b; Feldhaar et al. 2003; Molbo et al. 2003; Pellmyr 2003). Although breakdown of species-specificity has not been reported in the *Glochidion–Epicephala* system, the existence of multiple pollinator *Epicephala* species on a single *Glochidion* species can not be ruled out. If this is in fact the case with the *Glochidion–Epicephala* association, analysis using a single individual to represent each species would likely results in an oversimplification of the actual macroevolutionary pattern. In turn, such an underestimated diversity of *Epicephala* moths provides a comprehensive explanation for the observed incongruence between *Glochidion* and *Epicephala* phylogenies.

At present, plant–pollinator specificity in the *Glochidion–Epicephala* association has been tested morphologically and genetically for six species pairs, based on *Epicephala* moth specimens collected from six *Glochidion* species at 2–6 locations of southern Japan and Taiwan (Kato et al. 2003). Specificity is also confirmed for six species pairs in an analogous pollination mutualism recently found between *Epicephala* moths and New Caledonian *Phyllanthus* (Kawakita and Kato, 2004). Future analysis should incorporate a greater number of individuals per population of each species, preferably collected from a wide range of its distribution for a rigorous testing of the plausibility of one-to-one specificity in the *Glochidion–Epicephala* association.

In addition to the need for a critical examination of species diversity in *Epicephala*, a more thorough sampling of taxa would also add confidence to the estimates of macroevolutionary pattern in this association. Because my analyses included 18 of the >300 *Glochidion* species that are variously related to each other, the historical pattern of plant–moth diversification detected in this study may not be representative of the overall pattern. For example, a subset of the pollinators (or parasites) may be more likely to cospeciate with their hosts than others (Machado et al. 2001; Weiblen 2001, 2002; Taylor and Purvis 2003; Clayton et al. 2004), or cospeciation is more likely to be detected at higher taxonomic levels than at finer scales (Farrell and Mitter 1998; Demastes et al. 2003). Nevertheless, my results strongly suggest that both cospeciation and host shift have played an important role in organizing the current pattern of plant–pollinator association in this remarkable system, which is certainly true for the fig–fig wasp and yucca–yucca moth systems.

Chapter 3

Assessment of the diversity and species specificity of the mutualistic association between *Epicephala* moths and *Glochidion* trees

INTRODUCTION

Mutualisms are found at all levels of biological organization and are widely appreciated for their fundamental importance in the evolution and maintenance of biodiversity (Boucher 1985; Thompson 1994, 2005; Maynard Smith & Szathmary 1994; Herre *et al.* 1999). Although mutualisms can simply be viewed as reciprocally beneficial associations, the obvious fact of long-term persistence and intriguing coadaptations found among these interactions have offered topics of considerable ecological and evolutionary interest (Herre *et al.* 1999; Mueller 2002; Cook & Rasplus 2003; Heil & McKey 2003; Pellmyr 2003; Sachs *et al.* 2004). The obligate mutualisms between flowering plants and their seed-parasitic pollinators represent perhaps some of the most tightly integrated cases of interspecific mutualisms. The fig–fig wasp and yucca–yucca moth mutualisms are two classically known examples, which have been extensively studied as important model systems for the development and testing of theories of coevolution and mutualism stability (Weiblen 2002; Cook & Rasplus 2003; Pellmyr 2003). The high species diversity and reciprocal specificity found in the fig–fig wasp system have further lead to a proposition of strict coadaptation and parallel diversification between the partners (Herre *et al.* 1996; Machado *et al.* 2001; Weiblen & Bush 2002; Weiblen 2004), raising an interesting question of how host specificity in the pollinators affects patterns of speciation and reciprocal diversification in this association. Although the idea of cospeciation has been supported to some extent (Herre *et al.* 1996; Weiblen & Bush 2002; Weiblen 2004; Rønsted *et al.* 2005), cases of species-specificity breakdown are known (e.g., Wiebes 1979; Lopez-Vaamonde *et al.* 2002; Molbo *et al.* 2003) and suggested to have important consequences for the patterns of gene flow within a fig community (Machado *et al.* 2005)

The recently discovered obligate pollination mutualisms between seed-parasitic moths of the genus *Epicephala* (Gracillariidae) and their Phyllanthaceae hosts (*Glochidion*, *Phyllanthus*, and *Breynia*; Kato *et al.* 2003; Kawakita & Kato 2004a,b) provide novel opportunities to corroborate earlier findings in the fig and yucca systems. These systems are particularly suited for studying the pattern of speciation and diversification in obligate interactions, as the Phyllanthaceae–*Epicephala* associations are sufficiently diverse (>300 species in *Glochidion* alone; Govaerts *et al.* 2000), and preliminary tests have shown high plant–pollinator specificity in at least six East Asian *Glochidion* (Kato *et al.* 2003) and six New Caledonian *Phyllanthus* species (Kawakita & Kato 2004a). In these mutualisms, the female moth uses her specialized proboscis to actively collect and transport pollen between host flowers. She deposits the pollen in the stigmatic cavity of the female flower and subsequently inserts her long ovipositor to lay an egg in the flower she pollinates. The resultant seeds are the exclusive food for the *Epicephala* larva, but a fraction of the seeds is still viable for plant reproduction. Reciprocal plant specialization to *Epicephala* moth has led to obligate mutual dependence, such that neither of the partners can successfully reproduce in the absence of the other (Kato *et al.* 2003; Kawakita & Kato 2004a,b).

In ecological and evolutionary studies of mutualisms, it is of critical importance that the number of partner species and their associations are correctly known (Herre *et al.* 1999; Molbo *et al.* 2003), but in many cases determining the actual diversity in mutualisms has been problematic. Critical examination of morphology and recent application of molecular analyses have drastically changed our understanding of many mutualistic systems, including coral–zooxanthella symbiosis (Rowan & Knowlton 1995; Baker 2003), legume–rhizobium symbiosis (Moulin *et al.* 2001; Chen *et al.* 2003; Rasolomampianina *et al.* 2005), ant–myrmecophyte mutualism (Fiala *et al.* 1999; Ward 1999; Feldhaar *et al.* 2003), and obligate plant–pollinator mutualism (Pellmyr 1999; Pellmyr & Balcázar-Lara 2000; Molbo *et al.* 2003). In the fig–fig wasp association, Molbo *et al.* (2003) analyzed microsatellite markers and found that at least four species of neotropical figs are each associated with a pair of cryptic wasp pollinators. Likewise in the yucca moths, analysis of morphological, molecular, and biological data revealed considerable diversity of pollinator species, the majority of which had been previously regarded as one polyphagous species (Pellmyr 1999; Pellmyr & Balcázar-Lara 2000; Pellmyr & Segraves 2003). In addition, two derived species were found to have independently lost the pollinator habit and shifted to exploit fruits resulting from pollination by other species (Addicott 1996; Pellmyr *et al.* 1996; Pellmyr 1999). The refined species status in these model systems has greatly increased our understanding of the biological diversity and the consequences on

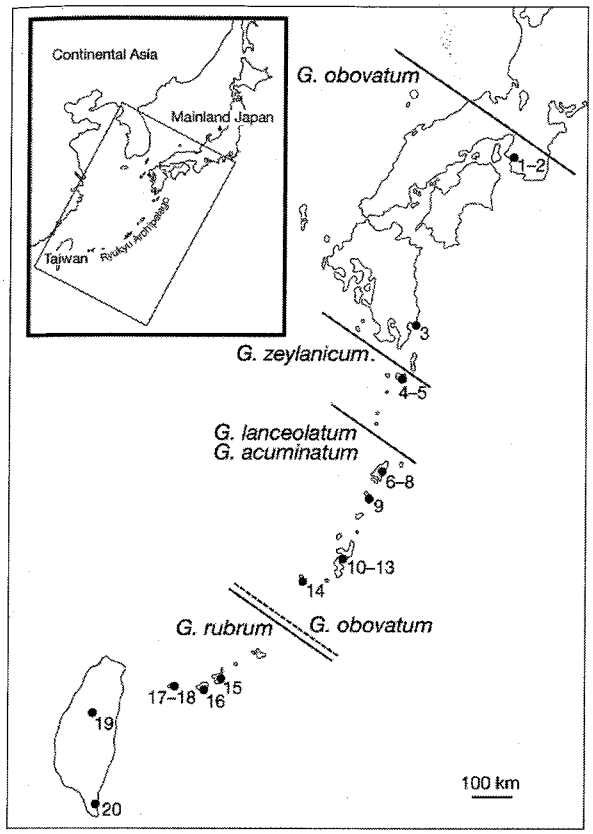


Fig. 1 Sampling localities of *Epicephala* moths collected from five *Glochidion* hosts in Japan and Taiwan. Numerals correspond to locality numbers in Table 1. The northern distribution limits of the five host *Glochidion* species are given by solid lines. All the studied *Glochidion* species are distributed as far south as tropical southeast Asia except *G. obovatum*, which is endemic to Japan (the southern limit shown by the hatched line).

ecological and evolutionary outcomes of the mutualism (Addicott & Bao 1999; Csotonyi & Addicott 2001; Marr *et al.* 2001; Shapiro & Addicott 2004; Segraves & Pellmyr 2004; Molbo *et al.* 2004; Segraves *et al.* 2005).

Previous studies in the mutualism between Phyllanthaceae and *Epicephala* have found that each plant species is pollinated by a morphologically distinct, undescribed moth species, thus assuming a strict one-to-one relationship between the plants and pollinators (Kato *et al.* 2003; Kawakita & Kato 2004a; Kawakita *et al.* 2004). Also, analyses of the mitochondrial cytochrome oxidase subunit 1 (COI) gene sequences in limited samples of *Epicephala* moths provided tentative support for the specific association between the two partners (Kato *et al.* 2003; Kawakita & Kato 2004a). However, the comparison of plant–pollinator phylogenies in 18 pairs of associated *Glochidion* and *Epicephala* species indicated several potential cases of pollinator host switch (Kawakita *et al.* 2004), suggesting possible coexistence of multiple *Epicephala* species on the same host resulting from colonization of novel *Glochidion* species. In this study, I test the previous assumption of the one-to-one relationship in the *Glochidion–Epicephala* mutualism using moth samples collected from five *Glochidion* hosts in Japan and Taiwan. To determine the number of pollinator species and the degree of genetic isolation among species, I analyze nucleotide sequences of three independent genetic loci, the mitochondrial COI and nuclear arginine kinase (ArgK) and elongation factor-1 alpha (EF-1α) genes, and link these results to morphological analysis of the genitalia. Using field observations and genetic data, I also evaluate whether or not any of the moth species sharing the same host has lost the pollinator habit, since some theories suggest that symbiont coexistence following colonization event can give rise to a transition from mutualism to parasitism (Maynard Smith & Szathmary 1995; Herre 1999; Herre *et al.* 1999; Yu 2001). Finally, I compare our results with situations in the fig–fig wasp and yucca–yucca moth systems and discuss their implications for the studies of the Phyllanthaceae–*Epicephala* mutualism.

MATERIALS AND METHODS

MOTH SAMPLING

I collected *Epicephala* moths from fruits of the five *Glochidion* species that occur in Japan, *G. acuminatum*, *G. lanceolatum*, *G. obovatum*, *G. rubrum*, and *G. zeylanicum*. Although intrageneric classification of *Glochidion* has not been well studied, a recent phylogenetic study indicates that the five *Glochidion* species can be grouped into three major intrageneric groups; *G. lanceolatum* and *G. zeylanicum*, and *G. obovatum* and *G. rubrum* form pairs of close relatives, while *G. acuminatum* occupies a distinct phylogenetic position and is equally related to the other four species (Kawakita *et al.* 2004). *Glochidion obovatum* is endemic to Japan, whereas the remaining four species are widely distributed throughout tropical Asia (Govaerts *et al.* 2000). Samples were collected from 2–8 populations covering a wide distribution range of each host species within Japan (Fig. 1). I also collected moths associated with *G. rubrum* at two additional populations in Taiwan. *Glochidion acuminatum*, *G. lanceolatum*, and *G. zeylanicum* are also known from Taiwan, but moth samples were not available for the study. Moths were either extracted from fruits as larvae or reared to adults, and stored in 99% alcohol for DNA sequencing. A total of 70 moths (larvae, males, or females) were sampled following this method. The adult males and females sampled for the molecular analysis were further used for morphological analysis of the genitalia and proboscis (see below). I sampled only one moth from a single *Glochidion* tree to minimize the possibility of examining multiple individuals that share the same

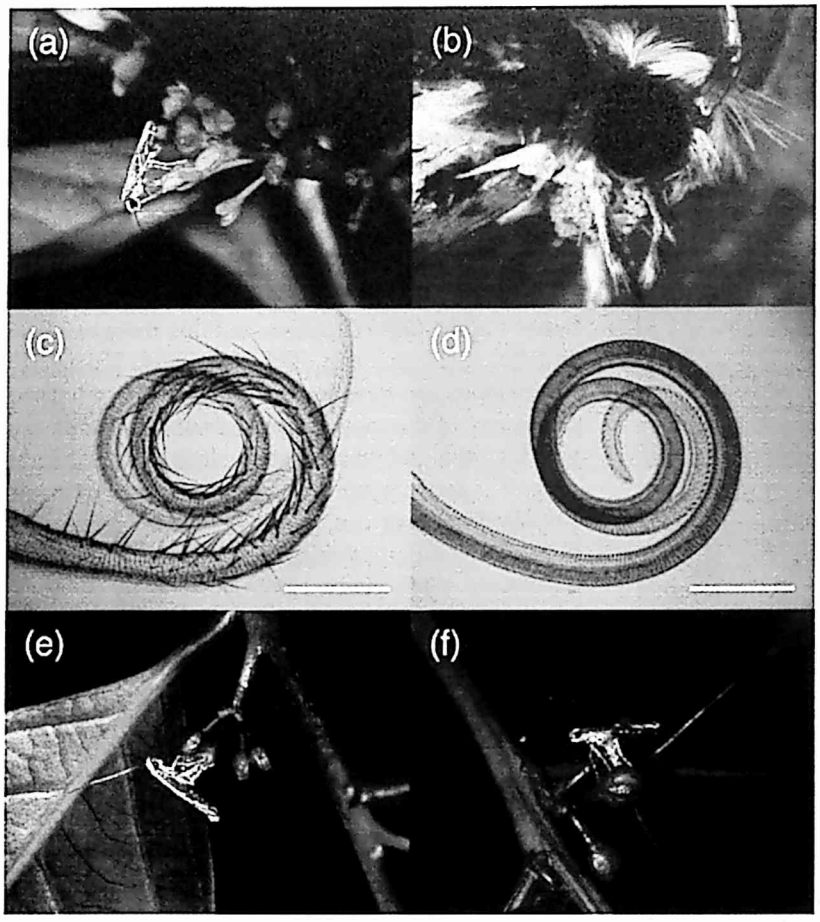


Fig. 2 (a) A female *Epicephala* moth actively pollinating *Glochidion* obovatum by probing the flower with her proboscis. (b) Pollen coat on the proboscis of a pollinating female. (c, d) The left galea, which pairs with the right galea to form the proboscis, of a female and male Epicephala moth, respectively. The sensilla in the females of pollinating *Epicephala* are a novel trait likely specialized for active collection and transportation of pollen. Bars = 0.1 mm. (e, f) Two *Epicephala* species ovipositing in *G. lanceolatum* flowers. The two species are specific to *G. lanceolatum* and coexist on the same host. The females of one species (C5 in Fig. 4) oviposit from the apical pit of female flower and lay the eggs in the stylar tissue, whereas those of the other species (C3) insert their ovipositor from the lateral ovary wall and place their eggs next to the ovules.

mother. However in *G. lanceolatum*, I found two moth lineages occurring in the same host population (see Results), so we additionally sampled multiple individuals collected on each of the three trees (two on Yonaguni Is. and one on Ishigaki Is.) to determine whether or not the two moths are associated with the same plants.

To determine whether or not each of the moth lineages serves as pollintors, I studied the behavior of 26 adult female moths on female flowers of the five *Glochidion* species (2–17 moths per *Glochidion* species). An actively pollinating female repeatedly extends and retracts its proboscis and probes the stigmatic cavity (Fig. 2a), thus the pollination behavior can unambiguously be distinguished from other activities. I recorded the presence/absence of active pollination behavior prior to oviposition at night using flashlights. The moths studied in the field were collected, stored in silica gel, and subsequently used for DNA sequencing to determine to which lineage they belong. The number of pollen grains on moth proboscis was counted under a light microscope before DNA extraction to confirm that pollination behavior actually resulted in deposition of pollen (Fig. 2b).

In total, 96 moths were newly sampled for the present study, either directly from *Glochidion* fruits or on the flowers after observation of pollination behavior. In addition, I used 15 genomic DNA samples from our previous analysis of the COI gene (Kato et al. 2003) to further sequence the nuclear loci. The number of larvae, males, and females sampled for each *Glochidion* species is given in Table 1.

Molecular methods

I extracted moth genomic DNA using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany). The head capsule of the larva or the head, wings, and abdomen of the adult were stored as vouchers and/or for subsequent morphological analyses. Using polymerase chain reactions (PCRs), I amplified fragments of the mitochondrial COI and nuclear ArgK and EF-1α genes. The primers used for amplifying and sequencing the COI region were 5′-ATAATTTTTTTTATAGTTATAC-3′ (Kato *et al.* 2003) and 5′-GATGGGCTCATACAATAAATCCTA-3′. PCR and sequencing primers for ArgK and EF-1α as well as PCR conditions for amplifying the three gene regions are provided in Kawakita *et al.* (2004). All PCR amplifications were aided by Ex Taq DNA polymerase (TaKaRa, Otsu, Japan) and carried out on GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). PCR products were purified using NucleoSpin Extract II (Macherey-Nagel, Düren,

Table 1. Locality information and sample sizes of male, female, and larval *Epicephala* moths sequenced in this study. Locality numbers correspond to those in Fig. 1. Numbers in parenthesis for the female moth indicate sample sizes of moths whose pollination/oviposition behavior was studied in the field.

Host	Locality	Locality No.	Male	Female (field-collected)	Larva
G. acuminatum	Tatsugo, Amami Is.	7	3	5 (3)	4
	Mt. Terukubi, Okinawa Is.	11	2	0	1
G. lanceolatum	Setsuko, Amami Is.	8	1	0	0
	Cape Hedo, Okinawa Is.	10	4	0	3
	Omoto, Ishigaki Is.	15	4	17 (13)	1
	Funaura, Iriomote Is.	16	1	2 (1)	1
	Kubura, Yonaguni Is.	18	6	4 (3)	1
G. obovatum	Tomogashima Is., Wakayama	1	2	4 (2)	1
	Yura, Wakayama	2	2	0	1
	Cape Toi, Miyazaki	3	1	0	1
	Nagata, Yaku Is.	4	1	0	1
	Kasari, Amami Is.	6	1	2	0
	Mikyo, Tokuno Is.	9	1	0	0
	Higashi, Okinawa Is.	12	1	2 (2)	0
	Ara, Kume Is.	14	1	0	0
G. rubrum	Omoto, Ishigaki Is.	15	1	0	2
	Funaura, Iriomote Is.	16	1	0	2
	Agarizaki, Yonaguni Is.	17	0	0	1
	Wushe, Taiwan	19	2	0	5
	Nanren, Taiwan	20	1	1	0
G. zeylanicum	Anbo, Yaku Is.	5	1	0	0
	Kasari, Amami Is.	6	2	1 (1)	0
	Mikyo, Tokuno Is.	9	1	0	2
	Henoko, Okinawa Is.	13	1	3 (1)	0
	Omoto, Ishigaki Is.	15	1	0	0
	Funaura, Iriomote Is.	16	1	0	0

Germany). Sequencing reaction was performed using the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer), and electrophoresis was conducted on an ABI 3100 sequencer (Perkin-Elmer).

Because the electrophoretic patterns were clear and simple for the COI sequences, I only sequenced one strand for each of the amplified COI fragments. However, ArgK and EF-1α frequently contained base polymorphisms that needed verification, so I sequenced both strands for all the ArgK and EF-1α fragments.

To determine the distribution of alternate bases within alleles of heterozygous ArgK and EF-1α sequences, I further cloned ArgK and EF-1α PCR products that were found to have more than one polymorphic base. Cloning was performed using the pGEM-T Easy Vector Systems (Promega, Madison, WI) and following the manufacturer's protocol. One colony containing the target PCR product (ca. 800 and 550 bp for ArgK and EF-1α, respectively) was sampled for each heterozygote, and the forward strand was sequenced as described above. The alternate allele was obtained by subtracting the cloned sequence from the corresponding heterozygous sequence. Some cloned sequences contained bases that were not found in the original heterozygous sequences; such bases were regarded as Taq errors and ignored in subsequent analyses.

PHYLOGENETIC ANALYSIS

Sequences of COI, ArgK, and EF-1α contained no introns, and the alignment was straightforward. The obtained sequences have been deposited in the GenBank database under accession numbers DQ298833–DQ299150 and DQ452149–DQ452295. In addition to the sequence data collected in this study, COI sequences from the previous study (Kato *et al.* 2003) were retrieved from GenBank and used for the analysis (AY221964–AY221974 and AY221976–AY221979).

Phylogenetic trees were estimated for each of the three genes using parsimony and Bayesian methods. Allelic sequences of the ArgK and EF-1α genes were treated separately for phylogenetic estimation. I used PAUP* version 4.0b10 (Swofford 2002) to reconstruct most parsimonious (MP) trees by heuristic searches with 100 random addition analyses and tree bisection-reconnection (TBR) branch-

Table 2. Characters of the valva used in the morphological analysis.

Cucullus	
1	Area of dense sclerotized hairs on ventral margin: absent (0); present (1).
2	Inner wall: flat (0); projected inwardly with an area of dense sclerotized hairs (1).
3	Acutely pointed edge with a single spine on ventral margin: absent (0); present (1).
4	Sparse spines on ventral half of inner wall: absent (0); present (1).
5	Distal margin: simple (0); ventrally concave (1).
Sacculus	
6	Dorsal margin of inner wall: simple (0); reflexed (1).
7	Row of more than 15 sclerotized hairs on distal margin of reflexed lobe: absent (0); present (1).
8	Triangular scales on distal end of inner wall: absent (0); present (1).
9	Distal margin: round (0); acute (1); concave (2).
10	Sclerotized hairs lining the ventral half of inner wall: absent (0); present (1).
11	Row of 3–5 sclerotized hairs on distal margin of reflexed lobe: absent (0); present (1).
12	Spine on distal end: absent (0); present (1).
13	Area of dense sclerotized hairs on reflexed lobe: absent (0); present (1).
14	Distal margin of reflexed lobe: simple (0); dorsally concave (1).

swapping, saving no more than 100 trees per analysis. The obtained trees were used as starting trees in an additional round of heuristic search with TBR branch-swapping, saving 10,000 MP trees at maximum. Robustness of the MP trees was validated with bootstrap analysis with 1,000 replications of heuristic searches with 10 random addition analysis and TBR branch-swapping, saving no more than 100 trees per analysis. Prior to Bayesian phylogenetic estimation, I used the program MrModelTest 2.2 (written by J. A. A. Nylander: http://www.ebc.uu.se/systzoo/staff/nylander.html) to select appropriate model of base substitution for each gene partition. Based on the selected model, I performed the Bayesian analyses using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Analyses consisted of running four simultaneous chains for 2×10^6 generations, sampling trees every 1,000 generations for a total of 2,001 trees. I plotted ln-likelihood of the sampled trees against generation time to identify the region of the analysis in which the parameter estimates were stable. I discarded the burn-in region (trees and parameter estimates obtained before equilibrium; the initial 501 trees), and the remaining 1,500 samples were used to estimate the tree topology, branch lengths, and substitution parameters. To ensure that analyses were not trapped on local optima, we carried out three separate runs and compared tree topologies and parameter estimates for consistency.

I assessed conflict of phylogenetic signals among gene partitions using incongruence length difference (ILD) test (Farris *et al.* 1994), as implemented in PAUP*. The test was performed based on 1,000 replications following the same heuristic search strategies as used in the parsimony bootstrap analysis. Because the ILD test did not indicate significant conflict among gene partitions (P = 0.392), I performed parsimony and Bayesian phylogenetic analyses simultaneously for the COI, ArgK, and EF-1α data sets. In the combined data set, allelic sequences of the ArgK and EF-1α genes were combined as single heterozygous sequences with polymorphic sites coded as missing data, because multiple combinations are possible for choosing between alternate alleles of ArgK and EF-1α within a single moth individual. Parsimony and Bayesian phylogenetic estimations were performed as described above for the individual partitions. For the Bayesian analysis, separate gene partitions were assigned different substitution models and treated as unlinked so that separate parameters estimates were obtained for each partition.

Because I found two pairs of *Epicephala* lineages that are each associated with the same *Glochidion* host, I statistically tested whether each pair of lineages is monophyletic using parsimony-based, one-tailed Kishino-Hasegawa tests (Kishino & Hasegawa 1989; Goldman *et al.* 2000; Felsenstein 2004). The alternative topologies for the tests were defined by constraining individuals associated with the same host as monophyletic and performing heuristic searches as described above.

MORPHOLOGICAL ANALYSIS

To determine whether distinct moth clades identified on the phylogenetic tree are associated with morphological difference, I investigated variations in genital morphology among the male moths used in the molecular analyses (N = 42; 5–11 moths per clade). There are only subtle differences in wing pattern and female genital characters among closely related species of *Epicephala*, but the male genitalia show sufficient morphological variation that can be reliably used to discriminate species. For specimen preparation, I removed the abdomen and boiled it in 10% potassium hydroxide for 5 min to remove

Table 3. Ranges (mean in parentheses) of uncorrected pairwise distances within and between *Epicephala* clades. Data in bold indicate that there are overlaps between intra- and inter-clade distance ranges. Clade numbers correspond to those in Figs. 3 and 4.

Locus Clade	Within clade	Between Clades
COI		
Clade 1	0–0.0412 (0.0181)	0.0430–0.0979 (0.0728)
Subclade 1	**0–0.0103 (0.0047)**	**0.0344–0.0979 (0.0702)**
Subclade 2	0 (0)	0.0344–0.0911 (0.0665)
Clade 2	0–0.0034 (0.0011)	0.0430–0.0773 (0.0607)
Clade 3	0–0.0052 (0.0016)	0.0567–0.0964 (0.0741)
Clade 4	0–0.0052 (0.0015)	0.0447–0.0842 (0.0666)
Clade 5	0–0.0017 (0.0003)	0.0533–0.0979 (0.0807)
Clade 6	0 (0)	0.0533–0.0964 (0.0767)
ArgK		
Clade 1	**0–0.0304 (0.0163)**	**0.0277–0.0636 (0.0449)**
Subclade 1	**0–0.0235 (0.0129)**	**0.0138–0.0636 (0.0440)**
Subclade 2	0–0.0069 (0.0029)	0.0138–0.0526 (0.0390)
Clade 2	0–0.0083 (0.0038)	0.0429–0.0692 (0.0569)
Clade 3	0–0.0152 (0.0064)	0.0277–0.0636 (0.0443)
Clade 4	0 (0)	0.0401–0.0636 (0.0518)
Clade 5	0–0.0166 (0.0072)	0.0194–0.0692 (0.0471)
Clade 6	0–0.0028 (0.0015)	0.0194–0.0609 (0.0405)
EF-1α		
Clade 1	0–0.0123 (0.0044)	0.0164–0.0431 (0.0294)
Subclade 1	**0–0.0123 (0.0045)**	**0–0.0431 (0.0285)**
Subclade 2	**0–0.0021 (0.0005)**	**0–0.0411 (0.0228)**
Clade 2	0–0.0082 (0.0031)	0.0164–0.0493 (0.0314)
Clade 3	0–0.0123 (0.0026)	0.0226–0.0493 (0.0325)
Clade 4	0 (0)	0.0267–0.0452 (0.0326)
Clade 5	0–0.0082 (0.0023)	0.0144–0.0431 (0.0319)
Clade 6	0–0.0103 (0.0032)	0.0144–0.0493 (0.0346)

adipose tissue. I then washed it with distilled water and subsequently 100% ethanol to clean out debris. After extraction of the genitalia, the specimen was mounted in Euparal (Waldeck, Münster, Germany) on a glass slide and observed under a microscope with transillumination.

Variations in genital morphology are most obvious in the valva (a piece of paired lobes used to grasp female abdomen during copulation). In *Epicephala*, each valva consists of two bursiform lobes (the dorsal cucullus and the ventral sacculus) that are connected to each other and variably modified by dense cluster of fine hair (setae) and sclerotized hairs and/or spines (Kuznetsov 1980). I looked for morphological characters of the valva that were variable among individuals and could be coded unambiguously as discrete states. Based on 14 such characters (Table 2), I constructed a character state matrix, which I used to test for an association between genetic variation and morphological difference.

I also investigated whether the females of each species possess morphological structures specialized for active pollination. The females of actively pollinating *Epicephala* moths possess numerous sensilla on the ventral surface of the proboscis, which are not found in the males (Fig. 2c, d). These structures are also absent in the females of related moth genera and non-pollinating species of *Epicephala* (A. Kawakita & M. Kato, unpublished results), indicating that the sensilla are a novel trait in the females likely specialized for active collection and deposition of pollen. I studied the proboscis of the laboratory-reared, adult females used in the molecular analyses (N = 15) and recorded the presence/absence of proboscidial sensilla. The proboscis was removed, mounted in Euparal on a glass slide, and observed under a microscope with transillumination.

RESULTS

PHYLOGENETIC ANALYSIS

The data matrices of COI, ArgK, and EF-1α consisted of 111, 196, and 173 non-heterozygous sequences and 582, 723, and 487 nucleotide sites, respectively, of which 99, 108, and 51 were

EF-1α
G. zaylanicum
G. lanceolatum
G. acuminatum
G. obovatum & G. rubrum
C1
C2
C3
C4
C5
C6
100/100
97/59
79/50
100/86
100/98
100/99
100/100
100/82
100/95
0.005 substitutions/site
ArgK (part)
S1
S2
100/92
100/97
99/68
65/–
(continue to left)
0.01 substitutions/site
ArgK (part)
C5
C6
C3
100/99
62/–
(continue to right)
0.01 substitutions/site
COI
S1
S2
C1
C2
C3
C4
C5
C6
100/100
63/–
77/–
0.01 substitutions/site

Fig. 3 Bayesian 50% majority-rule consensus phylograms obtained by individual analyses of COI, ArgK, and EF-1α. Terminal codes indicate specimen numbers which correspond to those in the GenBank accessions. Allelic sequences of single specimens are distinguished by different alphabets (a and b) following the specimen numbers. C1–C6 and S1–S2 are clade/subclade names referred to in the text. Posterior probabilities based on 1,500 post-burn-in trees (mean likelihood scores: COI, -1894.4024; ArgK, -3391.7296; EF-1α, -1540.4514) are shown below branches (left) followed by bootstrap values of the parsimony analyses (right; shown when >50%). The values are given only for the nodes C1–C6 and S1–S2 or above, because most apical nodes on the phylogeny are not consistent among the three data sets. The trees are unrooted. Host plant name(s) is given to the right of each clade.

Table 4. Character states for the 14 valval traits examined in the morphological analysis. Because all individuals belonging to the same clade had identical character states, data are summarized for each clade. Clade numbers correspond to those in Figs. 3 and 4. Dashes indicate that the states could not be inferred due to absence of particular structures.

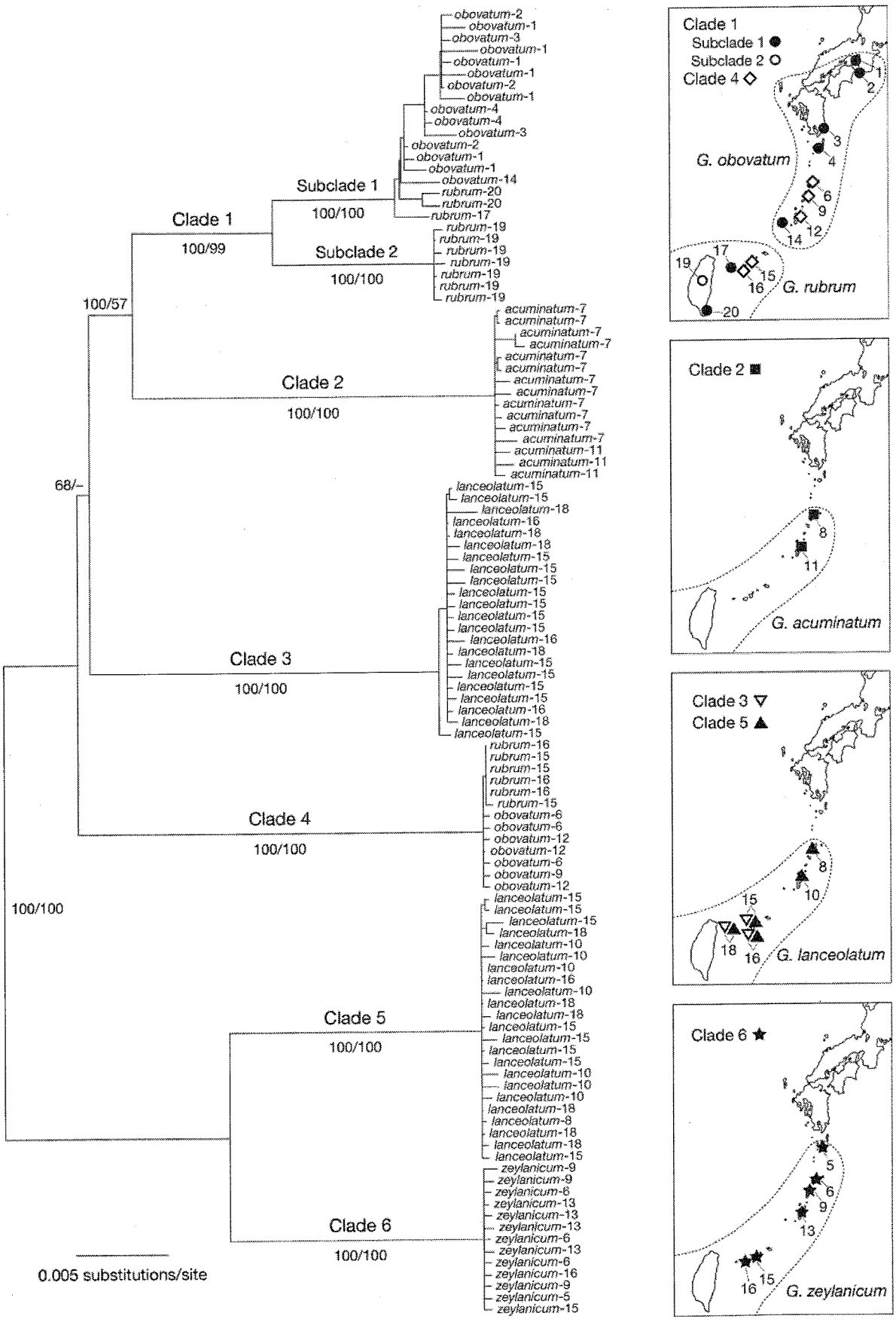
	Moths	Character No.													
Clade	examined	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Subclade 1	8	1	0	0	0	0	1	1	1	1	0	0	0	0	0
Subclade 2	8	1	0	0	0	0	1	1	1	1	0	0	0	0	0
Clade 2	5	0	0	0	0	0	1	0	1	1	0	0	0	0	0
Clade 3	5	0	1	0	0	0	0	–	0	0	1	–	0	–	–
Clade 4	5	0	0	1	0	0	1	0	0	2	0	1	1	0	0
Clade 5	11	0	0	0	1	0	1	0	0	1	0	0	0	1	1
Clade 6	7	0	0	0	1	1	1	0	0	0	0	0	0	1	0

Table 5. Results of behavioral and morphological analyses of female *Epicephala* moths. Clade numbers correspond to those in Figs. 3 and 4.

	Active pollination					Proboscidial sensilla	
	Moths	Pollination	Pollen grains			Moths	Sensilla
	examined	behavior	1–100	100–200	>200	Examined	present
Subclade 1	2	2			2	3	3
Subclade 2	0	–				0	–
Clade 2	3	3			3	2	2
Clade 3	10	10		1	9	4	4
Clade 4	2	2		1	1	2	2
Clade 5	7	7		2	5	2	2
Clade 6	2	2			2	2	2

parsimony-informative. 77 and 56% of the individuals analyzed were heterozygous at the ArgK and EF-1α loci, respectively, and each sequence had 4.07 (range, 0–14) and 1.08 (0–5) average polymorphic sites for each locus. Parsimony analysis of the COI and EF-1α data sets resulted in one and 96 MP trees, respectively, whereas the ArgK data set produced the maximum number of 10,000 MP trees (COI, 151 steps, consistency index [CI] = 0.755, retention index [RI] = 0.987; ArgK, 315 steps, CI = 0.474, RI = 0.957; EF-1α, 89 steps, CI = 0.679, RI = 0.981). Bayesian analyses of individual data sets produced trees that are largely similar with the MP trees, with all topological differences limited to nodes that are weakly supported (<70% parsimony bootstrap and <90% Bayesian posterior probability values); thus only the Bayesian trees are presented with parsimony bootstrap values included for shared branches (Fig. 3). The combined data set produced 10,000 MP trees (356 steps, CI = 0.745, RI = 0.984) and Bayesian trees with overall similar topologies with the individual-partition analyses (Fig. 4). All the data sets clearly recovered six distinct clades each consisting of the same set of individuals (clades C1–C6 in Figs. 3, 4). Because moths belonging to different clades occur in sympatry (Figs. 1, 4), these results strongly indicate reproductive isolation among the six clades. The COI and ArgK data sets further indicated that moths associated with *G. rubrum* in one population (Wushe, Taiwan) are genetically distinct from other individuals of the same clade (subclades S1 and S2 in Figs. 3, 4) with moderate to strong support (68–100% parsimony bootstrap and 99–100% Bayesian posterior probability values). However, monophyly of S1 and S2 was neither supported nor falsified in the EF-1α phylogeny (Fig. 3). Intra- and inter-clade comparisons of pairwise sequence differences are summarized in Table 3. Although the clades C2–C6 were genetically distinct, there were little overlaps between intra- and inter-clade distance ranges in C1, S1, and S2 for some of the analyzed loci.

Moths associated with *G. acuminatum* and *G. zeylanicum* each formed a well-defined monophyletic group, suggesting plant–pollinator specificity in these two species at least within my sampling range (Figs. 3, 4). On the other hand, moths collected from *G. lanceolatum* separated into two distinct lineages (C3


obovatum-2
obovatum-1
obovatum-3
obovatum-1
obovatum-1
obovatum-1
obovatum-2
obovatum-1
obovatum-4
obovatum-4
obovatum-3
obovatum-2
obovatum-1
obovatum-1
obovatum-14
rubrum-20
rubrum-20
rubrum-17
rubrum-19
rubrum-19
rubrum-19
rubrum-19
rubrum-19
rubrum-19
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-7
acuminatum-11
acuminatum-11
acuminatum-11
lanceolatum-15
lanceolatum-15
lanceolatum-18
lanceolatum-16
lanceolatum-18
lanceolatum-18
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-16
lanceolatum-18
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-16
lanceolatum-18
lanceolatum-15
rubrum-16
rubrum-15
rubrum-15
rubrum-16
rubrum-16
rubrum-15
obovatum-6
obovatum-6
obovatum-12
obovatum-12
obovatum-6
obovatum-9
obovatum-12
lanceolatum-15
lanceolatum-15
lanceolatum-15
lanceolatum-18
lanceolatum-10
lanceolatum-10
lanceolatum-10
lanceolatum-16
lanceolatum-10
lanceolatum-18
lanceolatum-18
lanceolatum-15
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lanceolatum-15
lanceolatum-15
lanceolatum-10
lanceolatum-10
lanceolatum-10
lanceolatum-18
lanceolatum-8
lanceolatum-18
lanceolatum-18
lanceolatum-15
zeylanicum-9
zeylanicum-9
zeylanicum-6
zeylanicum-13
zeylanicum-13
zeylanicum-13
zeylanicum-6
zeylanicum-13
zeylanicum-6
zeylanicum-16
zeylanicum-9
zeylanicum-5
zeylanicum-15
Subclade 1
100/100
Clade 1
100/99
Subclade 2
100/100
100/57
Clade 2
100/100
68/–
Clade 3
100/100
Clade 4
100/100
100/100
Clade 5
100/100
Clade 6
100/100
0.005 substitutions/site
Clade 1
Subclade 1
Subclade 2
Clade 4
G. obovatum
G. rubrum
Clade 2
G. acuminatum
Clade 3
Clade 5
G. lanceolatum
Clade 6
G. zeylanicum

Fig. 4 Bayesian 50% majority-rule consensus phylogram obtained by simultaneous analysis of COI, ArgK, and EF-1α. Each specimen is designated by its host species name followed by locality number as described in Fig. 1 and Table 1. Numbers below branches indicate Bayesian posterior probabilities based on 1,500 post-burn-in trees (mean likelihood score, -4839.2639) followed by parsimony bootstrap values (shown when >50%). The tree is unrooted. Geographic distribution of each clade/subclade is also shown on the map along with range of the host plant(s). The numbers on the map also refer to the locality numbers.

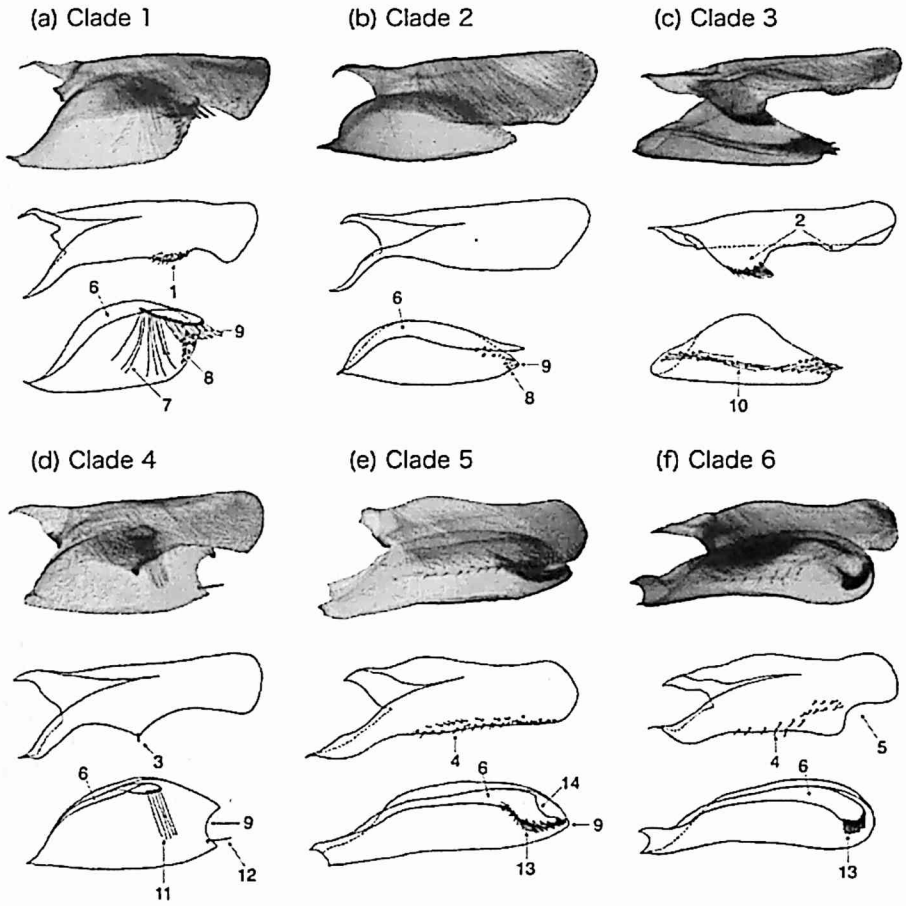


Fig. 5 Right valvae of male genitalia of the six *Epicephala* species identified in this study. (a)–(f) illustrate valvae of representative individuals of clade C1–C6, respectively. Clade numbers correspond to those in Figs. 3 and 4. The valva of *Epicephala* is composed of two lobes (upper cucullus and lower sacculus), which are separately drawn for each specimen. Valval traits used in the morphological analysis are indicated (the numbers correspond to those in Tables 2 and 4).

and C5; Figs. 3, 4). Multiple individuals collected on the same *G. lanceolatum* plant (three plants in two populations) also grouped separately, indicating coexistence of the two lineages on the same *G. lanceolatum* plants. Also, moths sampled from two parapatric hosts, *G. obovatum* and *G. rubrum*, formed two distinct, allopatric clades (C1 and C4; Figs. 3, 4) whose boundary does not correspond with that of their hosts. One of the lineages is confined to the southern region of the Ryukyu Archipelago in Japan, whereas the other was found in mainland Japan, Taiwan, and three small islands in southern Japan (Yaku, Kume, and Yonaguni Is.; Figs. 1, 4).

One-tailed Kishino-Hasegawa test rejected the hypothesis of monophyly of the two clades associated with *G. lanceolatum* (COI, $P = 0.004$; ArgK, $P = 0.085$; EF-1α, $P = 0.048$; Combined, $P < 0.001$), except for the ArgK data set which was marginally insignificant. The non-monophyly of the two lineages indicates that at least one of the *Epicephala* lineages had colonized a novel *Glochidion* host. However, monophyly was not rejected for the two clades associated with *G. obovatum* and *G. rubrum* (COI, $P = 0.159$; ArgK, $P = 0.342$; EF-1α, $P = 0.159$; Combined, $P = 0.129$), thus host shift is not required for explaining the current pattern of associations in these lineages.

MORPHOLOGICAL AND BEHAVIORAL ANALYSIS

Results of the male genital morphological analysis are summarized in Table 4, and variations in valval characters are illustrated in Fig. 5. Overall, individuals belonging to the same clade had identical states for the 14 characters examined, whereas those belonging to different clades could be distinguished by 2–7 characters (Table 4). Moths that grouped into two subclades within clade C1 (S1 and S2; Figs. 3, 4) had identical character states, indicating that the two lineages can not be discriminated morphologically at least based on the characters we used in the analysis.

All the female moths studied in the field exhibited the stereotypic pollination/oviposition behavior on the host female flowers and had more than 100 pollen grains on their proboscises, indicating that all the individuals are pollinators (Table 5). Each of the six clades on the phylogeny includes 2–10 of these moths,

suggesting that all six lineages possess the pollinating habit. Morphological examination of the laboratory-reared females (2–4 females per clade) suggested that they all have sensilla on proboscises that are associated with the active pollination behavior (Table 5).

Field observations of pollinating females further indicated that there is a behavioral difference between the two lineages associated with *G. lanceolatum*. The females of one lineage (C5; $N = 7$) inserted their ovipositor from the apical pit of the female flower and deposited the eggs in the stylar tissue, whereas the females of the other lineage (C3; $N = 10$) oviposited through the calyx lobe and lateral ovary wall of the female flower and placed the eggs within the interspace between the ovary wall and ovules (Fig. 2e, f). Moths belonging to the two clades were found pollinating the same tree, further supporting the co-occurrence of the two lineages on the same *G. lanceolatum* host.

DISCUSSION

The results of my molecular, morphological, and behavioral analyses clearly indicate that there are six well-defined pollinator *Epicephala* lineages that are associated with the five *Glochidion* species in Japan and Taiwan. The mitochondrial COI and two independent nuclear ArgK and EF-1α genes all grouped the same sets of individuals as distinct lineages, suggesting that there is no or, if any, extremely low level of gene flow between these groups. Although there was a little overlap between intra- and inter-clade distance ranges in C1 at the ArgK locus (Table 3), monophyly of this clade was strongly supported in the ArgK phylogeny (92% parsimony bootstrap and 100% Bayesian posterior probability values; Fig. 3). At the ArgK and EF-1α loci, 77 and 56% of the individuals were found to be heterozygous, respectively, but the two allelic sequences of each individual fell into the same clade, indicating that allelic differences are intraspecific genetic variations and not the result of interspecific hybridizations. Analysis of the male genitalia further revealed that the six lineages are morphologically distinct; moths belonging to the same lineage had identical states for the 14 valval characters that I studied, whereas those belonging to different lineages can be distinguished by 2–7 characters (Table 4; Fig. 5). Taken together, these results indicate that each of the six *Epicephala* lineages identified in this study represents a biological species. Sequence variation within some of the species was markedly lower in COI than ArgK and EF-1α (Table 3; Fig. 3), which contrasts with the generally higher rate of sequence evolution in mitochondrial than nuclear genes in insects (Lin & Danforth 2004). For example, mean intraspecific sequence difference in C5 was 24.0 and 7.7 times higher in ArgK and EF-1α than COI, respectively. One possible explanation is that such species have expanded their distribution relatively recently and rapidly, because the biparentally inherited nuclear alleles have four times the effective population size as the maternally inherited mitochondrial alleles (Birky *et al.* 1983, 1989; McCauley 1994; Levy & Neal 1999), and thus are expected to possess greater amount of ancestral polymorphisms.

Analyses of COI and ArgK further recovered two subclades within clade C1 (subclades S1 and S2). This pattern was not evident in the EF-1α phylogeny, which is likely due to limited sequence variation within the EF-1α loci. In contrast, the analysis of male genitalia indicated that individuals from the two subclades were indistinguishable based on the characters we studied. Given the allopatric distribution of S1 and S2 and tentative lack of morphological difference, available data are insufficient to evaluate specific status of the two subclades. S2 is restricted to a single isolated population in Wushe, Taiwan, which is located at 1,400 m a.s.l. Although the level of genetic divergence among *G. rubrum* populations still requires investigation, preliminary observation of morphology indicate that the plants of the Wushe population have distinctly shorter pedicels and larger fruits compared to the lowland populations in Taiwan and southern Japan (A. Kawakita, unpublished results). A further detailed genetic study is needed to determine whether or not this represents an incipient stage of simultaneous speciation in the plant and pollinator. Possible examples of ongoing parallel plant–pollinator speciation are known in the yucca–yucca moth (Pellmyr & Segraves 2004) and fig–fig wasp (Yokoyama 2003) mutualisms.

My results indicated that the southernmost populations of *G. lanceolatum* in Japan have two species of *Epicephala* pollinator that are genetically, morphologically, and behaviorally distinct (Figs. 2–5). The occurrence of the two species on the same *G. lanceolatum* plants further indicated the absence of cryptic plant divergence associated with the two pollinator species. Statistical analysis showed that these two species are not sister taxa, suggesting that a host shift has occurred at least once in the *Glochidion–Epicephala* association. These results provide a comprehensive explanation for the previously reported mismatch between *Glochidion* and *Epicephala* phylogenies (Kawakita *et al.* 2004), which parallels earlier cases in the fig–fig wasp (Molbo *et al.* 2003), ant–myrmecophyte (Fiala *et al.* 1999; Feldhaar *et al.* 2003), and coral–zooxanthella mutualisms (Rowan & Knowlton 1995; Baker 2003). Pollinator host shifts are difficult to interpret under the assumption of one pollinator per one host,

because host colonization inevitably leads to co-occurrence of two species on the new host (Kawakita *et al.* 2004). Theories suggest that mutualist coexistence following colonization event can give rise to transition from mutualism to parasitism (Maynard Smith & Szathmary 1995; Pellmyr *et al.* 1996; Herre 1999; Herre *et al.* 1999; Yu 2001). However, females of both species pollinated *G. laceolatum* in the field and had proboscidial sensilla that are associated with active pollination (Table 5), indicating that neither of the species has become a non-mutualistic cheater that exploits the pollination benefit by the other species. It should be noted that my results do not necessarily provide evidence against occurrence of cheaters among other *Epicephala* species that are associated with the >300 species of *Glochidion.* Whether the coexistence of two pollinators on a shared host is evolutionary stable is unknown, because the age at which the two species started to coexist can not be inferred from available data. Nevertheless, the prevalence of similar situations in many mutualisms (Fiala *et al.* 1999; Pellmyr 1989, 1999; Baker 2003; Molbo *et al.* 2003) may suggest that long-term coexistence on a shared host can occur.

In contrast, the two species associated with *G. obovatum* and *G. rubrum* (C1 and C4) were not found within the same population (Fig. 4). Although samples are limited for some populations, available data indicate allopatric distribution for these two species. The disjunct distribution of C1 in mainland Japan, Taiwan, and a few small islands (Fig. 4), coupled with relatively high level of intraspecific sequence variation (Table 3), may indicate ancient widespread distribution of C1 and subsequent extinction through competitive exclusion in most of the Ryukyu Archipelago. An alternative, but less likely explanation, is that C1 has expanded its range more recently by long-distance dispersal. In either case, these two species provide an additional opportunity to study the factors that may or may not lead to stable coexistence of two pollinators on a shared host.

Overall, the complex plant–pollinator association found in this study is similar to the situations found in other obligate pollination mutualisms. Yuccas are commonly pollinated by multiple, phylogenetically distant yucca moth species, either in sympatry or allopatry, and many pollinator moth species interact with two or more yuccas throughout their range (Pellmyr 1999; Pellmyr & Balcázar-Lara 2000; Pellmyr & Segraves 2003). Although more detailed studies are needed in the fig–fig wasp mutualism, the recent discoveries of cryptic wasp species in neotropical figs (Molbo *et al.* 2003; Machado *et al.* 2005), coupled with numerous similar observations in different biogeographic regions (Wiebes 1979; Compton 1990; Michaloud *et al.* 1996; Rasplus 1996; Kerdelhue *et al.* 1999; Lopez-Vaamonde *et al.* 2002; Cook & Rasplus 2003), likely indicate the general lack of the previously assumed one-to-one specificity between the figs and wasps. Furthermore, these complex associations parallel several recent findings in other mutualistic systems in which molecular techniques have revealed unsuspected diversity of associations between the partners (Sanders *et al.* 1996; Baker 2003; Feldhaar *et al.* 2003; Rasolomampianina *et al.* 2005). Thus, the pattern of plant–pollinator association found in the present study is characteristic of many mutualisms and contributes to a more refined understanding of how the partners are assembled and structured in mutualistic associations.

Appreciation of the complex plant–pollinator association has important consequences for the studies of coevolution and codiversification in these mutualisms. Because some *Epicephala* species are associated with more than one host (C1 and C4, although their hosts do not occur together; Fig. 4), it is possible that pollinator sharing between *Glochidion* species leads to hybridization or genetic introgression between the two plants. Also, occasional host shifts by the pollinator, as suggested in the present study, can also result in genetic mixing between the previous and novel hosts. Machado *et al.* (2005) studied the genetic structure of 17 species of closely related figs and their associated pollinators and found that shared use of host figs and colonization of novel hosts by the wasps may result in hybridization and genetic introgression across different fig species. Although the extent to which *Glochidion* trees hybridize is unknown, adult trees with hybrid characteristics have been observed in several populations (A. Kawakita, unpublished results), suggesting the need for further investigation of population genetic structure in *Glochidon.* Improved knowledge of plant–pollinator association patterns and their consequences on the genetic structure of host plant populations is critical for understanding the processes by which the two groups coevolve and codiversify (Herre *et al.* 1999; Thompson 2005). Future studies should ideally focus on larger sets of associated *Glochidion* and *Epicephala* species that are reasonably closely related, because the probability of pollinator sharing and genetic mixing in the hosts is expected to be higher among species that have diverged more recently (Machado *et al.* 2005).

Although the present study provided evidence for the complex association in the *Glochidion–Epicephala* mutualism, my sampling range was restricted to the northernmost edge of the distribution of the genus *Glochidion,* which comprises >300 species centered in the Indo-

Australian tropics (Govaerts *et al.* 2000). Four of the *Glochidion* species studied here are more or less widespread throughout tropical Asia and thus may have even more complex partner relationships with *Epicephala* pollinators. At the same time, distant populations of *Glochidion* may be exposed to different selection exerted by different sets of *Epicephala* species, creating a complex mosaic of genetic structure in host *Glochidion* populations. Thus, future progress in ecological and evolutionary studies of these mutualisms depends fundamentally on improved understanding of the diversity of each partner and their associations at both local and global geographic scales.

Chapter 4

Evolution of obligate pollination mutualism in New Caledonian *Phyllanthus* (Phyllanthaceae)

INTRODUCTION

Obligate, seed-parasitic pollination mutualisms have arisen repeatedly during the history of terrestrial ecosystems. Currently recognized examples of such interactions involve plant and insect groups of various lineages (Janzen 1979; Pellmyr 1989; Thompson and Pellmyr 1992; Pellmyr et al. 1996; Fleming and Holland 1998; Weiblen, 2002; Kato et al. 2003; Pellmyr 2003). These mutualisms range from highly coevolved (Janzen 1979; Weiblen 2002; Kato et al. 2003; Pellmyr 2003) to less specialized interactions (Pellmyr 1989; Thompson and Pellmyr 1992; Pellmyr et al. 1996; Fleming and Holland 1998), but the underlying principle is the same: pollination is accompanied by oviposition in flowers, and the larvae consume only a fraction of the seeds within a resulting fruit. In figs, there is only a single ovule per fruit, but the fig can be considered as an aggregate fruit containing many seeds, some of which are consumed by the pollinator larvae. The special case involves the functionally dioecious figs, in which the pollinator wasps occupy nearly all of the ovules within functionally male syconia (Galil 1973; Janzen 1979; Weiblen 2002).

Given the described principle in most obligate pollination interactions, it has been assumed that excessive exploitation of seeds by pollinators would confer a substantial cost to plants and would subsequently lead to a collapse of the mutualistic relationship (Bull and Rice 1991; Herre et al. 1999; Bronstein 2001; Holland and DeAngelis 2001). In this paper, we describe an obligate, seed-parasitic pollination mutualism in which a single larva of a pollinator moth consumes all seeds of the host fruit. This system, which involves New Caledonian *Phyllanthus* (subgenus *Gomphidium*) trees and gracillariid *Epicephala* moths, resembles the closely related *Glochidion–Epicephala* mutualism in terms of overall net outcome (Kato et al. 2003), but differs strikingly in the modes of interaction between the mutualists. These differences would allow for explicit comparative analyses on various aspects of interspecific mutualism and make these associations an important model system for general studies of coevolution.

Phyllanthus is a cosmopolitan genus of monoecious trees or herbs comprising more than 800 species (Govaerts et al. 2000). Although regarded as a nonmonophyletic group (Webster, 1994), it is the third largest genus of the family Euphorbiaceae (Govaerts et al. 2000). Among the 10 subgenera currently recognized, *Gomphidium* is a group of small trees comprising about 150 species restricted to Australia, New Guinea, and Polynesia (Holm-Nielsen 1979). Notably, this subgenus has undergone extensive diversification in New Caledonia (115 species) and now constitutes the largest genus on the islands (Schmid 1991). Most trees in this genus have a narrow distribution and use diverse habitats, ranging from rainforests to dry sclerophyllus scrubs, from calcareous to serpentinous soils, and from mangroves to high mountains. In New Caledonia, the subgenus is further divided into two sections, *Gomphidium* and *Adenoglochidion*; the former is distinguished from the latter by folded calyx lobes in the male flowers (Fig. 1). My analyses reveal that at least one species representing each section is actively and exclusively pollinated by host-specific seed-parasitic moths. Additional evidence of moth associations in other species and the overall similarity of the highly specialized flowers within the group further suggest that this mutualism can potentially be generalized to most, if not all, species of the subgenus *Gomphidium*.

MATERIALS AND METHODS

I studied the pollination biology of 25 *Phyllanthus* species at various localities in New Caledonia during 29–31 August 2001, 7–13 September 2001, 19–29 March 2002, and 29 October–8 November 2002. Insect flower visitors were observed for two *Phyllanthus* species: *P. bourgeoisii* at Chutes de Ba (21°3'S, 164°7'E) during 31 October–2 November 2002 and *P. aeneus* at Cap Bocage (21°2'S, 164°6'E) during 27–29 March 2002. *Phyllanthus bourgeoisii* is a common rheophyte on rocky riverbanks, and *P. aeneus* is a shrub that is typical of serpentine scrub habitats (Figs. 2, 3). *Phyllanthus aeneus* has open male flowers, whereas those of *P. bourgeoisii* have folded calyx lobes, which make the anthers inaccessible to facultative flower visitors (Fig. 1). Female flowers of both species are much reduced and consist of short, fused styles that are mostly covered with calyx lobes (Fig. 1). The flowers have three locules, each containing two ovules. The two species flowered and fruited throughout the period of our study, which suggests an extended duration of flower and fruit production that is typical in *Glochidion* and other closely related genera (A. Kawakita and M. Kato, unpublished data).

For each species, I spent a total of more than 30 h for diurnal and nocturnal observation of flower

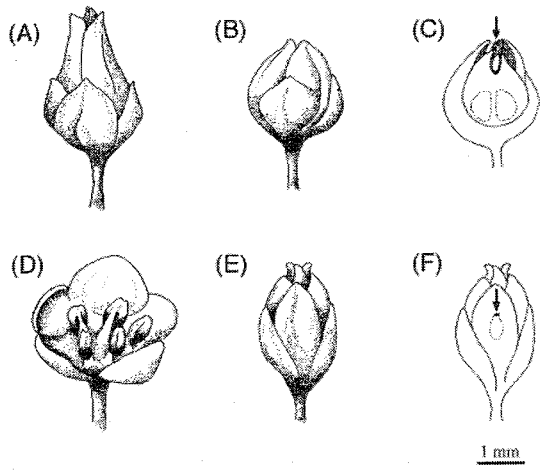


Fig. 1. Flowers of *Phyllanthus bourgeoisii* (A–C) and *P. aeneus* (D–F). (A) Male flower. (B) Female flower. (C) Longitudinal section of a female flower. The arrow indicates the location of an *Epicephala* moth egg. (D) Male flower. (E, F) Female flowers. *Epicephala* eggs are laid within the tissue of the calyx lobes (arrow).

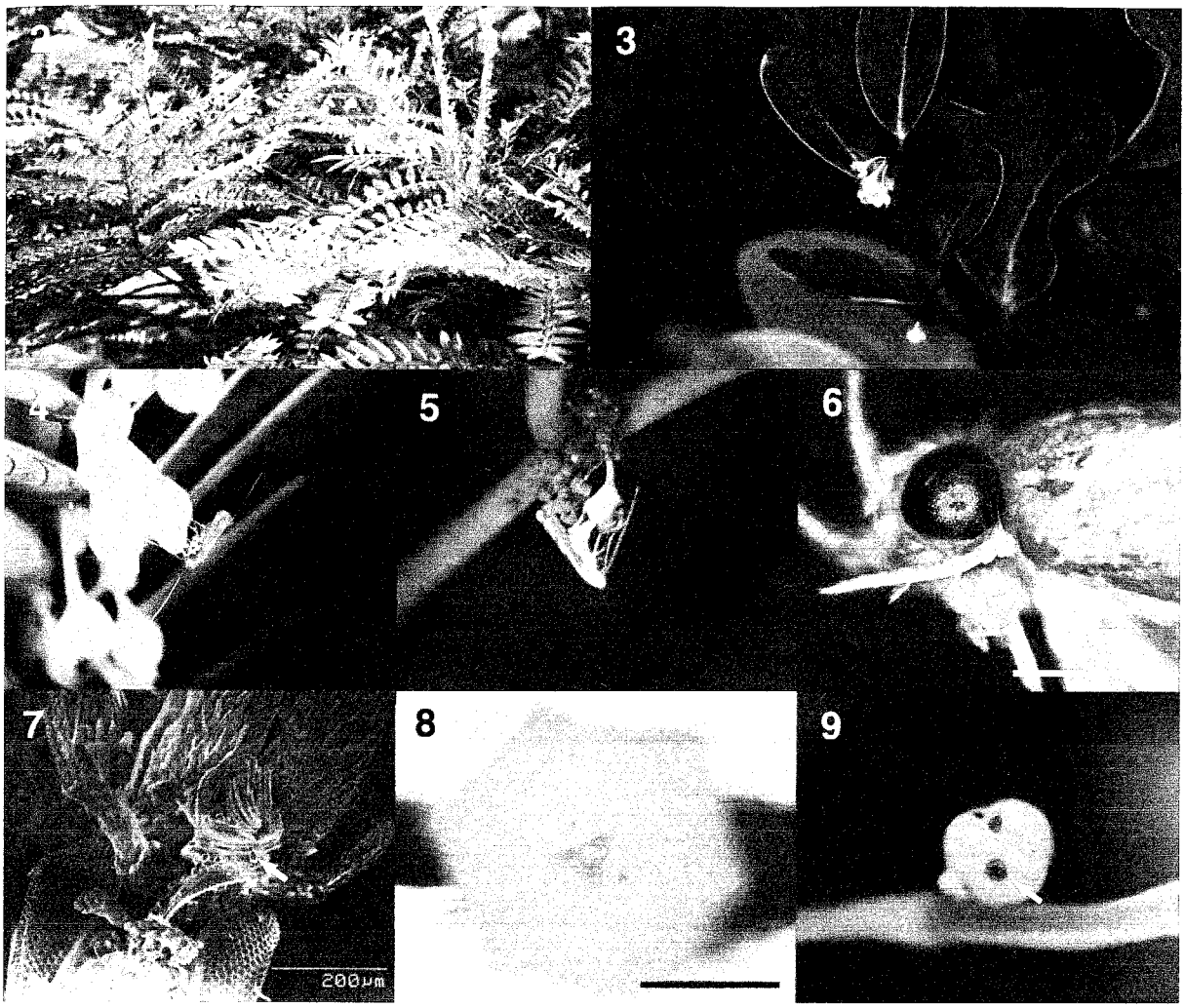
visitors. Particular effort was made so that my observation covers a wide range of the 24 h period. After field observations, I collected female flowers and mature fruits of the two species to assess the state of pollination and *Epicephala* moth oviposition and extent of seed infestation by the moth larvae. In the laboratory, I dissected the flowers using a light microscope and looked for the presence or absence of pollen grains on stigmas and *Epicephala* moth eggs in flowers. In the same way, I recorded the number of intact seeds within each mature fruit and assessed the cause of seed destruction for each fruit. Seed destruction was either caused by mature *Epicephala* larvae or by immature larvae that were parasitized by braconid wasps. For fruits from which moths/wasps had already emerged, I assessed the cause of seed destruction based on differences in exit-hole structure. In addition to the two species that I studied intensively, I also sampled female flowers of 10 *Phyllanthus* species and mature fruits of 23 species in order to infer the occurrence of moth oviposition in pollinated flowers and seed destruction by moth larvae in these plants. A list of species sampled and sample sizes of flowers and fruits examined are provided in Table 1.

To determine the extent to which the moths associated with different *Phyllanthus* hosts are genetically related, I analyzed nucleotide sequence variation within and among moth individuals reared from fruits of different *Phyllanthus* hosts: *P. bourgeoisii* (N = 7), *P. aeneus* (N = 5), *P. chamaecerasus* (N = 3), *P. tiebaghiensis* (N = 3), *P. guillauminii* (N = 5), and *P. mangenotii* (N = 2). Fruits used for rearing were collected from as many plant individuals as possible for each species in order to minimize the possibility that different moths shared the same mother (fruits were collected from the same plants used for examining seed infestation; see Table 1 for the number of individuals sampled). I extracted genomic DNA from ethanol-preserved larvae or adult moths reared from fruits of each species using DNeasy Tissue Kit (Qiagen, Valencia, CA). For each individual moth, I amplified c. 1.4-kb fragment of the mitochondrial cytochrome oxidase subunit 1 gene (COI) using polymerase chain reaction (PCR) and primers described by Kato et al. (2003). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen). The dye terminator cycle sequencing reaction was performed with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA) with the PCR primers and additional sequencing primers described by Kato et al. (2003) and electrophoresed on an ABI 3100 sequencer (Perkin-Elmer). Alignment of sequences was straightforward and required no gaps. Using PAUP* version 4.0b10 (Swofford, 2002), I calculated uncorrected pairwise distances within 1317 bp of the COI gene and estimated relative branch lengths using the neighbor-joining method. All sequences obtained in this study have been deposited in GenBank under accession numbers AY269392-AY269416.

RESULTS

During my field observation, *Epicephala* moths were the only visitors to *Phyllanthus* flowers. In the evening, females of undescribed *Epicephala* species used their proboscises to collect pollen from male *Phyllanthus* flowers (Fig. 4). I observed four visits by *Epicephala* moths to male flowers of *P. bourgeoisii* and one visit to male flowers of *P. aeneus*. On separate occasions, I recorded two visits by female *Epicephala* moths to female flowers of *P. bourgeoisii* and two visits to those of *P. aeneus*. All of these moths deliberately deposited pollen on the stigma with their proboscises and subsequently laid an egg (Fig. 5). Flower-visiting females consistently carried numerous pollen grains on their proboscises (Figs. 6, 7), and their behavior on flowers was similar to that observed in *Glochidion*-pollinating *Epicephala* moths (Kato et al., 2003). The low number of observations reflects the extreme difficulty of encountering moth visits even during the peak flowering period, which was also the case in *Glochidion*-pollinating *Epicephala* (A. Kawakita and M. Kato, personal observations).

In both *Phyllanthus* species, pollen grains were deposited on the inner surface of the fused styles (Fig. 8), which likely did not occur through passive pollination. In *P. bourgeoisii*, moth eggs were laid into the narrow pit of the style apex (Fig. 1), whereas in *P. aeneus*, eggs were laid directly into the tissue of



Figs. 2–9. Flowers and pollinators of *Phyllanthus bourgeoisii* and *P. aeneus*. **2.** Overview of *P. bourgeoisii*. **3.** Overview of *P. aeneus*. **4.** Female *Epicephala* moth collecting pollen from a male flower of *P. bourgeoisii*. **5.** Female *Epicephala* moth ovipositing in a female flower of *P. aeneus*. **6.** Lateral view of a female *Epicephala* moth collected on *P. aeneus* showing its pollen-coated proboscis. Bar = 1 mm. **7.** Scanning electron micrograph of a female *Epicephala* moth collected on *P. bourgeoisii*. The proboscis is dusted with pollen grains (indicated with an arrow). **8.** Apical view of a *P. bourgeoisii* female flower. Pollen grains are deposited on the inner surface of the fused styles enclosed by the calyx lobes. Bar = 1 mm. **9.** A *P. aeneus* fruit damaged by an *Epicephala* moth larva. The arrow indicates the exit hole.

the calyx lobes (Fig. 1). Surprisingly, not all pollinated flowers contained eggs; of 81 pollinated female flowers of *P. bourgeoisii*, only 49% had eggs, while of 45 pollinated *P. aeneus* flowers, 69% contained eggs. *Phyllanthus bourgeoisii* flowers used for oviposition invariably had one egg per flower, whereas 25% and 8% of infested *P. aeneus* flowers contained two and three eggs, respectively. Unpollinated flowers did not contain moth eggs (N = 32 and 15 for *P. bourgeoisii* and *P. aeneus*, respectively).

Of 136 mature fruits of *P. bourgeoisii*, 28% were infested by *Epicephala* larvae, and of 42 mature *P. aeneus* fruits, 40% were attacked (Fig. 10). Each larva consumed all six ovules to complete larval growth and emerged from the fruit to pupate on the host leaves or in litter (Fig. 9). In *P. bourgeoisii*, 58% of *Epicephala* larvae were parasitized by a braconid wasp species. These parasitoids had a significant positive effect on seed set by preventing further seed consumption by the moth larvae (Fig. 10).

Upon examining additional *Gomphidium* species, I found that *Epicephala* larvae infested the fruits of 20 of 25 species (Table 1) and that the seeds within these infested fruits were entirely destroyed. Of the 12 species from which I sampled female flowers, 11 contained moth eggs within pollinated flowers in proportions ranging from 25% (*P. poumensis*) to 95% (*P. buxoides*) (Table 1). In all cases, eggs were laid on the external surface of flowers, and thus oviposition by adult moths did not damage the ovary. These data indicate that the *Gomphidium–Epicephala* association is fairly widespread among other members of this subgenus.

I also obtained adult moths reared from the fruits of nine *Gomphidium* species, *P. bourgeoisii*, *P. aeneus*, *P. mangenotii*, *P. guillauminii*, *P. chamaecerasus*, *P. koniamboensis*, *P. pilifer*, *P.*

Table 1. A list of *Phyllanthus* species examined in the present study with information on locality and date of collection, presence/absence of *Epicephala* moths associated with fruits, and intensity of moth oviposition and fruit infestation. Intensity of fruit infestation for species with asterisks could not be determined due to degradation of the plant materials.

Genus Subgenus Section Species	Number of pollinated flowers examined (No. of individuals)	Proportion of pollinated flowers with moth eggs (%)	Number of fruits sampled (No. of individuals)	Presence (+) /absence (-) of infested fruits	Proportion of fruits infested by moths (%)	Locality	Date of collection
Phyllanthus							
Gomphidium							
Gomphidium							
bourgeoisii	81 (6)	49	136 (6)	+	28	Chutes de Ba	2 Nov. 2002
balansaenus	45 (6)	89	20 (6)	+	30	La Coulée	29 Oct. 2002
buxoides	38 (4)	95	24 (4)	+	54	Pouembout	2 Nov. 2002
faguetti	10 (2)	40	20 (2)	-	0	Poindimié	3 Nov. 2002
koumacensis	7 (1)	0	11 (1)	+	18	Koumac	4 Nov. 2002
mangenotii	13 (3)	46	21 (3)	+	43	Cap Bocage	2 Nov. 2002
pancherianus	11 (2)	45	12 (3)	+	33	Poro	1 Nov. 2002
poumensis	103 (11)	25	78 (15)	+	14	Paagouméne	4 Nov. 2002
tenuipedicellatus	40 (5)	58	37 (7)	+	14	Cap Bocage	2 Nov. 2002
tiebaghiensis	29 (4)	31	30 (4)	+	27	Tiébaghi	5 Nov. 2002
*caudatus**	—	—	13 (3)	+	—	Riviere Bleue	20 Mar. 2002
chamaecerasus	—	—	28 (2)	+	25	Chutes de Ba	2 Nov. 2002
*koghiensis**	—	—	15 (2)	+	—	Mont Koghi	6 Nov. 2002
*koniamboensis**	—	—	13 (3)	+	—	Tinip	23 Mar. 2002
*pilifer**	—	—	24 (2)	+	—	Voh	24 Mar. 2002
mouensis	—	—	19 (2)	-	0	Mont Mou	21 Mar. 2002
Adenoglochigion							
aeneus	45 (4)	69	42 (5)	+	40	Cap Bocage	2 Nov. 2002
montrozieri	8 (1)	38	16 (1)	+	13	St. Louis	6 Nov. 2002
gneissicus	—	—	33 (6)	+	18	Mont Panié	26 Mar. 2002
guillauminii	—	—	31 (6)	+	29	Tiébaghi	5 Nov. 2002
*peltatus**	—	—	18 (3)	+	—	Paagouméne	4 Nov. 2002
*vulcani**	—	—	64 (6)	+	—	Riviere Bleue	29 Oct. 2002
dorotheae	—	—	52 (12)	-	0	Plateau de Dogny	22 Mar. 2002
francii	—	—	37 (10)	-	0	Riviere Bleue	29 Oct. 2002
valeriae	—	—	15 (6)	-	0	Mont Panié	26 Mar. 2002

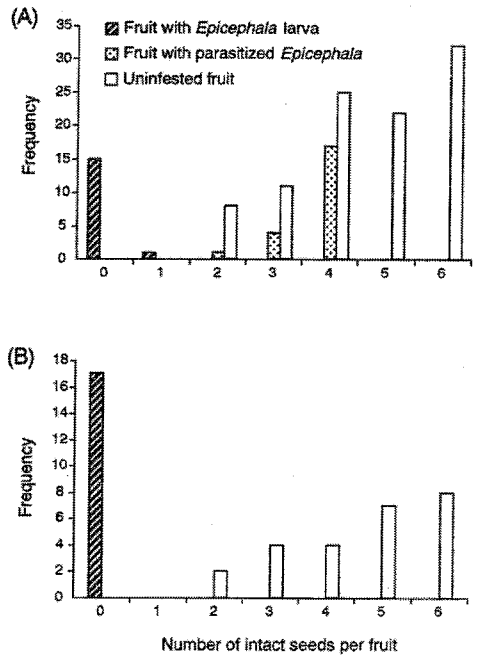


Fig. 10. Frequency distribution of the number of intact seeds per fruit. (A) *Phyllanthus bourgeoisii*. (B) *P. aeneus*. *Epicephala* larvae were parasitized by braconid wasps. Fruits from which moths/wasps had already emerged were assigned to each category based on differences in exit-hole structure. The number of intact seeds within uninfested fruits ranged from two to six due to the presence of unfertilized/aborted ovules and/or empty, sterile seeds.

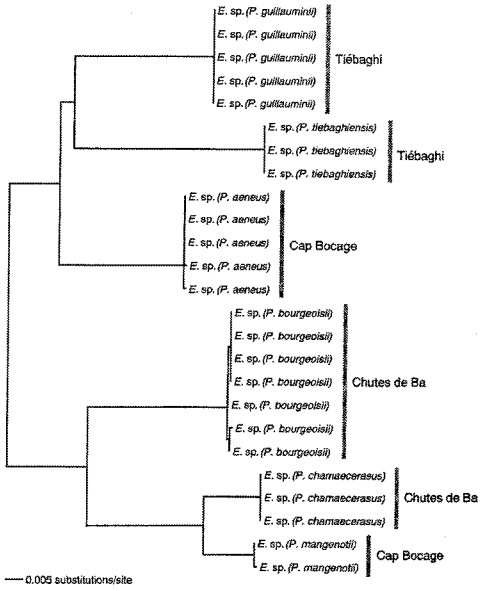


Fig. 11. Unrooted neighbor-joining phylogram depicting relative branch lengths within and among *Epicephala* moth individuals collected from different *Phyllanthus* host species. The tree is based on uncorrected pairwise distances within 1317 bp of the mitochondrial cytochrome oxidase subunit 1 gene (COI). All the moths used in the analysis are currently undescribed, and host affiliation of each individual moth is given in parentheses. Locality information is also provided to the right of shaded bars.

vulcani, and *P. pancherianus*. In most cases, individual moths that developed from different hosts were easily distinguishable by wing pattern and relative size, indicating that these moths are specific to a single *Phyllanthus* host. The host-specificity of the moths was further supported by nucleotide sequence variations within 1317 bp of the COI gene (Fig. 11). Pairwise sequence differences between individuals collected from different hosts averaged 12% (range: 3–15%), whereas differences were < 0.3% among individuals parasitizing the same host, despite regional co-occurrence of the host plants (*P. bourgeoisii* and *P. chamaecerasus* at Chutes de Ba, *P. aeneus* and *P. mangenotii* at Cap Bocage, and *P. tiebaghiensis* and *P. guillauminii* at Tiébaghi).

Discussion

My analyses revealed that at least two species of New Caledonian *Phyllanthus* are pollinated actively and exclusively by *Epicephala* moths. The larvae of these moths consume all six ovules of the developing fruit, while leaving a fraction of the fruits intact (Fig. 10). Similar association of *Epicephala* moths found in majority of the *Phyllanthus* species examined (Table 1) suggests that the interaction between species of *Phyllanthus* and *Epicephala* is a coevolved pollination mutualism that is potentially widespread among diverse species of the subgenus, *Gomphidium*. *Epicephala* moths were shown to be highly specific to a single *Phyllanthus* host based on both morphology and genetic variation (Fig. 11). Although pairwise sequence differences among individuals of the same species were extremely low (0–0.3%), the level of intra- and interspecific genetic variation is comparable to that reported for *Glochidion*-pollinating *Epicephala* moths (Kato et al. 2003).

The most critical factor underlying the *Gomphidium–Epicephala* mutualism is that a fraction of the fruits is left untouched by the moths. This is most likely brought about by the absence of moth eggs in a fraction of pollinated flowers, although I have not ruled out asexual seed production (apomixis), which may also account for the occurrence of uninfested fruits. One possible explanation for the described pattern of egg distribution is that *Epicephala* eggs may be lost from some flowers, possibly by egg predation or strong desiccation. In some yucca–yucca moth interactions, high mortality of eggs and/or early instar larvae is an important process for limiting seed consumption by the moths (Addicott and Bao 1999; Csotonyi and Addicott 2001; Shapiro and Addicott 2003). However, in *P. aeneus*, moths oviposit directly into the tissue of the calyx lobes, thereby scarring the surface of the

lobes. Such scars were not observed in flowers without moth eggs, which may allow exclusion of egg mortality as an explanation.

Another possibility is that the moths do not always oviposit in flowers that they pollinate, although this cannot be concluded from my limited number of observations. Such behavior seems paradoxical, because the moths do not benefit from the pollinating behavior itself. This seemingly altruistic pollination behavior may be advantageous to the moth because presence of uninfested fruits might force the braconid parasitoid to spend excessive time in detecting a host, thus decreasing the probability of detecting and parasitizing moth larva. Weiblen et al. (2001) suggested that in functionally dioecious figs, the presence of seed figs reduces search efficiency of the parasitoids that attack pollinator wasps and hypothesized that functional dioecy leads to increased pollinator production. More detailed examinations of moth pollination and oviposition behavior as well as parasitoid searching strategy are clearly needed before this hypothesis can be evaluated robustly.

Empirical studies have demonstrated that in some obligate pollination–seed-parasitic interactions, plants selectively abscise flowers that contain large numbers of eggs, thereby preventing excessive seed destruction (Pellmyr and Huth 1994; Ritcher and Weis 1995; Wilson and Addicott 1998; Addicott and Bao 1999). In light of this, it is paradoxical that *Gomphidium* trees do not abscise flowers containing moth eggs, despite the substantial cost imposed by the larvae. This may be a primary source of evolutionary instability because the extent of larval damage should vary among populations and between years (Addicott 1986; Thompson 1994; Pellmyr and Thompson 1996; Thompson and Cunningham 2002), and excessive exploitation by pollinators should lead to insufficient plant reproduction. One explanation for the lack of selective abscission in *Gomphidium* is that the potential for such a mechanism is weak because the available resources do not limit seed set and thus need not be allocated to high-quality fruits. However, as hypothesized for some yuccas (Addicott and Bao 1999), *Gomphidium* flowers may not have proximate cues to predict whether their ovules are infested, because oviposition by *Epicephala* moths does not directly damage the ovary. Selective abscission may be more likely involved in the *Glochidion–Epicephala* mutualism, in which the ovipositor of the moth directly cuts through the ovary and/or style tissue, and the reproductive success of the plant strongly depends on the number of eggs laid per flower (Kato et al. 2003).

Given that *Gomphidium* plants do not possess a mechanism by which to prevent excessive exploitation by *Epicephala* moths, there is also no means by which the pollinators can retaliate against being overexploited by the plant. Once a plant acquires the ability to selectively abscise flowers containing moth eggs, it attains higher relative fitness, which would rapidly lead to pollinator extinction. Importantly, such a pathway leading to the breakdown of the system is inherently avoided in other obligate mutualisms, because the exclusive pollinators of the plants consistently infest the flowers (or syconia) that they pollinate.

Theoretical studies have predicted that cooperative interactions are evolutionarily stable only when both participants possess mechanisms to prevent overexploitation by the other (Axelrod and Hamilton 1981; Bull and Rice 1991; Bronstein 2001). It is therefore intriguing that a seemingly unstable interaction between *Gomphidium* and *Epicephala* has persisted through evolutionary time and has undergone extensive reciprocal diversification. The underlying principle of this system implies that mechanisms inherent to the mutualists are not necessarily responsible for the evolutionary stability of obligate interactions. Recent empirical studies on yucca–yucca moth and *Trollius–Chiastocheta* systems have also shown that various ecological factors, such as density-dependent mortality of moth larvae, may be more important in determining the overall costs and benefits of the mutualism (Wilson and Addicott 1998; Addicott and Bao 1999; Csotonyi and Addicott 2001; Jaeger et al. 2001).

Although the proximal process generating seed set in *Gomphidium* plants is currently unknown, my results show that there are major differences in feeding patterns between *Epicephala* moths associated with *Gomphidium* and *Glochidion* fruits and that different mechanisms may be responsible for the evolutionary stability of these specialized interactions. Future studies should rigorously determine the processes regulating the costs and benefits of these mutualisms as well as factors contributing to the observed differences in modes of interaction between the two systems. Within the family Euphorbiaceae, there are several other genera that are closely related to *Glochidion* and *Phyllanthus*, such as *Breynia*, *Sauropus*, *Flueggea*, and *Margaritaria* (Webster 1994). Knowledge on pollination systems of these related plant groups, combined with robust phylogenetic hypotheses of both plant and moth lineages would further add to our understandings of the evolutionary dynamics of pollination mutualisms involving euphorbiaceous trees and *Epicephala* moths.

Chapter 5

Obligate pollination mutualism in *Breynia* (Phyllanthaceae): Further documentation of pollination mutualism involving *Epicephala* moths (Gracillariidae)

INTRODUCTION

The classically known obligate pollination mutualisms between figs–fig wasps and yuccas–yucca moths are among the most fascinating examples of pollination mutualisms known (Janzen 1979; Weiblen 2002; Pellmyr 2003). In these systems, figs and yuccas depend exclusively on adult female wasps and moths for pollination, respectively, while the adult females depend on the developing seeds for nourishment of their offspring. These mutualisms are unusual both in the diversity of species involved and host-specificity of the pollinators (Pellmyr 1999; Weiblen 2002, Molbo et al. 2003). In addition, these interactions involve highly coevolved traits, such as active pollination behavior and specialized floral structures, topics of general biological interest (Kjellberg et al. 2001; Pellmyr and Krenn 2002; Jousselin et al. 2003). Furthermore, costs and benefits of the interaction for the plant (seed production and seed consumption) are relatively easy to measure, thus facilitating ecological analysis of the outcome of mutualism in these plants (Addicott 1986; Pellmyr and Huth 1994; Herre and West 1997; Addicott and Bao 1999; Patel and Hossaert-McKey 2000). Together, these attributes of the fig–fig wasp and yucca–yucca moth interactions provide model systems for various analyses of coevolutionary processes and ecological dynamics of mutualism.

The recently discovered obligate pollination mutualism between *Epicephala* moths and trees of the family Phyllanthaceae (formerly Euphorbiaceae; see APG 2003) possesses striking similarities with the fig–fig wasp and yucca–yucca moth systems and potentially provides a model system for studies of coevolution and mutualism (Kato et al. 2003; Kawakita and Kato 2004). In these associations, trees of the genera *Glochidion* and *Phyllanthus* (subgenus *Gomphidium*) are pollinated exclusively by the females of species-specific *Epicephala* moths that actively collect and transport pollen with their proboscises. The moths lay eggs in female flowers, and their offspring consume 28–74% of the developing seeds while leaving the rest intact (Kato et al. 2003; Kawakita and Kato 2004). These associations are extremely diverse with *Glochidion* and *Gomphidium* together comprising more than 450 species (Govaerts et al. 2000), while high host-specificity of the pollinator moths indicate that a comparable number of *Epicephala* moths also exist (Kato et al. 2003; Kawakita and Kato 2004). In addition, there are multiple sources of variation in modes of the plant–moth interaction (e.g., difference in the number of ovules per fruit; partial or total destruction of fruit by a single moth larva; Kato et al. 2003; Kawakita and Kato 2004), allowing comparative approaches for the studies of evolutionary and ecological dynamics of the mutualism.

While previously reported mutualisms between *Glochidion*/*Gomphidium* and *Epicephala* provide novel opportunity for studies of pollination mutualisms and coevolutionary processes, the pollination systems in closely related plant genera have yet to be investigated. Here, I provide evidence for obligate pollination mutualism in two species of *Breynia* (*B. vitis-idea* and *B. fruticosa*), which are closely related to *Glochidon* and *Gomphidium* (Webster 1994; Govaerts et al. 2000). The genus *Breynia* comprises 35 species of monoecious shrubs, distributed in tropical and subtropical regions of Asia, Australia, and the Pacific Islands (Webster 1994; Govaerts 2000). In this paper, I determine whether *Epicephala* moths associated with *Breynia* plants constitute the primary pollinators in the two species. I also investigate if there is variation in the cost of mutualism (i.e., seeds comsumed by pollinator larvae) between the two species, which potentially affects the outcome of the interaction. Finally, I compare the results with those of the *Glochidion* and *Gomphdium* systems and discuss major variants in the mode of interaction within the Phyllanthaceae–*Epicephala* mutualism.

MATERIALS AND METHODS

BREYNIA VITIS-IDEA

Breynia vitis-idea is a monoecious shrub that occurs in forest margins of tropical and subtropical forests in Asia (Fig. 1). The species is distributed from Pakistan to the southern part of Japan, including most parts of tropical Southeast Asia (Govaerts et al. 2000). The flowers lack petals and are dimorphic, with male flowers arranged toward the base and female flowers at the apex of each branch (Fig. 2). Typically, only one or two flowers are born on axils. Male flowers have fused calyx lobes with inflexed apical ends that make the stamens unlikely to be accessible to opportunistic flower visitors (Fig. 3). Female flowers are campanulate with three short styles fused at the center of the upper surface of the ovary (Fig. 4). Female flowers have three locules,

each containing two ovules. Fruits are produced shortly after pollination within 3–4 weeks. In the course of fruit development, pedicels become erect, and fruit coat eventually turn red to dark purple (Fig. 5). Flowering and fruiting occur throughout the year but typically peak in spring (March to May) and early fall (August to October) at our study sites.

I studied insect flower visitors of *B. vitis-idea* during 27 September–1 October 2002 and 9–13 May 2003 at Kasari, Amami Island (28°28′N, 129°41′E), 29 September–2 October 2003 at Banna, Ishigaki Island (24°22′N, 124°10′E), and 3–5 October 2003 at Funaura, Iriomote Island (24°24′N, 123°48′E), Japan. Preliminary observations on flowering and fruiting phenologies were made at various localities in southern Japan in 2001–2003. I made diurnal and nocturnal observations of flower visitors for a total of more than 60 h during the study periods. Particular effort was made for nocturnal observations to study flower visitation by *Epicephala* moths. *Epicephala* moths that visited flowers were collected after they became inactive on branches or leaves. I also collected other insect visitors after they left the flowers. The collected insects were identified and examined for pollen attachment with a light microscope.

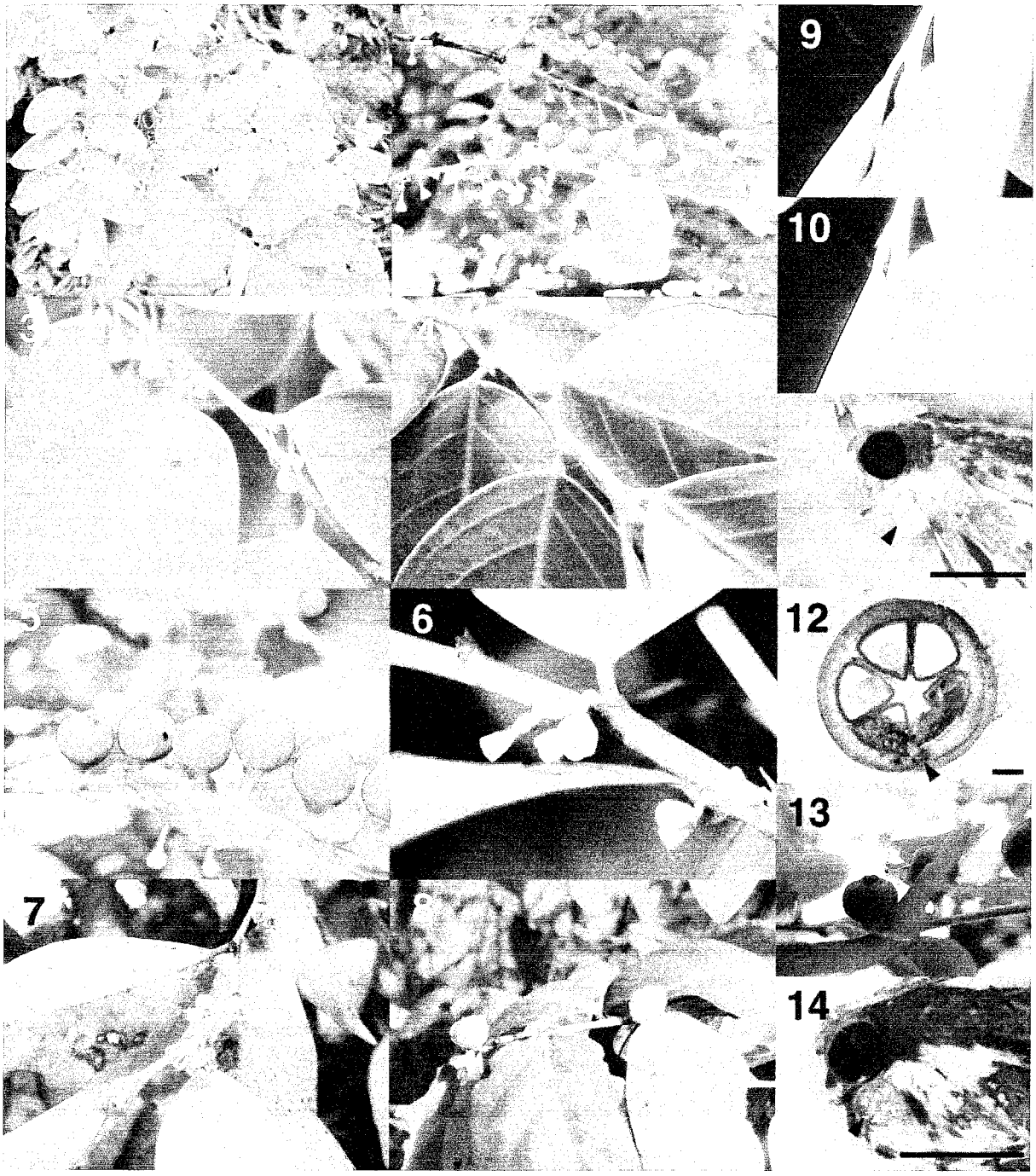
Because flowers of *B. vitis-idea* produced a small amount of nectar, I determined whether nectar production occurred during the day or at night using 10 marked female flowers on each of four individual plants. I covered the marked flowers with fine netting (0.25-mm mesh; Wataya, Kyoto, Japan) to exclude nectar foragers and sampled nectar at 0600 h and 1800 h during 11–13 May 2003 using microcapillaries (Drummond, Broomall, Pennsylvania, USA). We also measured sugar concentration of sampled nectar using a pocket refractometer (Bellingham & Stanley, Kent, UK) to monitor temporal changes in sugar concentration of nectar. I did not use male flowers for nectar measurements because of the difficulty of sampling nectar from enclosed male flowers without severe destruction to the flowers.

After field studies, I collected female flowers of *B. vitis-idea*, counted the number of pollen grains on stigmas, and examined the presence or absence of *Epicephala* moth eggs in flowers under a light microscope. Totals of 179 and 274 female flowers were sampled from three and four individuals at Kasari on 15 May 2003 and at Banna on 8 October 2003, respectively. To determine the extent of seed infestation by seed-parasitic moths, I also sampled 365 and 39 mature fruits from seven and two individuals at Kasari on 15 May and Funaura on 5 October 2003, respectively. For each fruit, I counted the number of destroyed seeds, intact seeds, and unfertilized ovules. In addition, whenever uninfested fruits were encountered, I looked for remains of *Epicephala* moth eggs to infer whether the moths had oviposited on the fruits. For the fruit samples collected at Kasari, I also assessed the cause of seed destruction for each fruit. Seeds were primarily destroyed by *Epicephala* larvae, but immature larvae were occasionally parasitized by a braconid wasp, and a nonpollinating carposinid moth, *Paramorpha* sp. also infested the seeds. I therefore determined the causes of seed destruction based on differences in the structure of feces and exit holes left by the insects. Also, the number of moths and braconid wasps that occupied each fruit was determined based on the number of exit holes on surface of each fruit. Preliminary rearing of moths and wasps has indicated that these insects always bore an exit hole upon leaving the fruit; thus, the number of exit holes can be reliably used to estimate the number of insects emerged.

Breynia fruticosa

Breynia fruticosa is a monoecious shrub that is typical in forest margins of Indochina and southern China (Govearts et al. 2000; van Welzen et al. 2000). Typically, two to four flowers are born on each axil with male flowers arranged towards the base of each branch. Male flowers have fused calyx lobes with inflexed apical ends as those of *B. vitis-idea* (Fig. 6). Female flowers are not as specialized as in *B. vitis-idea* and have free calyx lobes and free styles (Fig. 7). The styles are split in upper half and are likely accessible to opportunistic flower visitors (Fig. 7). Female flowers have three locules, each containing two ovules. Once pollinated, female flowers become erect, and fruits are produced within three to four weeks after pollination (Y. Kosaka, Kyoto Univeristy, Japan, personal communication) (Fig. 8). Flowering and fruiting occurs year-round with several peaks per year (Y. Kosaka, personal communication).

I studied insect flower visitors of *B. fruticosa* during 9–11 March and 14–16 September 2003 at Dongmakhai (17°58′N, 102°36′E) and 17–18 September 2003 at Thakhaek (17°24′N, 104°48′E), Laos. I studied diurnal and nocturnal flower visitors for a total of 26 h during the study period with emphasis on nocturnal observations of *Epicephala* moths. All insect visitors were collected and examined for pollen attachment as described. In addition, I collected *Epicephala* moths that rested on leaves to check for pollen grains on their proboscises. After field observations, I collected totals of 210 flowers and 43 fruits from six *B. fruticosa* individuals to study pollen load on stigmas, presence or absence of *Epicephala* moth oviposition, and extent of seed destruction by moth larvae as described for *B. vitis-idea*. For fruits that were not infested by *Epicephala* moths, I looked for indications of moth oviposition



Figs. 1–14. Flowers, fruits, and associated insects of *Breynia vitis-idea* and *B. fruticosa*. **1.** *B. vitis-idea* plant. **2.** Branch of *B. vitis-idea* with male flowers toward base (to left) and female flowers toward apex (right) **3.** Male and **4.** female flowers of *B. vitis-idea*. **5.** Fruits of *B. vitis-idea*. One of the fruits has an exit hole of *Epicephala* moth larva (arrowhead). **6.** Male flowers, **7.** female flowers, and **8.** fruits of *B. fruticosa*. **9.** Female *Epicephala* moth actively pollinating a *B. vitis-idea* female flower with its proboscis. **10.** Female *Epicephala* moth ovipositing in a female *B. vitis-idea* flower. **11.** Female *Epicephala* moth collected from a *B. vitis-idea* flower. Proboscis of moth is covered with pollen grains (arrowhead). Bar = 1 mm. **12.** Cross section of *B. vitis-idea* fruit. The fruit has six seeds, three of which were destroyed by an *Epicephala* larva. The larva had emerged through the exit hole (arrowhead). Bar = 1 mm. **13.** Female braconid wasp probing a *B. vitis-idea* fruit with its ovipositor. **14.** Female *Epicephala* moth collected from a *B. fruticosa* plant having a pollen-coated proboscis (arrowhead). Bar = 1 mm.

Table 1. Amount and sugar concentration of nectar produced by female *Breynia vitis-idea* flowers during 11–13 May 2003. Values are means ± SE across four plant individuals (average value of 10 flowers for each plant).

Time	Nectar amount (μl)	Sugar concentration (%)
1800–600 h 11–12 May	0.57 ± 0.13	14.10 ± 7.17
600–1800 h 12 May	0	–[a]
1800–600 h 12–13 May	0.11 ± 0.02	41.43 ± 4.72
600–1800 h 13 May	0	–[a]

[a]Sugar concentration could not be measured in these samples because there was no nectar production.

Table 2. Patterns of seed infestation by seed-parasitic moths in *Breynia vitis-idea* at two locations in Japan.

Locality	Fruits examined (individuals)	Damaged seeds[a]	Intact seeds[a]	Unfertilized ovule[a]	Intact fruit (%)	Intact fruit with egg (%)
Kasari	365 (7)	3.02 ± 0.13	2.41 ± 0.11	0.57 ± 0.05	30.14	43.59
Funaura	39 (2)	2.26 ± 0.41	3.67 ± 0.41	0.08 ± 0.04	43.64	64.71

[a]Mean ± SE

(i.e., oviposition scars) to infer whether the *Epicephala* moths had oviposited on fruit. Flowers and fruits were collected at Dongmakhai on 20 September 2003.

RESULTS

BREYNIA VITIS-IDEA

Nectar was produced at night on female flowers of *B. vitis-idea* (Table 1). The marked differences in the amount of nectar produced during the two nights was likely from the difference in air humidity during the 2 days (humid and dry on 12 and 13 May, respectively), which was reflected in sugar concentration of nectar (Table 1).

Observed visitors to flowers of *B. vitis-idea* were the ant *Anoplolepis longipes* and an undescribed species of *Epicephala* moth. Workers of *A. longipes* visited female flowers of *B. vitis-idea* during the day but mainly at night to forage nectar. However, these ants were not observed on male flowers, and none carried pollen grains (N = 19). At night, I observed female *Epicephala* moths visiting female *B. vitis-idea* flowers, depositing pollen grains with their proboscises (Fig. 9), and subsequently laying an egg within the interspace between clayx lobes and ovary (Fig. 10). I observed four female *Epicephala* moths visiting female flowers, which all had the same stereotypic behavior on flowers. In addition, one of these moths repeated the pollination–oviposition behavior twice on the same flower. Although I did not observe *Epicephala* moths collecting pollen on male flowers, all the moths that visited female flowers carried numerous pollen grains on their proboscises (Fig. 11). The low frequency of moth visits reflects the rare occurrence of moth visitation and the short time *Epicephala* moths spent on flowers, which was also the case in *Glochidion*- and *Gomphidium*-pollinating *Epicephala* moths (Kato et al. 2003; Kawakita and Kato 2004 and personal observations).

Examination of pollen load and *Epicephala* moth eggs in female flowers revealed that nearly all pollinated flowers had moth eggs, whereas unpollinated flowers only rarely had eggs (Fig. 15). The mean number of pollen grains on female flowers with moth eggs was 12.0 ± 0.6 (mean ± SE; N = 116) and 9.8 ± 0.9 (N = 38) at Banna and Kasari, respectively, which was significantly greater than that on female flowers without moth eggs (0.4 ± 0.2, N = 158 and 0.1 ± 0.1, N = 141, Mann-Whitney U test; U = 409 and 23, $P < 0.0001$). These data indicate that *Epicephala* moths are likely exclusive pollinators of *B. vitis-idea*. Pollen grains were aggregated at the stigmatic part of female flowers as in *Glochidion* and *Gomphidium* (Kato et al. 2003; Kawakita and Kato 2004), which is unlikely to occur through passive pollination. Eggs were laid between the ovary and calyx lobes, and on average, egg-loaded flowers had 1.58 and 1.53 eggs per flower at Banna and Kasari, respectively (N = 116 and 38, range: 1–4).

The mean number of intact seeds per fruit was 2.4 ± 0.1 (N = 365) and 3.7 ± 0.4 (N = 39) at Kasari and Funaura, respectively, and seed-parasitic moths destroyed 3.0 ± 0.1 and 2.3 ± 0.4 seeds (Table 2). However, 30.1% of the fruits sampled at Kasari and 43.6% at Funaura were not infested by the moths (Table 2). Of these uninfested fruits, 43.6% and 64.7% had remains of *Epicephala* moth eggs (Table 2), indicating egg/larval death of *Epicephala* moths in these fruits. Seed destruction was mainly caused by *Epicephala* larvae (Fig. 16). Normally, a single moth larva did not consume all seeds within a fruit (Figs. 12, 16), but two moth larvae were enough to destroy all seeds of a fruit (Fig. 16). Braconid wasps parasitized early instar *Epicephala* larvae by probing the fruit (Fig. 13); this parasitism had a significant positive effect on seed set by preventing further seed consumption by *Epicephala* larvae (Mann-Whitney U test; U = 135, $P < 0.0001$; Fig. 16).

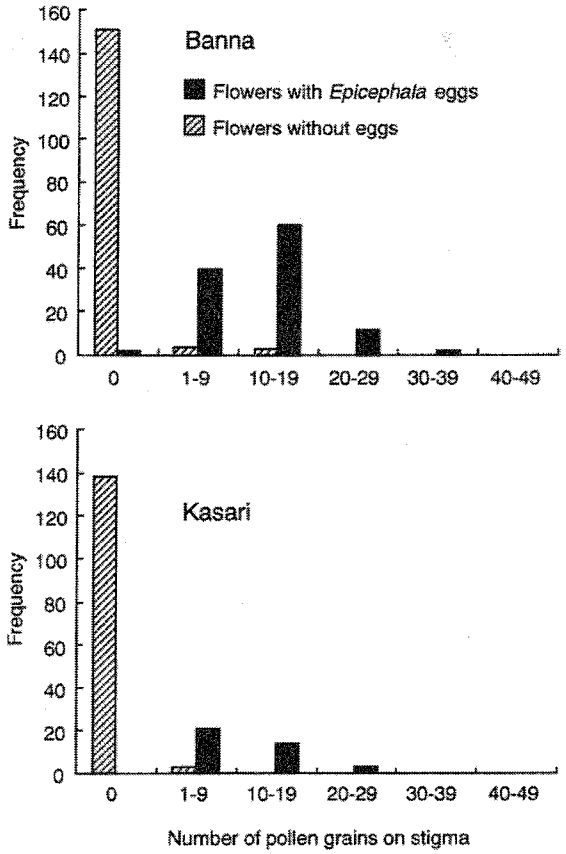


Fig. 15. Frequency distributions of the number of pollen grains attached to stigmas of female *Breynia vitis-idea* flowers with and without *Epicephala* moth eggs at Banna and Kasari. The mean number of pollen grains attached to flowers with and without moth eggs was 12.01 ± 0.56 (mean ± SE; N = 116) and 0.37 ± 0.16 (N = 158) at Banna and 9.76 ± 0.93 (N = 38) and 0.11 ± 0.07 (N = 141) at Kasari, respectively.

Breynia fruticosa

Although I did not observe *Epicephala* moths visiting flowers in *B. fruticosa*, *Epicephala* females collected on leaves of *B. fruticosa* carried numerous pollen grains on proboscises as those pollinating *B. vitis-idea* (Fig. 14), which indicates that these moths are active pollinators. I also observed a gall midge, *Clinodiplosis* sp., resting at the entrance of male flowers or on the styles of female flowers. Of the 15 midges that I collected, two had a few pollen grains on legs and heads, suggesting that these gall midges may also contribute to pollination.

The pattern of relationship between pollination and *Epicephala* oviposition was similar to that observed in *B. vitis-idea* (Fig. 17), indicating that *Epicephala* moths are the primary pollinators of *B. fruticosa*. The mean number of pollen grains on female flowers with moth eggs was 14.6 ± 1.0 (N = 141), significantly greater than that on flowers without moth eggs (1.1 ± 0.4, N = 69, Mann-Whitney U test; U = 477.5, $P < 0.001$). *Epicephala* moth eggs were laid at the basal part of the ovary, and oviposition occasionally damaged the ovule. Moth

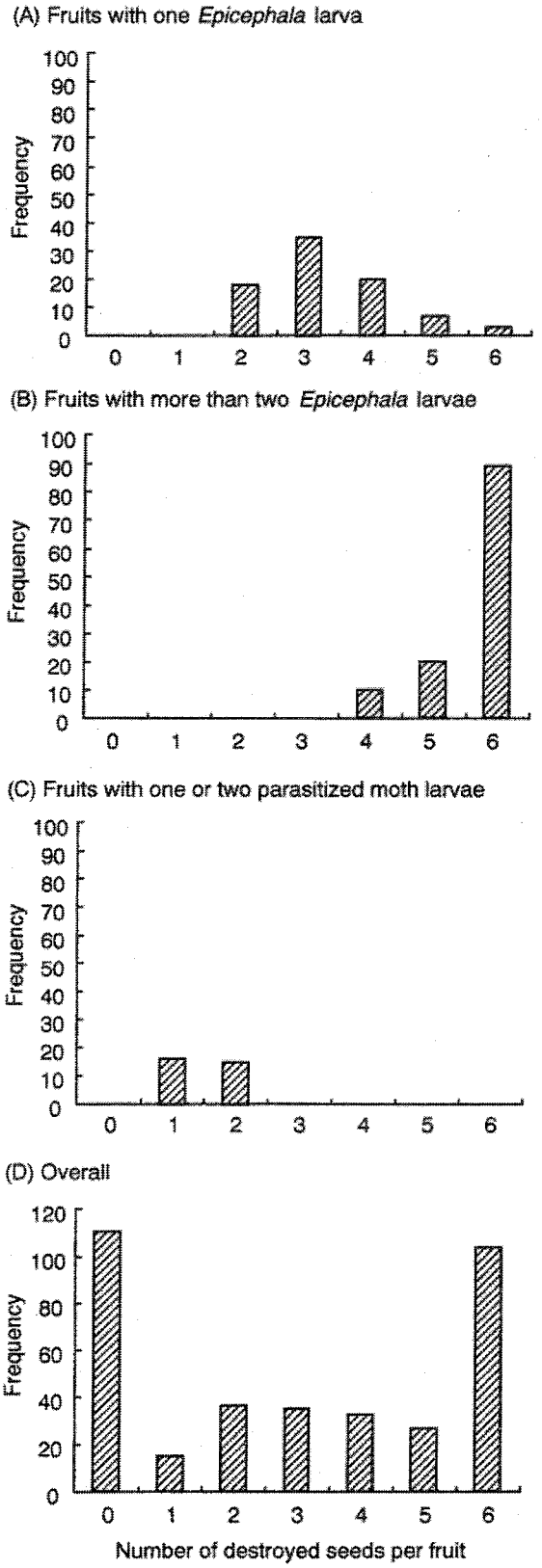


Fig. 16. Frequency distributions of the number of destroyed seeds per fruit in *Breynia vitis-idea* at Kasari. Fruits of *B. vitis-idea* invariably have six ovules. Immature larvae of *Epicephala* moths were occasionally parasitized by braconid wasps. The mean number of seeds destroyed was (A) 3.30 ± 0.11 (mean ± SE; N = 83), (B) 5.66 ± 0.06 (N = 119), (C) 1.48 ± 0.09 (N = 31), and (D) 3.02 ± 0.13 (N = 365).

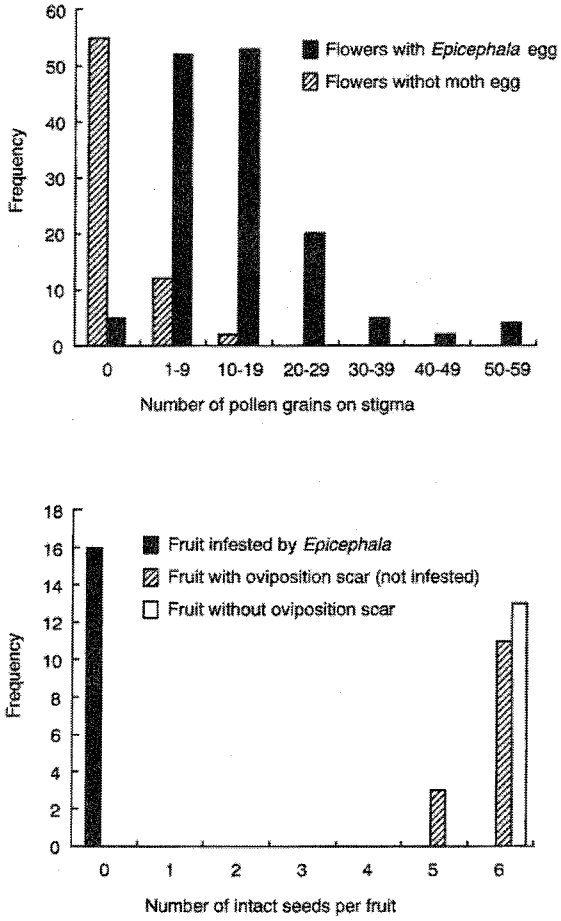


Fig. 17. Frequency distributions of the number of pollen grains attached to stigmas of female flowers (upper) and number of intact seeds per fruit (lower) in *Breynia fruticosa* at Dongmakhai. The mean number of pollen grains attached to female flowers with and without moth eggs was 14.58 ± 1.00 (mean ± SE; N = 141) and 1.07 ± 0.37 (N = 69), respectively.

oviposition cut through the calyx lobes and the ovary wall, thereby leaving oviposition scars. These scars were reliable indicators of moth oviposition; the presence of scars and moth eggs had a one-to-one correspondence (N = 180). An egg-loaded flower had an average of 1.28 eggs (N = 141, range: 1–3).

Seed destruction was caused exclusively by *Epicephala* moths, and a single larva destroyed all six seeds of a developing fruit (Fig. 17). However, 66.0% of the fruits remained uninfested (N = 43; Fig. 17). Of these intact fruits, 51.6% had oviposition scars, indicating that the eggs or early instar larvae of the moth did not develop in these fruits.

DISCUSSION

OBLIGATE POLLINATION MUTUALISM IN *BREYNIA*

This study revealed that at least two species of *Breynia* are pollinated by *Epicephala* moths that actively transport pollen between flowers. Although I did not observe moth visits in *B. fruticosa*, the presence of moth eggs in the majority of pollinated female flowers (Fig. 17), combined with possession of heavy pollen load on the proboscises of the female moths (Fig. 14) strongly indicates that *Epicephala* moths associated with *B. fruticosa* are the pollinators of their hosts. In both *B. vitis-idea* and *B. fruticosa*, larvae of the moths consumed developing seeds, but in total, a fraction of the seed crop was left intact, thus imposing a net benefit to plant reproduction (Table 2; Fig. 17).

Although my data showed that pollinated flowers of the two species normally had moth eggs (Figs. 15, 17), a fraction of fruits was not infested by *Epicephala* moths (Table, 2; Fig. 17). In most cases, this is brought about by egg/larval mortality of *Epicephala* moths, as inferred from indication of moth oviposition (egg remains in *B. vitis-idea* and oviposition scars in *B. fruticosa*) in these fruits. Yet, some fruits seemed not to have been oviposited by *Epicephala* (Table 2; Fig. 17). In *B. vitis-idea*, the proportion of intact fruits with moth oviposition was likely underestimated because egg remains are not always detectable and may be lost in the course of fruit development. Therefore, the possibility that the fruits normally had been oviposited cannot be ruled out in *B. vitis-idea*. In *B. fruticosa*, on the other hand, scars on calyx lobes and ovary walls are reliable markers of moth oviposition; thus, a fraction of intact fruits probably had not been oviposited. Pollination in these fruits may have been caused abiotically or by other potential copollinators such as gall midges. It is also possible that these fruits were produced by *Epicephala* moths that pollinated flowers but failed to lay eggs, as suggested for the mutualism between *Gomphidium* and *Epicephala* (Kawakita and Kato 2004). In any case, a detailed study of moth oviposition and demographic pattern of moth egg/larvae are needed to fully understand the factors limiting the cost and benefit of the mutualism in these systems.

In some yucca–yucca moth interactions, yuccas selectively abscise flowers with high egg loads, thereby limiting seed destruction by yucca moths and allocating available resources to increased seed production (Pellmyr and Huth 1994; Ritcher and Weis 1995; Wilson and Addicott 1998; Addicott and Bao 1999). However, such a mechanism is not likely in the systems we studied, nor in the *Gomphidium–Epicephala* system (Kawakita and Kato 2004), because the plants did not abort flowers with the level of egg load that most likely led to total destruction of seeds (two and one eggs in *B. vitis-idea* and *B. fruticosa*, respectively). Instead, egg/larval mortality of *Epicephala* moths played an important role in limiting seed consumption in the two systems. The importance of egg/larval mortality as a factor limiting excessive consumption is also noticed in other seed-parasitic pollination mutualisms including yucca–yucca moths (Addicott and Bao 1999; Shapiro

and Addicott 2003), globeflower–globeflower flies (Jaeger et al. 2001), and senita cactus–senita moth mutualisms (Fleming and Holland 1998; Holland and Fleming 1999), as well as in an ecologically analogous fly–fungus mutualism (Bultman et al. 2000). Whether egg/larval mortality is host-induced (i.e., retaliation on overexploitation by the mutualist) or caused by factors independent of hosts is unknown. These issues should also be addressed in the future.

The genus *Breynia* currently comprises 35 species distributed in tropical regions of Asia, Australia, and the Pacific Islands (Govaerts et al. 2000). Plants of this genus are characterized by the fused, obconic or turbinate calyx lobes in male flowers and minute styles that are more or less fused in female flowers (Figs. 3, 4, 6, 7; Chakrabarty and Gangopadhyay 1996). These structures likely prevent effective contact with anthers and stigmas by facultative flower visitors and suggest that the specialized *Epicephala* moth pollination is potentially widespread within the genus. Fruits of *B. distica* in New Caledonia and *B. cernua* and *B. oblongifolia* in Australia are also infested by *Epicephala* moths (A. Kawakita and M. Kato, personal observations), which further supports the widespread occurrence of obligate pollination mutualism in the genus *Breynia*.

Obligate pollination mutualism in Phyllanthaceae

Obligate pollination mutualism in Phyllanthaceae was first discovered between *Glochidion* trees and *Epicephala* moths (Kato et al. 2003). In this association, female *Epicephala* moths actively pollinate flowers and lay eggs in female flowers. The moth larvae consume the developing seeds, but on average, 20–54% of the seeds are left intact in each fruit (Kato et al. 2003). Species of *Gomphidium* are also pollinated actively by female *Epicephala* moths that oviposit in flowers (Kawakita and Kato 2004). In this association, however, a single moth larva consumes the entire seeds of the developing fruit. Instead, 60–78% of the fruits are left untouched by the moth, probably because egg/larval mortality is high in these species or the moths do not always oviposit in flowers that they pollinate (Kawakita and Kato 2004). The *Breynia–Epicephala* mutualism reported in this study is similar to the *Gomphidium–Epicephala* system in that the moth larvae frequently consume all seeds of the developing fruit (Figs. 16, 17). We showed that in this association, egg/larval mortality of *Epicephala* is an important, yet not exclusive, factor limiting seed destruction by the moths (Table 2, Fig. 17).

The different modes of plant–moth association found in *Glochidion*, *Gomphidium*, and *Breynia* provide multiple sources of variation in the factors affecting the interaction between *Epicephala* moths and their hosts. For example, ovule number per flower and fruit size typically varies among plant genera (e.g., six ovules per flower in *Gomphidium* and *Breynia* and 6–12 in *Glochidion*), which may be associated with the proportion of seeds that a single moth larva destroys. Also, oviposition methods vary among *Epicephala* moths infesting different host species (e.g., whether oviposition penetrates the ovary or not), and these differences may correspond to differential abilities of the host plants in detecting moth oviposition and selectively aborting heavily infested fruits, as suggested for some yucca species (Addicott and Bao 1999; Marr and Pellmyr 2003; Shapiro and Addicott 2003). Thus, these variations allow comparative approaches in various ecological and evolutionary studies of plant–insect mutualisms. Furthermore, the interaction between the plant of a closely related genus *Flueggea* and its seed-parasitic *Epicephala* moth likely represent a plesiomorphic, antagonistic condition for the mutualism (Kawakita and Kato, unpublished data). Together, these attributes of the association between Phyllanthaceae plants and *Epicephala* moths provide an increasingly fascinating model system for studies of mutualisms and coevolutionary processes.

Chapter 6

Systematic survey of fruit parasitism by *Epicephala* moths (Gracillariidae) and its relevance to pollination in Phyllantheae (Phyllanthaceae)

INTRODUCTION

The obligate mutualisms between figs and fig wasps, and yuccas and yucca moths are among the most elaborate mechanisms of pollination known in angiosperms (Janzen 1979; Weiblen 2002; Pellmyr 2003). The females of fig wasps and yucca moths actively collect and transport pollen between fig and yucca flowers, respectively, while the plants sacrifice a subset of the resulting seeds for the nourishment of pollinator larvae. Recent studies have found that species of *Glochidion*, *Breynia*, and *Phyllanthus* (subgenus *Gomphidium*) in the tribe Phyllantheae (Phyllanthaceae) have established analogous mutualisms with seed-parasitic *Epicpehala* moths (Gracillariidae; Kato et al. 2003; Kawakita and Kato 2004a,b). In these mutualisms, the female moth uses her proboscis to actively pollinate the flower and lays an egg in the flower she pollinates. The hatched larva feeds solely on the developing seeds, but a fraction of the crop is still viable for plant reproduction. Because the plants depend exclusively on *Epicephala* for pollination, neither of the partners can successfully reproduce in the absence of the other.

Phyllantheae is a pantropical tribe of more than 1200 species, consisting of monoecious or dioecious shrubs, trees, herbs, rarely climbers, scramblers, succulents, or aquatics (Hoffmann et al. 2006). The high species number and minute, inconspicuous flowers have made this group a particularly difficult taxon to classify, but recent molecular phylogenetic studies have established robust taxonomic limit and generic classification for the tribe (Kathriarachchi et al. 2005, 2006; Samuel et al. 2005). Currently, six well-supported clades are recognized at generic rank, *Phyllanthus* s. l. (including *Glochidion*, *Breynia*, *Sauropus*, and *Reverchonia*), *Plagiocladus*, *Flueggea*, *Lingelsheimia*, *Margaritaria*, and *Savia* section *Heterosalvia* (Hoffmann et al. 2006). The latter five genera form a grade leading to the large, paraphyletic genus *Phyllanthus* with embedded *Glochidion*, *Breynia*, *Sauropus*, and *Reverchonia*. *Phyllanthus* is further divided into ca. 10 subgenera and numerous sections, but many of the subgenera are found to be non-monophyletic (Kathriarachchi et al. 2006).

Although information is rapidly becoming available on the classification of Phyllantheae, much less is known about pollination biology and ecological associations with *Epicephala* moths within the tribe. Aside from previous reports of *Epicephala* pollination (Kato et al. 2003; Kawakita and Kato 2004a,b), studies of pollination ecology in Phyllantheae are limited to two economically important fruit crops in Southeast Asia (*P. emblica* and *P. acidus*) and one dioecious tropical tree (*P. pinnatus*). Reddi and Reddi (1984) hypothesized wind-pollination for *P. emblica* and *P. acidus* based on high densities of air-borne pollen. Reddi and Reddi (1985) conducted field observation and bagging experiment and suggested that *P. pinnatus* is pollinated both by wind and diurnal insect visitors, such as bees, ants, and flies.

Because Phyllantheae has a remarkable diversity of habitat, growth form, and floral morphology (Fig. 1), pollination systems are probably highly divergent. Knowledge about pollination biology and ecological association with *Epicephala* in various lineages within the tribe thus provides important basic information for studying the origins of the highly specialized mutualism and conditions that led to its evolution. In this paper, I study 25 species spanning broad taxonomic range within Phyllantheae (Table 1) to determine whether or not each species is associated with seed-parasitic *Epicephala* moths. I also conduct field observation and pollination experiment to identify potential pollinator fauna for each species. The sampled species cover various ecological habitats (temperate to subtropical forests, seasonal tropical forest, montane cloud forest, and desert sand dune) and a wide geographic range (Japan, Taiwan, Laos, Madagascar, and the USA), thus represent much of the diversity observed within the tribe.

MATERIALS AND METHODS

SPECIES STUDIED

Flueggea

Flueggea is one of the earliest lineages to have diverged within Phyllantheae (Kathriarachchi et al. 2005, 2006; Samuel et al. 2005) and comprises 14 species having a relictual, pantropical distribution (Webster 1984). The two species sampled in this study (*F. suffruticosa* and *F. virosa*) are dioecious shrubs common in temperate to subtropical vegetations of eastern Asia. Flowers are borne in axillary clusters (Fig. 1a), and the male flowers have five free stamens with anthers subtended by long, slender filaments (Fig. 2a). The female flowers have three styles that are basally connate and distally free, bifid, and dilated (Fig. 2m).

Phyllanthus subgenus *Isocladus*

Subgenus *Isocladus* is one of the basal members within *Phyllanthus* s. l. and comprise nearly 70

Table 1. List of Phyllantheae species studied.

Genus	Subgenus	Section	Species	Study sites	Study dates
Flueggea			*F. suffruticosa*	Takedao, Hyogo, Japan	July, October 2002
				Akakina, Amami Is., Japan	June 2003,
				Manzamou, Okinawa, Japan	June 2003, 2004
				Makiyama, Irabu Is., Japan	September 2004
			F. virosa	Vieng Xai, Houaphan, Laos	August 2005
				Fangliao, Pingtung, Taiwan	August 2004
Phyllanthus	*Isocladus*	*Macraea*	*P. ussuriensis*	Machida, Tokyo, Japan	October 2004, September 2005
				Kameoka, Kyoto, Japan	September 2006
			P. virgatus	Dongmakhai, Vientiane, Laos	September 2003, August 2005
	Kirganelia	*Kirganelia*	*P. flexuosus*	Kibune, Kyoto, Japan	May–June 2003, April–June 2004
				Takedao, Hyogo, Japan	June 2004
				Inohae, Miyazaki, Japan	June 2003
			P. oligospermus	Kubura, Yonaguni, Japan	September 2004, October 2006
		Anisonema	*P. reticulatus*	Henchun, Pingtung, Taiwan	August 2004
			P. sp.	Lakxao, Bolikhamxai, Laos	January 2005
		Pentandra	*P. tenellus*	Oura, Okinawa, Japan	April 2005
	Cicca	*Cicca*	*P. acidus*	Vientiane, Laos (cultivated)	March 2003
	Emblica	*Emblica*	*P. emblica*	Ban Chomesy, Xiengkhouang, Laos	August 2005
	Phyllanthus	*Urinaria*	*P. lepidocarpus*	Kyoto University, Kyoto, Japan	September–October 2004
				Nohara, Miyako Is., Japan	September 2004
				Omoto, Ishigaki Is. Japan	September 2004
		Phyllanthus	*P. amarus*	Ohama, Ishigaki Is., Japan	September 2003, 2004
				Thakhaek, Khammouane, Laos	April 2005
			P. debilis	Ohama, Ishigaki Is., Japan	September 2003, 2004
	Eriococcus	*Eriococcus*	*P. liukiuensis*	Manzamou, Okinawa, Japan	April, June 2004, May 2005
			P. pulcheroides	Mahaxai, Bolikhamxai, Laos	January 2005
	Phyllanthodendron		*P. roseus*	Phialat, Vientiane, Laos	March 2003, April 2005
	Not placed		*P. marojejiensis*	Mt. Marojeji, Antsiranana, Madagascar	December 2003, November 2005
			P. humbertii	Mt. Marojeji, Antsiranana, Madagascar	December 2003, November 2005
Reverchonia			*R. arenaria*	Bingham, New Mexico, USA	September 2005, August 2006
Breynia			*B. retusa*	Dongsanghin, Vientiane, Laos	March 2003, March, June 2004
Sauropus			*S. quadangularis*	Dongmakhai, Vientiane, Laos	March, June 2004,
			S. brevipes	Dongmakhai, Vientiane, Laos	March, June 2004,
			S. androgynus	Thakhaek, Khammouane, Laos	March, June 2004
			S. granulosus	Dongmakhai, Vientiane, Laos	June 2004
				Ban Thonkhan, Vientiane, Laos	June 2004

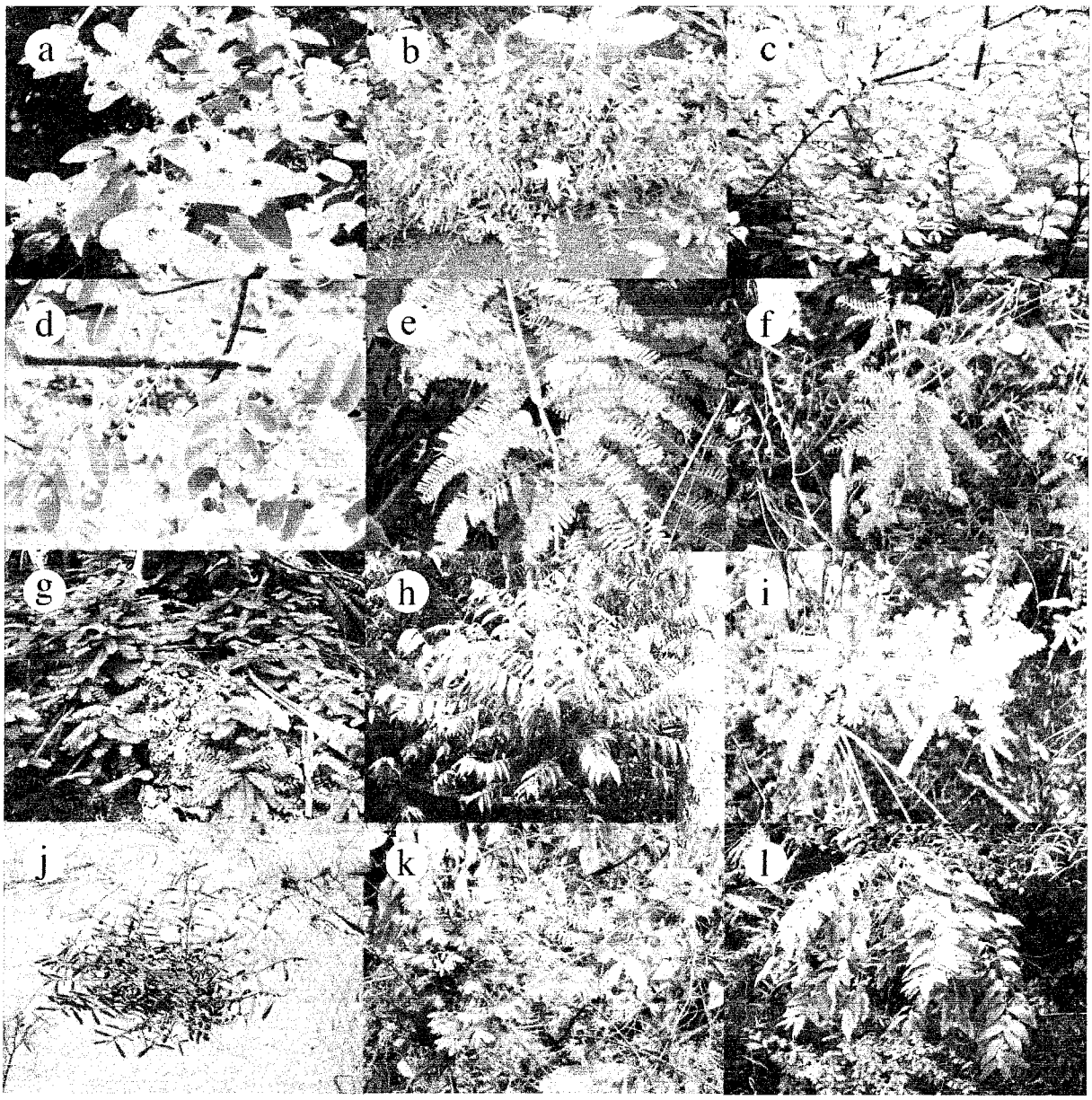


Fig. 1. Variations in growth form and habitat among Phyllantheae plants. **a.** *F. suffruticosa*. **b.** *P. virgatus*. **c.** *P. flexuosus*. **d.** *P.* sp. **e.** *P. emblica*. **f.** *P. debilis*. **g.** *P. liukiuensis*. **h.** *P. roseus*. **i.** *P. marojejiensis*. **j.** *R. arenaria*. **k.** *B. retusa*. **l.** *S. androgynus*.

species in five sections. *Isocladus* has a pantropical distribution, but a recent molecular phylogenetic work has suggested that the subgenus is polyphyletic (Kathriarachchi et al. 2006). The two species included here (*P. ussuriensis* and *P. virgatus*; Fig. 1b) belong to section *Macraea* and are herbs that occupy rather disturbed, weed-like habitats. The flowers are borne on axils with one to three male flowers arranged basally and single female flowers distally within a branch. The male flowers have two stamens that are connected into a column (Fig. 2b), and the female flowers have three styles that are free and deeply bifid (Fig. 2n).

Phyllanthus subgenus *Kirganelia*

Subgenus *Kirganelia* consists of herbs, shrubs, and trees with a variety of habitat and floral morphology (Webster 1957). It comprises nearly 50 species of paleotropical distribution but is also polyphyletic (Kathriarachchi et al. 2006). The two shrub species sampled from section *Kirganelia* (*P. flexuosus* and *P. oligospermus*; Fig. 1c) are infrequently found in understory or forest margins of temperate and subtropical vegetation in western Japan and Taiwan. The male flowers are aggregated on the axils and have three to five free stamens (Fig. 2c). The female flowers are arranged singly on axils and have three, entire or slightly bifid styles (Fig. 2o).

Section *Anisonema* is centered in the tropical regions of Madagascar and continental Africa, but one species (*P. reticulatus*) is widely distributed throughout the Old World tropics as for east as Taiwan (Govaerts et al. 2000). During the course of this study, I found that plants currently referred to as *P. reticulatus* contains two distinct forms that can be clearly distinguished based on floral characters (not corresponding to the pubescent and glabrous forms as discussed elsewhere). In one of the forms, the male flowers are ovoid and have five or six, oblong calyx lobes that are narrowly imbricate and reddish abaxially at the base (Fig. 2d). The female flowers have the ovary almost entirely invested by five or six, near orbicular calyx lobes, and 6–10, entire or apically bifid styles that are irregularly inflexed and forming a fleshy clump of stigmatic tissue (Fig. 2p). In the other form (Fgi. 1d), the male flowers are more or less spheroidal, having whitish, imbricate calyx lobes (Fig. 2e). The calyx lobes of the female flowers merely invest the basal half of the ovary, and the styles are reduced and almost completely fused into the spheroidal structure of the ovary, forming a stigmatic pit at the apex of the flower (Fig. 2q). These two forms are found at various localities in Taiwan and Laos, indicating that these morphological differences are well established and widespread. Furthermore, they are pollinated by different species of *Epicephala* that can be clearly distinguished by male genital morphology and egg-laying behavior (see RESULTS), suggesting that the two forms are reproductively isolated. I therefore treated the two forms as distinct species in this study. I refer to the first form as *P. reticulatus* and the second as *P.* sp., as the former more closely matches published floral descriptions for the species (Webster 1957).

Subgenus *Pentandra* includes three species of herbs or a shrub that are primarily distributed in the tropical regions of Africa. The species included here, *P. tenellus*, is a native herb of the Mascarene Islands (Webster 1957), but is now a common weed in tropical and subtropical regions worldwide (Kurosawa 2001). The flowers are morphologically simple with five free stamens in the male flowers and three bifid styles in the female flowers.

Phyllanthus subgenus *Cicca*

Subgenus *Cicca* contains some of the most distinctive plants in the genus and comprises four species of the New World origin. *Phyllanthus acidus* is a medium-sized tree of up to 10 m high and commonly cultivated in tropical areas worldwide for its acid fruits. Both male and female flowers are bone together in dense cymules on modified deciduous branchlets. The male flowers have four slender stamens, and the female flowers have three, deeply bifid styles.

Phyllanthus subgenus *Emblica*

Subgenus *Emblica* includes less than 10 tree or shrub species native to the Asian tropics and subtropics. The species studied here, *P. emblica* (Fig. 1e), is a moderately tall tree, widely cultivated for its edible fruits. The flowers are borne on cymules, which are subtended by reduced leaves, with single apical female flowers and several lateral males. The three stamens of the male flowers are connected to a terete column. The female flowers have three, basally connate and distally free styles that are twice bifid.

Phyllanthus subgenus *Phyllanthus*

Subgenus *Phyllanthus* contains the most divergent groups within the genus and comprises about eight sections and subsections of non-monophyletic origins (Kathriarachchi et al. 2006). The species are mostly herbs (Fig. 1f) and rarely small shrubs, widely distributed in the tropics of the world. Several species are widespread weeds in tropical regions worldwide, including *P. debilis* and *P. amarus* (native to India and Ceylon, and tropical America, respectively) that are sampled here (Kurosawa 2001). *Phyllanthus lepidocarpus* is distributed throughout eastern and southern Asia. The flowers of these species are more or less similar in having three stamens connate into a column and three deeply bifid styles (Fig. 2f,r). One to three male

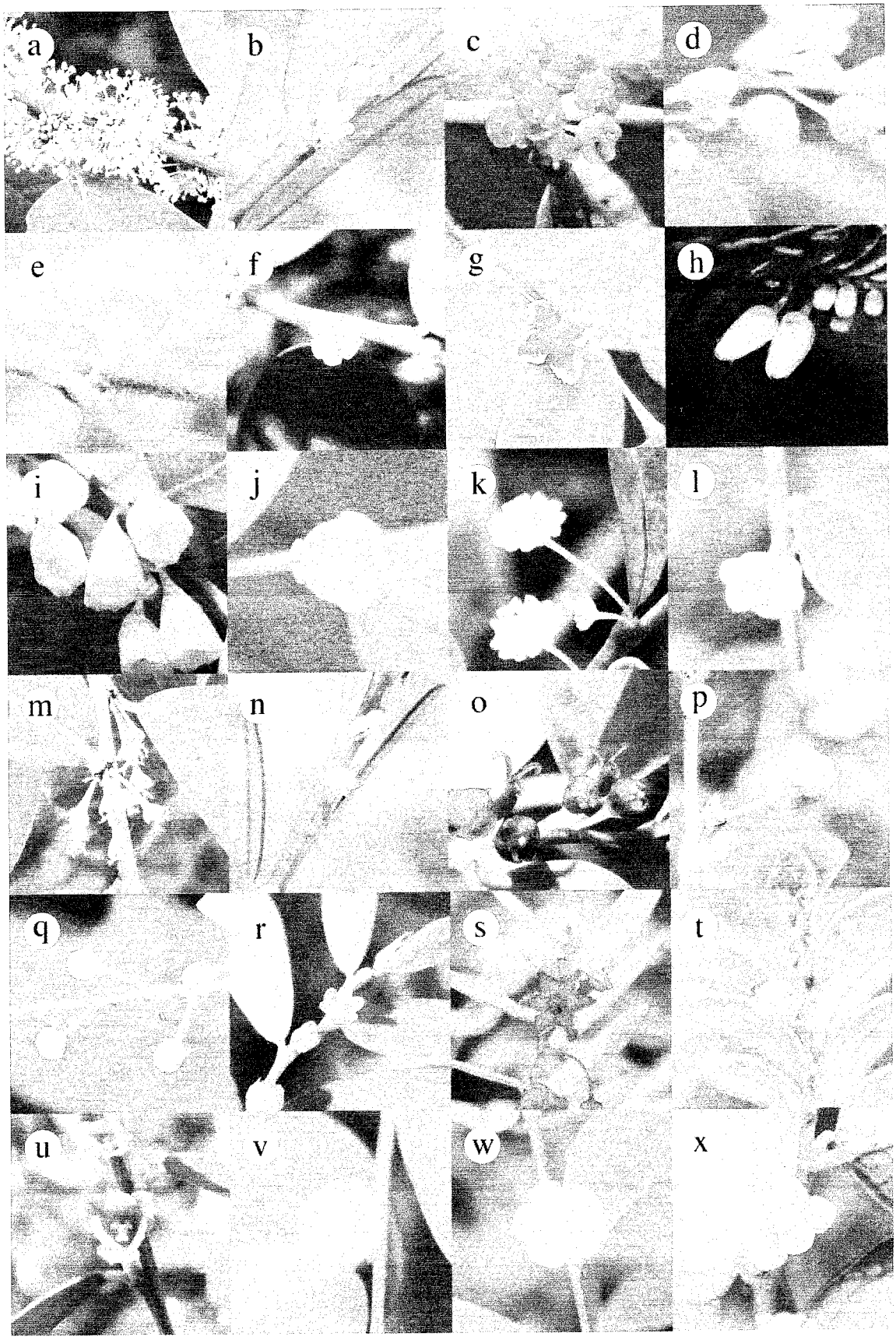


Fig. 2. Male (a–l) and female (m–x) flowers of various species of Phyllantheae. **a.** *F. suffruticosa*. **b.** *P. ussuriensis*. **c.** *P. flexuosus*. **d.** *P. reticulatus*. **e.** *P.* sp. **f.** *P. debilis*. **g.** *P. liukiuensis*. **h.** *P. marojejiensis*. **i.** *B. retusa*. **j.** *S. quadangularis*. **k.** *S. brevipes*. **l.** *S. androgynus*. Figures m–x correspond to the same species as a–l, respectively.

flowers and single female flowers are borne together on the axils.

Phyllanthus subgenus *Eriococcus*

Subgenus *Eriococcus* is a well-defined natural group of ca. 30 species distributed from southern India and Ceylon to Japan and the Philippines. The species of this subgenus is easily distinguished from other groups by virtue of lacerate, often purplish-red calyx lobes and characteristic androecium of two connate stamens (Fig. 2g; Webster 1957). The styles are more or less free and bifid (Fig. 2s). Two species, *P. liukiuensis* (Fig. 1g)and *P. pulcheroides*, are sampled for this study.

Phyllanthus subgenus *Phyllanthodendron*

Subgenus *Phyllanthodendron* is a small Asian group of trees and shrubs, characterized by bell-shaped flowers with apically reflexed, acuminate calyx lobes and connate stamens with minute subulate prolongation. The styles are fleshy, basally connate, and slightly bifid. The species sampled here, *P. roseus* (Fig. 1h), is a common tree in evergreen forests of the southeast Asian tropics.

Phyllanthus marojejiensis and *P. humbertii*

Phyllanthus marojejiensis and *P. humbertii* belong to a group of seven species endemic to Madagascar, which have previously placed in the genus *Glochidion*. They are now considered distinct from the true, entirely Asia-Australian *Glochidion* based on morphological examination (Hoffmann and McPherson 2003), although their position within *Phyllanthus* is still unknown. The two species are restricted to montane cloud forests in northeastern Madagascar. The male flowers have five narrowly imbricate calyx lobes and three connate stamens (Fig. 2h). The styles are entire but more or less reduced, and three of them are fused on top of the ovary to form fleshy stigmatic tissue (Fgi. 2t).

Reverchonia

Reverchonia is a monotypic genus of a highly specialized desert annual (*R. arenaria*; Fig. 1j)) found in sand dune areas of the southwestern United States and northeastern Mexico (Webster 1963). Although the genus has a series of distinctive floral characters and an unusual habitat, it is deeply nested within *Phyllanthus* s. l., based on a molecular phylogenetic analysis (Kathriarachchi et al. 2006). The flowers are dark reddish and borne on axillary cymules, each with single female and several male flowers. The clayx lobes are slender, obtuse, and constricted in both sexes. The male flowers have two free stamens, and the females three, dilated and slightly bifid styles.

Breynia

Breynia comprises ca. 35 species of shrubs or trees occurring from India and Sri Lanka to Japan, Australia, and the Pacific. Most species of the genus have obconic or turbinate male flowers with completely fused, apically inflexed calyx, and female flowers with medially fused, minute styles (Chakrabarty and Gangopadhyay 1996). These floral characteristics are most likely associated with specialization to *Epicpehala* pollination (Kawakita and Kato, 2004b). However, a few *Breynia* species have less specialized floral morphologies including fused but apically simple male calyx, and conspicuous and bifid styles. I sampled one such species, *B. retusa* (Fig. 1k), distributed widely in the Indo-Malesian tropics. The plants have yellow male flowers with connate stamens (Fig. 2i) and greenish female flowers with three, basally connate and distally bifid styles (Fig. 2u). The female flowers are borne singly on axillary cymules among several male flowers.

Sauropus

Sauropus is also an entirely Old World genus of herbs, shrubs, and rarely tall trees. There are ca. 80 species distributed from India and Sri Lanka to Southeast Asia and Australia, but the group is probably unnatural with respect to the Australian taxa. The Asian species are further paraphyletic with respect to embedded *Breynia*. Most of the species, including the three species studied here (*S. quadangularis*, *S. brevipes*, and *S. androgynus*; Fig. 1l), are characterized by the fused, discoid calyx in the male flowers with connate, flat filaments bearing anthers horizontally or downwardly at the corners of the triangular connective (Fig. 2j–l), and the bifid and mostly horizontal styles in the female flowers (Fig. 2v–x).

PREVALENCE OF *EPICEPHALA* ASSOCIATES IN PHYLLANTHEAE

To determine whether or not each species is associated with seed-parasitic *Epicephala* moths, I collected fruits that are fully developed in size, and examined the intensity of *Epicephala* infestation under a light microscope. Larval feeding is usually complete or near complete at this stage, so the proportion of the seeds destroyed in each fruit can be reliably measured. Larvae that emerged from the fruit samples were kept in plastic containers under room temperature (25°C) to rear out adult *Epicephala* moths. Information on locality and study period is given in Table 1, and sample sizes are given in Table 2.

To assess whether or not *Epicephala* is involved in pollination, I also collected the flowers and examined the ratios of pollinated and non-pollinated

flowers with *Epicephlala* oviposition. Actively pollinating female *Epicephala* always lays an egg in the flower that she pollinates (Kato et al. 2003; Kawakita and Kato 2004a,b), thus the link between successful pollination and oviposition is a required condition in *Epicephala*-pollinated plants. I examined each female flower under a light microscope to check for pollination status, and searched for *Epicephala* eggs in the following three-step procedure. First, I searched for eggs laid superficially on the floral surface, especially within the interspace between the ovary and calyx lobes, or underneath the horizontally dilated styles. Second, I looked for oviposition scars on floral surface, which would be left as obvious black dots if the eggs were laid internally. Lastly, I cut the flowers vertically through the median line to check for eggs laid from the floral apex, in which case the oviposition scar is usually difficult to locate. These criteria encompass all the known patterns of egg deposition in *Epicephala* (Kawakita and Kato, unpublished), thus are sufficient to detect or rule out the presence of oviposition. Sample sizes of flowers are given for each species in Table 3.

Identification of potential pollinator fauna

To study potential pollinator fauna for each species, I sampled flower visiting insects and assessed the amount of pollen attached to their bodies. Observations were made between 0900 and 1600 h under fine weather condition, and all flower visitors were captured by netting. For species that were found to have associations with *Epicephala*, I also made night observations between 1900 and 2300 h to study the oviposition site and presence/absence of pollination behavior. Visitation rate was calculated for each insect species as the number of individuals collected per hour per plant. For each insect specimen, I counted the number of pollen grains attached to the body under a light microscope. Pollen was checked against that of the focal plant for its size, shape, and color to ensure that I counted pollen grains of the correct species.

Test for effectiveness of ant pollination

In several species of herbaceous *Phyllanthus*, ants were frequently observed on the flowers (see RESULTS). Because some studies show that antibiotic substances secreted from metapleural glands of ants have inhibitory effect on pollen germination (Beattie et al., 1985), I experimentally tested whether or not ant visitation results in fruit set in *P. lepidocarpus*. For this purpose, 50 plants of *P. lepidocarpus* were collected from a wild population in September 2004 at the Kyoto University campus and transplanted individually in 500 ml pots. I kept the plants indoors for one week and marked 15–39 female flowers per plant that newly opened during this period. I then applied the following four treatments: 1) the plants were caged with fine netting (cage, 50 cm × 80 cm × 60 cm; mesh size, 0.25 mm) to exclude all flower visitors (N = 15), 2) caged as above, but ants were allowed to freely forage through a slit (width, 5 mm) at the bottom of the cage (N = 15), 3) caged and self-pollinated by hand (N = 5), and 4) left uncaged as control (N = 15). The experimental pots were placed in a greenhouse where ants were abundant for a period of one week, and proportion of the marked flowers that developed into fruits was calculated for each plant. During the experiment, I monitored the 'ants-only cage' at various times of the day between 800 h and 2200 h for a total of 12 h and confirmed that no insects other than ants (*Leptothorax* sp.) were present in the cage.

Results

Associations with *Epicephala* moths in Phyllantheae

Inspection of field-collected fruits indicated that seed parasitism by *Epicephala* moth occurred in the following eight species: *F. suffruticosa* from Takedao and Makiyama, *P. ussuriensis*, *P. reticulatus*, *P.* sp., *P. lepidocarpus* from Nohara and Omoto, *P. amarus* from Thakhaek, *P. marojejiensis*, and *P. humbertii* (Table 2). *Epicephala* moths emerged from the eight species were all undescribed, and those collected on different hosts were clearly distinct from each other based on adult size, wing pattern, and male genital morphology. This indicates that the eight species are associated with different *Epicephala* species, each having a very narrow host range. The rate of fruit parasitism by *Epicephala* varied greatly among species and populations (data were not available for *P.* sp. and *P. amarus* from Thakhaek, due to degradation of the fruit samples). The proportion of infested fruits varied from 17.4 % in *P. lepidocarpus* from Nohara to 89.5 % in *F. suffruticosa* from Makiyama. Within infested fruits, *Epicephala* larvae destroyed nearly all the seeds, except in *P. reticulatus* in which *Epicephala* destroyed 35.2 % of the seeds on average.

In addition to *Epicephala* moths, I also found braconid wasps, *Bracon* spp., that fed on the seeds in fruits of *P. reticulatus*, *P.* sp. *P. pucheroides*, and *P. roseus*. The wasps emerged from fruits as adults after pupating in the seeds. The proportions of fruits infested by *Bracon* spp. were 79.2 % and 75.5 % in *P. pulcheroides* and *P. roseus*, respectively, and within each infested fruit, 57.5 % and 43.0 % of the seeds were destroyed on average. The intensity of seed damage was not determined for *P. reticulatus* because it was not possible to discriminate between damages caused by *Epicephala* and *Bracon*. A carposinid moth, *Paramorpha* sp., also infested 12.3 % of the fruits in *B. retusa*, whose larva each destroyed all the seeds in one fruit.

Table 2. Presence/absence and intensity of fruit parasitism by *Epicephala*.

Genus	Species	Locality	Fruits examined (individuals)	Ovules per fruit[a]	Fruits infested by *Epicephala* (%)	Seeds destroyed per infested fruit[b] (%)
Flueggea	*F. suffruticosa*	Takedao	461 (4)	6	51.0	94.6 ± 10.7
		Akakina	115 (2)	6	0	—
		Manzamou	263 (5)	6	0	—
		Makiyama	143 (2)	6	89.5	100.0 ± 0.0
	F. virosa	Vieng Xai	243 (3)	6	0	—
Phyllanthus	*P. (Isocladus) ussuriensis*	Machida	29 (12)	6	44.8	100.0 ± 0.0
		Kameoka	148 (6)	6	0	—
	P. (Isocladus) virgatus	Dongmakhai	233 (31)	6	0	—
	P. (Kirganelia) flexuosus	Kibune	193 (4)	6	0	—
		Takedao	51 (1)	6	0	—
		Inohae	79 (2)	6	0	—
	P. (Kirganelia) oligospermus	Kubura	123 (5)	6	0	—
	P. (Kirganelia) reticulatus	Henchun	61 (3)	16.4 (12–20)	78.7	35.2 ± 16.0
	P. (Kirganelia) sp.		n. a.	n. a.	n. a.	n. a.
	P. (Kirganelia) tenellus	Oura	322 (7)	6	0	—
	P. (Cicca) acidus	Vientiane	200 (2)	6	0	—
	P. (Emblica) emblica	Ban Chome Sy	111 (2)	6	0	—
	P. (Phyllanthus) lepidocarpus	Kyoto	100 (5)	6	0	—
		Nohara	184 (10)	6	17.4	100.0 ± 0.0
		Omoto	207 (10)	6	49.8	100.0 ± 0.0
	P. (Phyllanthus) amarus	Ohama	212 (6)	6	0	—
	P. (Phyllanthus) debilis	Ohama	76 (7)	6	0	—
	P. (Eriococcus) liukiuensis	Manzamou	139 (16)	6	0	—
	P. (Eriococcus) pulcheroides	Mahaxai	53 (6)	6	0	—
	P. (Phyllanthodendron) roseus	Phialath	53 (3)	6	0	—
	P. marojejiensis	Mt. Marojeji	49 (6)	6.2 (6–8)	77.8	97.5 ± 6.8
	P. humbertii	Mt. Marojeji	27 (5)	6	88.9	97.9 ± 5.6
Reverchonia	*R. arenaria*	Bingham	266 (18)	6	0	—
Breynia	*B. retusa*	Dongsanghin	138 (4)	6	0	—
Sauropus	*S. quadangularis*	Dongmakhai	43 (11)	6	0	—
	S. brevipes	Dongmakhai	19 (8)	6	0	—
	S. androgynus	Thakheak	78 (6)	6	0	—
	S. granulosus		n. a.	n. a.	n. a.	n. a.

[a]Mean and range in parentheses for species with variable ovule number.
[b]Mean ± SD.

Table 3. Presence/absence of *Epicephala* oviposition in female flowers.

Genus	Species	Locality	Flowers examined (individuals)	Flowers pollinated	Pollinated flowers with eggs (%)	Non-pollinated flowers with eggs (%)
Flueggea	*F. suffruticosa*		n. a.	n. a.	n. a.	n. a.
	F. virosa		n. a.	n. a.	n. a.	n. a.
Phyllanthus	*P. (Isocladus) ussuriensis*	Kameoka	275 (6)	217	0	0
	P. (Isocladus) virgatus		n. a.	n. a.	n. a.	n. a.
	P. (Kirganelia) flexuosus	Kibune	81 (4)	72	0	0
	P. (Kirganelia) oligospermus	Kubura	182 (3)	167	0	0
	P. (Kirganelia) reticulatus	Henchun	276 (3)	120	89.2	7.7
	P. (Kirganelia) sp.	Lakxao	170 (2)	54	94.4	0
	P. (Kirganelia) tenellus	Oura	190 (10)	169	0	0
	P. (Cicca) acidus		n. a.	n. a.	n. a.	n. a.
	P. (Emblica) emblica		n. a.	n. a.	n. a.	n. a.
	P. (Phyllanthus) lepidocarpus	Kyoto	106 (5)	104	0	0
		Nobaru	129 (10)	114	0	0
		Omoto	176 (10)	162	0	0
	P. (Phyllanthus) amarus	Ohama	171 (6)	148	0	0
	P. (Phyllanthus) debilis	Ohama	68 (7)	62	0	0
	P. (Eriococcus) liukiuensis	Manzamou	82 (11)	53	0	0
	P. (Eriococcus) pulcheroides	Mahaxai	38 (4)	17	0	0
	P. (Phyllanthodendron) roseus	Phialat	158 (3)	120	0	0
	P. marojejiensis	Mt. Marojeji	173 (15)	90	80.0	0
	P. humbertii		n. a.	n. a.	n. a.	n. a.
Reverchonia	*R. arenaria*		n. a.	n. a.	n. a.	n. a.
Breynia	*B. retusa*	Dongsanghin	226 (4)	112	0	0
Sauropus	*S. quadangularis*	Dongmakhai	128 (13)	73	0	0
	S. brevipes	Dongmakhai	88 (11)	37	0	0
	S. androgynus	Thakheak	70 (6)	34	0	0
	S. granulosus	Dongmakhai	25 (5)	15	0	0

Table 4. List of insect flower visitors in various species of Phyllantheae.

Genus Species	Study sites	Hours observed Day	Hours observed Night	Insect species	Family: order	Visits / h	Pollen attachment
Flueggea							
F. suffruticosa	Takedao	0.2	11.0	*Adoretus tenuimaculatus*	Sca: Col	5.0	++
				Eucetonia roelofsi	Sca: Col	5.0	+++
				Betasyrphus serarius	Syr: Dip	5.0	+++
				Phytomya zonata	Syr: Dip	5.0	+++
				Spaerophoria philanthus	Syr: Dip	25.0	+++
				Odontomyia garatas	Str: Dip	5.0	+++
				Stomorhina obsoleta	Cal: Dip	130.0	+++
				Phasia crassipennis	Pha: Dip	5.0	+++
				Lasioglossum sp. 1	Hal: Hym	10.0	+++
				Lasioglossum sp. 2	Hal: Hym	45.0	+++
				Lasioglossum sp. 3	Hal: Hym	70.0	+++
				Apis cerana	Api: Hym	65.0	+++
				Epicephala sp. 1	Gra: Lep	3.5	
F. suffruticosa	Akakina	0.3		*Stomorhina obsoleta*	Cal: Dip	50.0	+++
				sp. 1	Mus: Dip	3.3	+
				sp. 1	Dro: Dip	3.3	+++
				Polistes jokahamae	Ves: Hym	6.7	+++
				Lasioglossum sp. 4	Hal: Hym	16.7	+++
F. virosa	Fangliao	0.3		sp.	Pla: Hem	3.3	++
				Aiolocaria sp.	Coc: Col	3.3	++
				Eristalinus quinquestriatus	Syr: Dip	6.7	+++
				Ischiodon scutellaris	Syr: Dip	6.7	++
				Paragus sp.	Syr: Dip	10.0	++
				Stomorhina obsoleta	Cal: Dip	50.0	+++
				Chrysomya sp. 1	Cal: Dip	3.3	+++
				Chrysomya sp. 2	Cal: Dip	10.0	+++
				sp. 2	Mus: Dip	6.7	+++
				sp. 3	Mus: Dip	10.0	+++
				sp. 4	Mus: Dip	20.0	+++
				Braunsapis sp.	Api: Hym	3.3	+++
				Apis cerana	Api: Hym	20.0	+++
				Apis mellifera	Api: Hym	16.7	+++
				Amata sp.	Arc: Lep	3.3	+++
				Princeps demoleus	Pap: Lep	3.3	+++
				Eurema hecabe	Pie: Lep	3.3	+

				Precis lemonias	Nym: Lep	3.3	+
				Precis iphita	Nym: Lep	3.3	+++
				Hypolimnas misippus	Nym: Lep	3.3	+
Phyllanthus							
P. ussuriensis	Kameoka	1.0		*Tetramorium* sp. 1	For: Hym	5.0	+
				Tetramorium sp. 2	For: Hym	2.0	
P. virgatus	Dongmakhai	0.3		*Paragus* sp.	Syr: Dip	6.7	++
				Camponotus sp.	For: Hym	13.3	+
				Solenopsis sp.	For: Hym	10.0	+
P. flexuosus	Kibune	8.0		*Pidonia puziloi*	Cer: Col	0.5	+++
				Pidonia simillima	Cer: Col	0.3	+++
				Ezonacerda sp.	Oed: Col	0.1	+++
				sp. 1	Cec: Dip	2.4	++
				Rhamphomyia formidabilis	Emp: Dip	0.3	++
				Episyrphus balteatus	Syr: Dip	0.1	+++
				Sphegina sp.	Syr: Dip	0.1	+++
				sp. 1	Syr: Dip	0.1	++
				sp. 2	Dro: Dip	0.1	++
				sp.	Ich: Hym	0.1	+
P. oligospermus	Kubura	3.0		*Episyrphus* sp.	Syr: Dip	0.7	++
				Chrysomya sp. 1	Cal: Dip	0.3	+++
				sp. 1	Cal: Dip	0.3	+
				Lasioglossum sp. 5	Hal: Hym	0.3	+++
P. reticulatus	Henchun	5.0	7.0	*Epicephala* sp. 2	Gra: Lep	2.9	+++
P. tenellus	Oura	0.5		—			
P. lepidocarpus	Kyoto	0.5		*Formica japonica*	For: Hym	22.0	+++
				Formica sp.	For: Hym	24.0	++
	Nohara	0.3	2.0	*Tetramorium* sp. 3	For: Hym	24.0	++
				Anoplolepis gracilipes	For: Hym	3.3	+
				Epicephala sp. 3 (on young fruit)	Gra: Lep	6.5	
	Omoto	0.3		*Technomyrmex* sp.	For: Hym	10.0	++
				Tapinoma sp.	For: Hym	10.0	++
				Tetramorium sp. 4	For: Hym	26.7	++
P. amarus	Ohama	0.3		*Monomorium* sp. 1	For: Hym	20.0	++
	Thakhaek		1.5	*Epicephala* sp. 4 (on young fruit)	Gra: Lep	4.0	
P. debilis	Ohama	0.3		*Monomorium* sp. 2	For: Hym	16.7	++
P. liukiuensis	Manzamou	3.0		sp. 2	Cec: Dip	0.7	+
P. roseus	Phialath	2.0		sp.	Cur: Col	0.5	++
				Stomorhina obsoleta	Cal: Dip	2.5	+++

				sp.	Lau: Dip	0.5	++
P. marojejiensis	Mt. Marojeji	4.0	4.0	*Epicephala* sp. 5 (on leaf)	Gra: Lep	0.3	+++
Reverchonia							
R. arenaria	Bingham	3.0		sp.	Nit: Col	0.3	
				sp. 5	Mus: Dip	1.0	+
				Lasioglossum sp. 6	Hal: Hym	0.3	+
Breynia							
B. retusa	Dongsanghin	2.0		*Clinodiplosis* sp.	Cec: Dip	4.5	++
Sauropus							
S. quadangularis	Dongmakhai	8.0		*Paragus* sp.	Syr: Dip	0.5	+++
S. brevipes	Dongmakhai	4.0		—			
S. androgynus	Thakhaek	1.0		—			
S. granulosus	Ban Thonkhan	2.0		*Halictus* sp.	Hal: Hym	0.5	+++

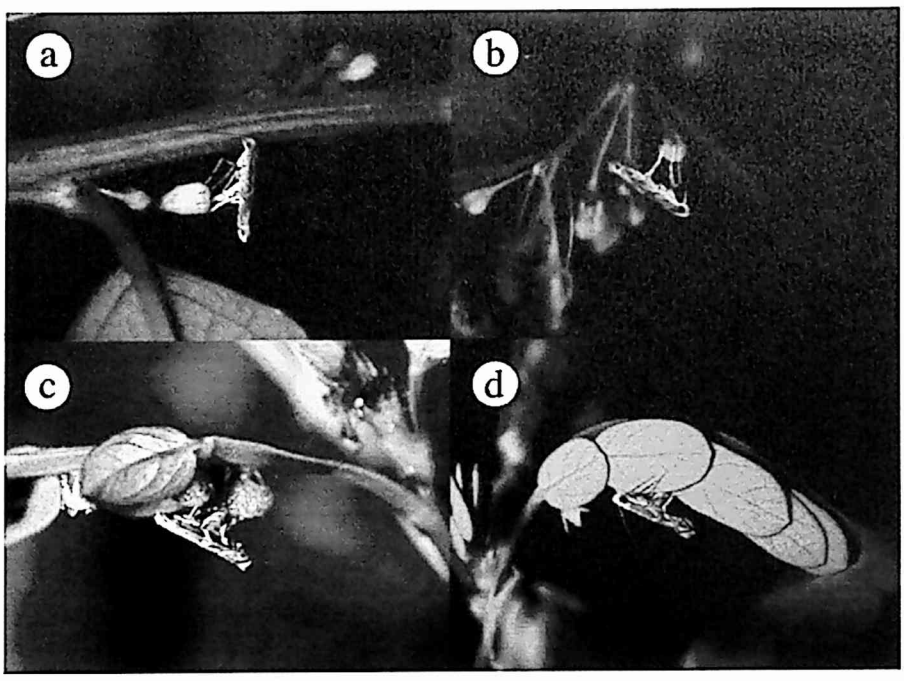


Fig. 3. Female *Epicephala* moths associated with Phyllantheae plants. a. *E.* sp. 1 ovipositing in a flower bud of *Flueggea suffruticosa*. b. *E.* sp. 2 pollinating a female flower of *Phyllanthus reticulatus*. c. *E.* sp. 3 ovipositing in a young fruit of *P. lepidocarpus*. d. *E.* sp. 4 ovipositing in a young fruit of *P. amarus*.

Table 4. Results of pollination experiment in *Phyllanthus lepidocarpus*. Fruit sets in the four treatments were significantly different (Kruskal-Wallis test, $\chi^2 = 40.01$, df = 3, $P < 0.001$). Letters with different superscript letters indicate significant difference.

	Plants	Flowers per plant	Fruit set
Control	15	20–32	99.4[a]
Caged with fine mesh	15	26–38	0.0[b]
Only ants allowed to forage	15	22–39	99.1[a]
Hand self-pollinated	5	15	100.0[a]

Epicephala eggs were found in flowers of *P. reticulatus*, *P.* sp., and *P. marojejiensis* (Table 3). In these species, eggs were laid almost exclusively in pollinated female flowers, indicating that *Epicephala* oviposition is closely linked to pollination. In *P. reticulatus*, the eggs were laid superficially underneath the epidermal tissue in pedicels or calyx lobes, whereas in *P.* sp., the eggs were laid through the apical stylar pit and embedded within the ovary tissue. In *P. marojejiensis*, the eggs were laid internally through the lateral ovary wall and placed within the interspace between the ovary wall and ovules. Floral data were not available for some species or populations, due to unavailability of flowers during the study period or degradation of the materials during transportation.

Flower visitor fauna in Phyllantheae

A list of flower-visiting insects collected during this study is given in Table 4. Overall, the most common diurnal visitors were syrphid and calliphorid flies and ants, but the composition of flower visitor fauna varied greatly among species. The flowers of *Flueggea* attracted a wide variety of insects including bees, wasps, flies, beetles, and butterflies. In contrast, the herbaceous *Phyllanthus* species were visited exclusively by ants, except in *P. virgatus* which were visited by syrphid flies in addition to ants. Visitation rates were relatively higher in *Flueggea* and herbaceous *Phyllanthus* as compared to the rest of the species. Most diurnal visitors carried pollen grains on their bodies, indicating that they act as pollinators at least to some extent.

Epicephala moths were also found visiting the plants at night. In *F. suffruticosa*, I observed the females of *Epicephala* laying eggs in flowers (N = 32), flower buds (Fig. 3a; N = 5), and developing fruits (N = 1). The moth did not pollinate the flowers as in actively pollinating *Epicephala* species, and none of them (N = 38) carried pollen grains on their proboscis or on any other parts of their bodies. In *P. reticulatus*, I observed female *Epicephala* pollinating the flowers with their proboscis (Fig. 3b) and subsequently ovipositing into the flowers (N = 20). All the moths collected on *P. reticulatus* flowers carried loads of pollen on their proboscises. In *P. lepidocarpus* and *P. amarus*, female *Epicephala* moths oviposited in immature young fruits without pollinating (Fig. 3c,d; N = 13 and 6 for *P. lepidocarpus* and *P. amarus*, respectively), and none

of them carried pollen grains on their bodies. Although I did not observe *Epicephala* oviposition behavior in *P. ussuriensis*, *P. marojejiensis*, and *P. humbertii*, inspection of fruits at various developmental stages in *P. ussuriensis* indicated that eggs were laid in immature young fruits ($N = 4$) as in *P. lepidocarpus* and *P. amarus*. In *P. marojejiensis*, I collected one *Epicephala* moth resting on a leaf at night, which carried a pollen load on the proboscis, suggesting that the moths associated with *P. marojejiensis* also has the actively pollinating habit.

Effectiveness of ant pollination

The results of pollination experiment in *P. lepidocarpus* are summarized in Table 5. These results clearly indicate that ants are capable of effectively pollinating the flowers in *P. lepidocarpus*. Each fruit contained six fully matured seeds in all treatments, indicating that negative effect of ant metapleural secretion is negligible at most. I also showed that the plants are self-compatible, indicating that within-plant movement by ants can also result in fruit set.

Discussion

Variations in pollinator fauna and modes of association with *Epicephala* in Phyllantheae

I have shown that there is potentially a very large variation regarding pollination system and association with *Epicephala* moths in the tribe Phyllantheae. Below I evaluate most likely pollinators and modes of association with *Epicephala* moths in different groups.

Flueggea

The two species of *Flueggea* were visited frequently by a wide array of insects, which most likely cause effective pollination. *Epicepahla* moths found in *F. suffruticosa* in Takedao and Makiyama are not involved in pollination because none of the ovipositing females I observed carried pollen on their bodies. I did not investigate whether or not *Epicephala* moths prefer to lay eggs in pollinated flowers than non-pollinated flowers, due to lack of floral samples. However, our observation that females laid eggs at various floral stages including flower buds likely indicates that they do not choose oviposition sites based on pollination status.

Phyllanthus section *Anisonema* (subg. *Kirganelia*)

The two species of section *Anisonema*, *P. reticulatus* and *P.* sp., were both associated with *Epicephala* moths specific to each species. These moths are likely exclusive pollinators of their hosts because (1) *Epicephala* oviposition was closely linked to successful pollination in both species (Table 3), (2) the moths were observed pollinating the flowers at least in *P. reticulatus* (Fig. 3b), and (3) no other flower visitors were present during the study period (Table 4). The proportion of seeds destroyed in each fruit was limited to 35.2 % on average (Table 3), indicating that there is an overall net benefit of the interaction for the plant. Both *Phyllanthus* species occur in Taiwan and Laos and are each associated with the same specific *Epicephala* moth in the two geographic areas (A. Kawakita and M. Kato, personal observations), indicating that these pollination mutualisms are well established and widespread throughout much of eastern Asia.

Herbaceous *Phyllanthus* (sections *Macraea*, *Pentandra*, *Phyllanthus*, and *Urinaria*)

All the herbaceous species of *Phyllanthus* were visited by ants and less frequently syrphid flies that carried pollen grains on the bodies, except in *P. tenellus* (section *Pentandra*) which I could not record any visitors. Cage experiment in *P. lepidocarpus* indicated that the plants are self-compatible and ants are capable of fertilizing the flowers. These observations suggest that herbaceous species of *Phyllanthus* are most likely pollinated by ants. In some populations of *P. ussuriensis*, *P. lepidocarpus*, and *P. amarus*, fruits were parasitized by host-specific *Epicephala* moths. These moths are not involved in pollination because they laid eggs in immature fruits (Fig. 3c,d), and ovipositing females did not carry pollen on their bodies. Their larvae almost invariably destroyed all the seeds within single fruits (Table 3).

Malagasy *Phyllanthus* (*P. marojejiensis* and *P. humbertii*)

The flowers of the two Malagasy species were not visited by any insect during the study, but several lines of evidence suggest that they are also pollinated exclusively by actively pollinating *Epicephala* moths. First, there was a high correspondence between pollination status and presence of *Epicephala* oviposition in *P. marojejiensis*. Second, fruits of the two species were parasitized by species-specific *Epicephala* moths, and a field-collected female of one of these species was found bearing a load of pollen on the proboscis. Lastly, the floral morphology of the two species shares several features with that of other *Epicephala*-pollinated plants, including the imbricate male calyx lobes with narrow entrance and the reduced, medially fused styles (Fig. 2h,t; Kato et al., 2003; Kawakita and Kato, 2004a,b). *Epicephala* larvae destroyed nearly all the seeds in fruits that are infested, but other fruits were left untouched (Table 2). The mechanism responsible for this pattern is unknown, but the mortality of eggs and/or early instar larvae is probably important, as shown in a different *Epicephala* pollinated species (Kawakita and Kato, 2004b) and another obligate seed-parasitic pollination system (Fleming and Holland, 1999), with similar seed destruction patterns.

Sections *Kirganelia*, *Cicca*, *Emblica*, *Eriococcus*, and subgenus *Phyllanthodendron*

The remaining groups of *Phyllanthus* were visited by various diurnal insects but at a relatively lower frequency as compared to *Flueggea* or herbaceous taxa. The fruits of these species were not infested by *Epicephala* moths, which at least indicates that they are not involved in specialized mutualisms with *Epicephala*. The insects collected on flowers mostly carried pollen grains on the bodies, indicating that they are strong candidates as pollinators. Previous study hypothesized that *P. acidus* (section *Cicca*) and *P. emblica* (*Emblica*) are wind-pollinated, based on high abundance of air-borne pollen. I was not able to test this hypothesis because the flowers of the two species were not available during the study. The other work (Reddi and Reddi, 1985) suggested that *P. pinnatus* (section *Chorisandra*), a close relative of *P. acidus* (Kathriarachchi et al., 2006), are visited by bees, ants, and flies, which points to the possibility that *P. acidus* also has animal pollinators as well.

Reverchonia, *Breynia*, and *Sauropus*

These non-*Phyllanthus* taxa are morphologically distinct but phylogenetically embedded in *Phyllanthus* s. l. The species studied here also did not have associations with *Epicephala*, as shown by the absence of *Epicephala* infestation in large fruit samples (Table 2). Pollination systems of the species are difficult to determine, as they were scarcely visited by insects. Some diurnal insects bearing pollen were collected (Table 4), but their effectiveness as pollinators must be studied more in detail. A previous study showed that two *Breynia* species (*B. vitis-idea* and *B. fruticosa*) are actively pollinated by seed-parasitic *Epicephala* (Kawakita and Kato 2004). In addition, at least three other *Breynia* species, *B. cernua* and *B. oblongifolia* in Australia and *B. disticha* in New Caledonia, are probably involved in similar mutualisms, because they are infested by host-specific *Epicephala* species and share the specialized floral morphology (A. Kawakita and M. Kato, unpublished data). However, the absence of *Epicephala* infestation and floral specialization (Table 2, Fig. 2i,u) likely indicate that *B. retusa* is pollinated by a non-*Epicephala* insect. The gall midge, *Clinodiplosis* sp., was also observed on *B. fruticosa* flowers (Kawakita and Kato, 2004), suggesting a strong association of this insect to *Breynia* plants and hence potential as a pollinator.

NON-*EPICEPHALA* INSECTS INFESTING FRUITS IN PHYLLANTHEAE

In addition to *Epicpehala* moths, I also found that braconid wasps, *Bracon* spp., are associated with fruits of Phyllantheae plants. These wasps were treated as parasitoids of *Epicephala* moths in previous studies (Kawakita and Kato, 2004a,b). However, a closer examination of larval habit suggested that the wasps do not parasitize *Epicephala* but instead feed on the seeds. This is supported by the fact that they emerged from species that do not have associations with *Epicephala* moths, such as *P. pulcheroides* and *P. roseus*. However, a further work is needed to study their larval biology in more detail and effects they may have on reproductive output of Phyllantheae plants. Also, a carposinid moth, *Paramorpha* sp. was found infesting the fruits of *B. retusa*. This species was also found in *B. vitis-idea* and *B. fruticosa* (Kawakita and Kato, 2004b and unpublished data). However, its adult habit and oviposition behavior are still unknown, which require further investigation.

ASSOCIATION BETWEEN FLORAL SPECIALIZATION AND POLLINATION BY *EPICEPHALA* MOTH

The results of this study and earlier works (Kato et al, 2003; Kawakita and Kato, 2004a,b) can be combined to evaluate patterns of floral evolution in the tribe Phyllantheae. The most prominent characteristic unique to *Epicephala*-pollinated plants is the fusion of the styles (Fig. 2p,q,t), which most likely facilitates effective receipt of pollen during active pollination by *Epicephala* females. This characteristic is seen in all the known species that are pollinated by *Epicephala* (*Glochidion*, *Breynia* excluding *B. retusa*, subgenus *Gomphidium*, section *Anisonema*, and Malagasy *Phyllanthus*), except in *B. fruticosa* which has free, erect styles. Another important feature is the reduction of the styles, most typically into non-lobed, simple projection but rarely into specialized stylar pit. However, the styles of *B. fruticosa* are bifid, and some individuals of *P. reticulatus* have weakly bilobed styles. Reduction of styles is not necessarily unique to *Epicephala*-pollinated plants, as species of section *Kirganelia* also have entire styles (Fig. 2o), although these are conspicuously extended and spreading.

Specialization in male flowers is more variable among different groups. The most intriguing modification is the narrowly imbricate or fused calyx lobes found in *Breynia*, *Gomphidium*, *Anisonema* (Fig. 2d,e), and Malagasy *Phyllanthus* (Fig. 2h). The narrow entrance of the flowers probably serves to prevent undesired loss of pollen by facultative flower visitors. However, species of *Glochidion* and nearly half the species of *Gomphidium* (section *Adenoglochidon*) have free calyx lobes, suggesting that this characteristic is also not shared among all the *Epicephala*-pollinated taxa. In addition, similar constriction of calyx occurs in *Reverchonia* and *Phyllanthodendron*, thus the trait is not unique to plants pollinated by *Epicephala*.

CONCLUSIONS

Overall, the results of this study indicated that there is considerable variation in pollination system and mode of association *Epicephala* moths in Phyllantheae. I have shown that two additional *Phyllanthus* lineages have established pollination mutualisms with seed-parasitic *Epicephala*, whereas some species of *Flueggea* and herbaceous *Phyllanthus* are associated with previously undocumented antagonistic *Epicephala*. Although robust classification and phylogenetic relationships are rapidly becoming available for Phyllantheae, a better understanding of evolutionary history of the Phyllantheae–*Epicephala* association would come from phylogenetic analyses targeted at species with known life histories. Such analyses may reveal the frequency and timing of the evolution of the specialized mutualism with *Epicephala*, as well as evolutionary conditions or trait changes that may have been involved. In addition to phylogenetic issues, there are still a large number of taxa for which pollination systems are unknown. Examination of herbarium specimens at University of California, Davis indicates that species of the Central American subgenus *Xylophylla* and Neotropical section *Nothoclema* are also infested by *Epicephala* (judged based on exit holes and characteristic pupal cocoons; A. Kawakita, personal observations), thus the phylogenetic and geographic distribution of *Epicephala*-pollinated species may even be broader. Future progresses in evolutionary studies of Phyllantheae–*Epicephala* association would certainly depend on reconstruction of a well-resolved phylogeny and improved knowledge of pollination system in various lineages.

Chapter 7

Multiple origins of obligate pollination mutualism in the Phyllanthaceae–*Epicephala* association

INTRODUCTION

Mutualisms form an integral part of many communities and often play an important role in the maintenance and promotion of biodiversity (Herre et al. 1999; Thompson 2005). The obligate mutualisms between flowering plants and their seed-parasitic pollinators represent perhaps some of the most tightly integrated cases of interspecific mutualisms (Janzen 1979; Weiblen 2002; Pellmyr 2003). The fig–fig wasp and yucca–yucca moth mutualisms are two classically known examples, which have provided important model systems for understanding the origin, persistence, and coevolutionary process of obligate mutualisms (Cook and Rasplus 2003; Pellmyr 2003). Several recent studies have found intriguing new examples (Fleming and Holland 1998; Kato et al. 2003; Kawakita and Kato 2004a,b), providing promising new opportunities to study various ecological and evolutionary attributes of mutualisms.

Flowering plants commonly depend on insects for pollination, and many insects specialize on developing seeds for their diet. However, origins of pollination mutualism between plants and their seed parasites are exceedingly rare in nature (Thompson 1994, 2005; Pellmyr 1997). Previous studies have explored factors that limit or promote the evolution of mutualisms and found that various ecological conditions, such as host specificity, absence of co-pollinators, limited seed destruction, and high pollinating ability are critical to such events (Thompson and Pellmyr 1992; Pellmyr et al. 1996; Westerbergh and Westerbergh 2001; Westerbergh 2004). Although these conditions are rarely met in most plant–seed parasite interactions (Kephart et al. 2006), empirical and theoretical studies suggest that obligate mutualisms can quickly evolve among lineages with necessary life history traits, sometimes resulting in multiple origins of similar mutualisms within the same association (Thompson 1994, 2005; Pellmyr et al. 1992; Pellmyr et al. 1996).

The recently discovered association between Phyllanthaceae plants and seed-parasitic *Epicephala* moths is particularly useful for testing this prediction, because obligate mutualisms are reported in several different genera or subgenera (Kato et al. 2003; Kawakita and Kato 2004a,b), while many other related species are pollinated by non-*Epicephala* insects (Chapter 6). Mutualism in this association is similar to those found in figs and yuccas in that the plants are pollinated actively and exclusively by the ovipositing females of *Epicephala* moths, whose larvae in turn consume some of the developing seeds. In addition, the association involves parasitic *Epicephala* species that do not pollinate flowers, thus provides interesting opportunities to explore evolutionary pathways leading to transition between mutualism and antagonism. In this chapter, I conduct molecular phylogenetic analysis of Phyllanthaceae plants and *Epicephala* moths to determine the phylogenetic origin of obligate pollination mutualism in this association. The present results indicate striking convergence of obligate mutualisms in this association, which have arose repeatedly as much as five times in the plant lineage. The emerging picture of historical diversification in this obligate mutualism is provides wealth of opportunities to test specific predictions regarding the origin of mutualism and historical role of coevolutionary processes in shaping reciprocal diversification.

MATERIALS AND METHODS

PHYLOGENETIC ANALYSIS

Molecular phylogenetic analysis was focused on the tribe Phyllantheae, which includes several lineages that have established mutualisms with *Epicephala* moths. The analysis included a total of 49 species of Phyllantheae and 25 species representing the remaining seven tribes in the family (Hoffmann et al. 2006). The phylogeny was rooted with two species of Picrodendraceae, which represents putative sister group to Phyllanthaceae (Wurdack et al. 2004). The analysis was based on a ca. 1.3 kb stretch of chloroplst DNA encoding the maturase K (*matK*) gene, which has previously been shown to be particularly useful for phylogenetic analysis of the family and tribe (Samuel et al. 2005; Kathriarachchi et al. 2005, 2006). Sequences of Phyllantheae plants were newly obtained for this study; the remaining sequences were adopted form previous studies (Kathriarachchi et al. 2005).

For *Epicephala*, we sampled a total of 38 species obtained from fruits of various species of Phyllantheae. The species treated here are currently all undescribed, but the sampled individuals each represent distinct species based on adult size, wing pattern, and male genitalic morphology (A. Kawakita, unpublished data). For outgroups, eight species from four putatively closely related genera were sampled and sequenced. Phylogenetic analysis was based on the elongation factor-1 alpha (EF-1α) gene, which is widely used in higher-level phylogenetics in insects (Cho et al. 1995; Danforth 2002).

Protocols for DNA extraction, polymerase chain reaction (PCR), and sequenceing follow those

Table 1. Nodal age constraints used to estimate divergence times in Phyllanthaceae

Node	Fossil taxon	Age (mya)	Reference
Actephila–Leptopus	*Actephila*	37	Gruas-Cavagnetto and Köhler (1992)
Bischofia–Scepeae	*Bischofia*	39	Gruas-Cavagnetto and Köhler (1992)
Phyllantheae stem group	*Phyllanthus*[a]	54	Gruas-Cavagnetto and Köhler (1992)
Phyllanthaceae–Picrodendraceae	—[b]	108	Davis et al. (2005)

[a]This fossil is considered here as stem group Phyllantheae, as *Phyllanthus* in the broadest sense encompasses the whole tribe.

[b]Based on maximum age of the family obtained in a previous study.

described in Kawakita et al. (2004). For PCR and sequencing of the *matK* gene, the following primers were used (located in order from 5′- to 3′-end): matk6 (forward) 5′-TA AAR CGT TTA ATT ACT CG-3′, matk3 (forward) 5′-GR TTY TTT CTT CAC GAG TAT TG-3′, matk4 (reverse) 5′-C GAG RGA YTG YTT CGA TAA TG-3′, and matk2 (reverse) 5′-ACA AAY AAT ATC MAA ATA CC-3′. The primers used for EF-1α are the same as those in Kawakita et al. (2004).

The alignment of *matK* sequences was conducted using Clustal X (Thompson et al. 1997) with manual adjustments of obvious misalignments. Sequences of EF-1α had not length variation, thus the alignment was straightforward. For each data set, I obtained most-parsimonious (MP) trees by heuristic searches with 100 random addition analyses and tree bisection-reconnection (TBR) branch swapping using PAUP* ver. 4.0b10 (Swofford 2002). Robustness of the MP trees was validated with bootstrap analysis with 1000 replications and decay indices (Bremer 1994). Command file for calculating decay indices was generated using TreeRot ver. 2 (Sorenson 1999).

We also conducted Bayesian phylogenetic reconstruction using mrbayes 3.1.2 (Ronquist and Huelsenbeck 2003). Prior to the analysis, we used the program MrModeltest 2.2 (written by J. A. A. Nylander: http://www.ebc.uu.se/systzoo/staff/nylander.html) to select an appropriate model of base substitution for each gene partition. Bayesian analysis consisted of running four simultaneous chains for 2 million generations, sampling trees every 1000 generations for a total of 2001 trees. We plotted ln-likelihood of the sampled trees against generation time to identify the burn-in period at which the parameter estimates reached a plateau. Accordingly, we discarded the initial 501 trees and the remaining 1500 samples were used to estimate the tree topology. To ensure that the analyses were not trapped in local optima, we carried out three separate runs and compared parameter estimates for consistency.

Reconstruction of ancestral character states

To infer the phylogenetic origin of obligate pollination mutualism in Phyllanthaceae and *Epicephala*, we mapped onto the obtained phylogenies the presence/absence of *Epicephala* pollination in Phyllantheae plants and active pollination behavior in *Epicephala*. Specialized *Epicephala* pollination is present in species of *Glochidion*, *Breynia*, *Phyllanthus* subgenus *Gomphidium*, section *Anisonema*, and an unclassified group of Malagasy endemics (Kawakita and Kato et al. 2004a,b; Chapter 6). Although we do not have sufficient ecological data for all the included species for these groups, obvious similarity of reduced floral morphology and confirmation of the presence of seed-feeding *Epicephala* (A. Kawakita and M. Kato, unpublished data) provide rationale for coding these species as having the specialized pollination system. The only exception is *B. retusa*, which has markedly different floral morphology and lacks the association with *Epicephala* (Chapter 6). Moths associated with the above five groups were coded as active pollinators, while species that are associated with *F. suffruticosa* and herbaceous *Phyllanthus* are coded as non-pollinators (Chapter 6). Only species within Phyllantheae and *Epicephala* were included in the analysis to prevent possible interference by outgroup character states on overall reconstruction.

To infer the ancestral pollination habit of Phyllantheae and *Epicephala*, I optimized character states onto the Bayesian tree with the highest likelihood score using the program Multistate (Pagel 1999). This program estimates the likelihood of alternative states by taking into account branch lengths and state transformation rate parameters estimated from data. I used the general Mk1 model of Lewis (2001), which was judged by likelihood ratio test as not significantly worse than a more complex model ($P > 0.1$).

Divergence time estimates

Analysis of ancestral character reconstruction indicated that obligate mutualism arose multiple times in Phyllantheae (see Results). However, considering that such analyses can often produce equivocal results, support for a particular hypothesis can be strengthened by independent analyses. Assuming that the mutualism evolved repeatedly in Phyllantheae, it is possible that colonization of Phyllantheae by *Epicephala* occurred much later than the initial diversification of the most recent common ancestor (MCRA) of *Epicephala*-pollinated species. I therfore tested whether or not the origin of active

pollination behavior in *Epicephala* postdates the initial divergence of the MCRA of *Epicephala* pollinated lineages.

To infer divergence time estimates for the two groups, I chose the Bayesian tree from above to test for rate constancy among lineages. Branch lenghts and associated likelihood scores were calculated on this tree in PAUP* under the optimal model and parameters with and without a molecular clock enfored, which resulted in rejection of global rate constancy ($P < 0.05$). Consequently, divergence times were estimated on this tree using non-parametric rate smoothing (NPRS; Sanderson 1997) method as implemented in r8s ver. 1.71, which relaxes the assumption of molecular clock by optimizing rate changes among neighboring branches. To estimate standard errors associated with divergence times, we used the non-parametric bootstrapping strategy: 100 bootstrapped data sets were generated, and branch lenghts were estimated using the Bayesian topology and parameter estimates with the highest likelihood using PAUP*. The resulting branch lengths were used to calculate the 95 % confidence interval in divergence time estimates.

To calibrate the Phyllanthaceae tree, we used three palynofossils from the Tertiary (Gruas-Cavagnetto and Köhler 1992) as reliable minimun age constraints for several internal nodes, and the estimated maximum date (108 mya; Davis et al. 2005) of the divergence between Phyllanthaceae and Picrodendraceae as the maximum age constraint (Table 1). Pollen fossils of *Phyllanthus* were considered conservatively to represent the stem group Phyllantheae, because circumscription of the genus in the broadest sense encompasses the entire tribe (Kathriarachchi et al. 2006).

In contrast, there are no reliable fossils for *Epicephala* or related moth genera within Gracillariidae, which prevents the estimation of moth divergence times using geological evidence. I therefore used ecological associations between the plants and moths to constrain several internal nodes on the *Epicephala* phyogeny. Specifically, we assumed that the crown group ages of well-defined, species-rich *Epicephala* clades should not predate those of associated host clades, because the moths are highly specific to and obligately dependent on their hosts and thus are unlikely to have diverged in the absence of host diversification. I used the estimated optimal crown group ages of *Glochidion*, *Breynia*, and section *Gomphidium* as maximum age constraints for the crown group node of associated *Epicephala* pollinators. Because the r8s program requires both maximum and minimum constraint, or at least one node to have a fixed age, I estimated relative node ages by fixing the root node to have the age of one (as suggested by the program), and subsequently imposed the constraints to estimate maximum possible ages for the focal nodes.

In addition, I also used the stem group age of the three host clades to account for possible effect of incomplete sampling on overall estimates. This option most likely provides overestimates for the maximum age of *Epicephala*, but is highly conservative when testing the hypothesis of delayed host colonization. The maximum possible ages of the pollinators should reside in between the two estimated divergence times.

RESULTS

The obtained phylogenies for the plants and moths are given in Figs. 1 and 2, respecitvely. Overall, parsimony and likelihood analyses gave nearly identical topologies. Most nodes on the plant phylogeny are well supported, and the results are highly consistent with previous studies (Kathriarachchi et al. 2006). *Phyllanthus* is shown to be paraphyletic with respect to embedded *Reverchonia*, *Sauropus*, *Breynia*, and *Glochidion*. The five groups that have mutualistic associations with *Epicephala* pollinators (*Glochidion*, *Breynia*, *Gomphidium*, *Anisonema*, and Malagasy *Phyllanthus*) are clearly non-monophyletic, suggesting that multiple shifts in pollination systems have taken place. The phylogenetic tree of *Epicephala* revealed that major moth clades are generally specific to single plant taxonomic groups. However, the overall relationships are not concordant with those of the plants, indicating that multiple host colonizations have taken place. The actively pollinating lineages do not form a monophyletic group, and species parasitizing herbaceous *Phyllanthus* grouped together on the phylogeny, despite their associations with divergent host taxa.

The results of ancestral character reconstructions indicated that several critical nodes on the phylogeny are robustly supported as having non-*Epicephala* pollination system (Table 2; Fig. 3). The inferred absence of mutualism in MRCA of *Sauropus* and *Breynia*, and that of *Glochidion*, *Phyllanthodendron*, *Saurpous*, and *Breynia* clearly indicate that mutualisms arose independently in *Glochidon* and *Breynia*, with a single loss of mutualism in *B. retusa*. Significance was not found for the remaining nodes, but the overall high probability of non-*Epicephala* pollination is consistent with the hypothesis of recurrent independent evolution of mutualism in Phyllantheae. In total, the analysis of ancestral character reconstruction indicates that the mutualism originated at least three times and lost once within Phyllantheae. Character reconstruction in *Epicephala* gave more consistent results (Fig. 3), which indicate that active pollination behavior evolved once and subsequently lost at least once, although it is uncertain whether or not the *Epicephala* species associated with *Flueggea* represents the ancestral

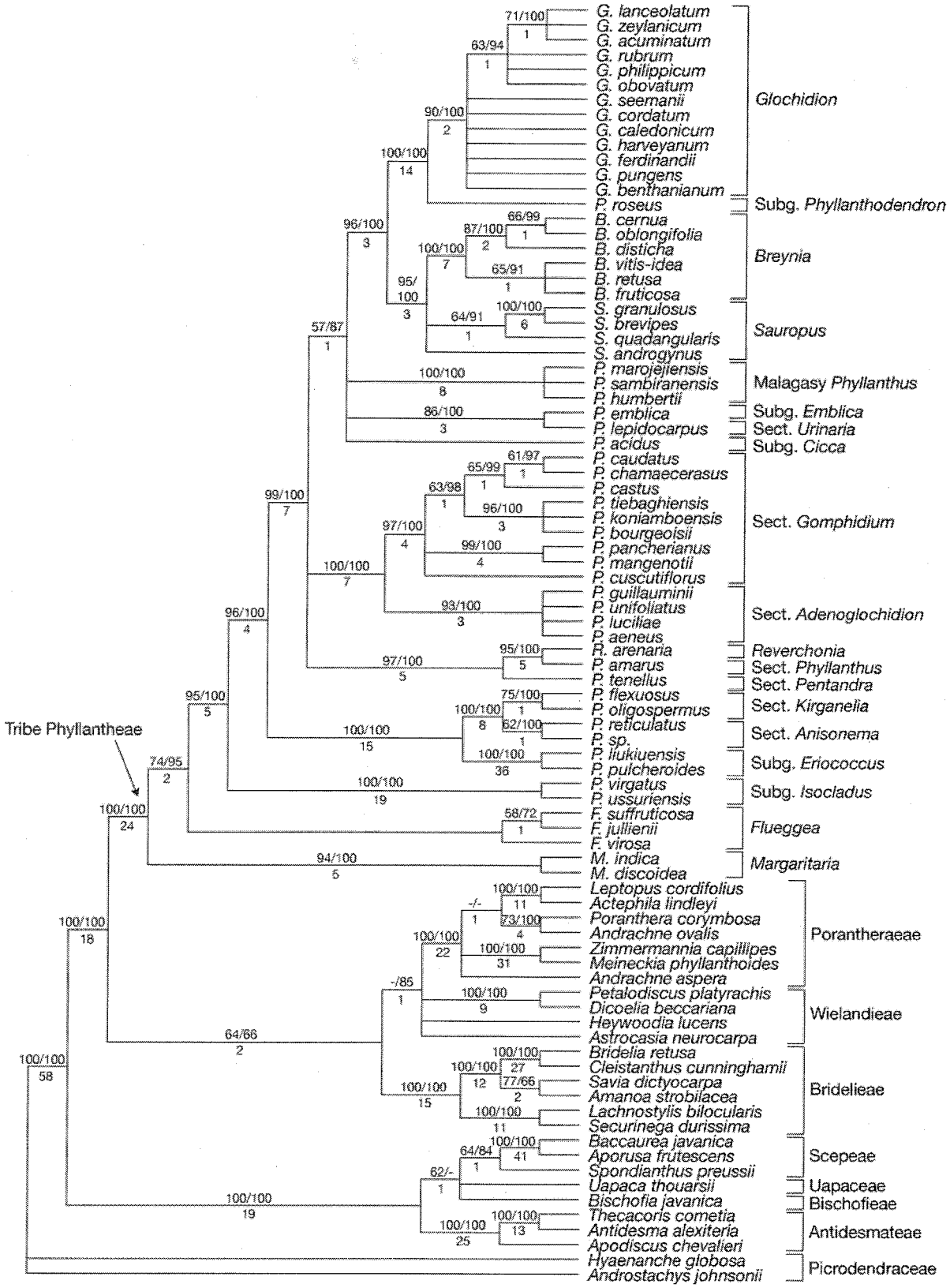


Fig. 1. Strict consensus tree of 1146 most-parsimoniuos trees (length, 1847; CI = 0.60; RI = 0.81) based on 1.3 kb of chloroplast *matk* gene. Numbers above nodes indicate parsimony bootstrap values followed by Bayesian posterior probabilities. Those below branches are decay indices.

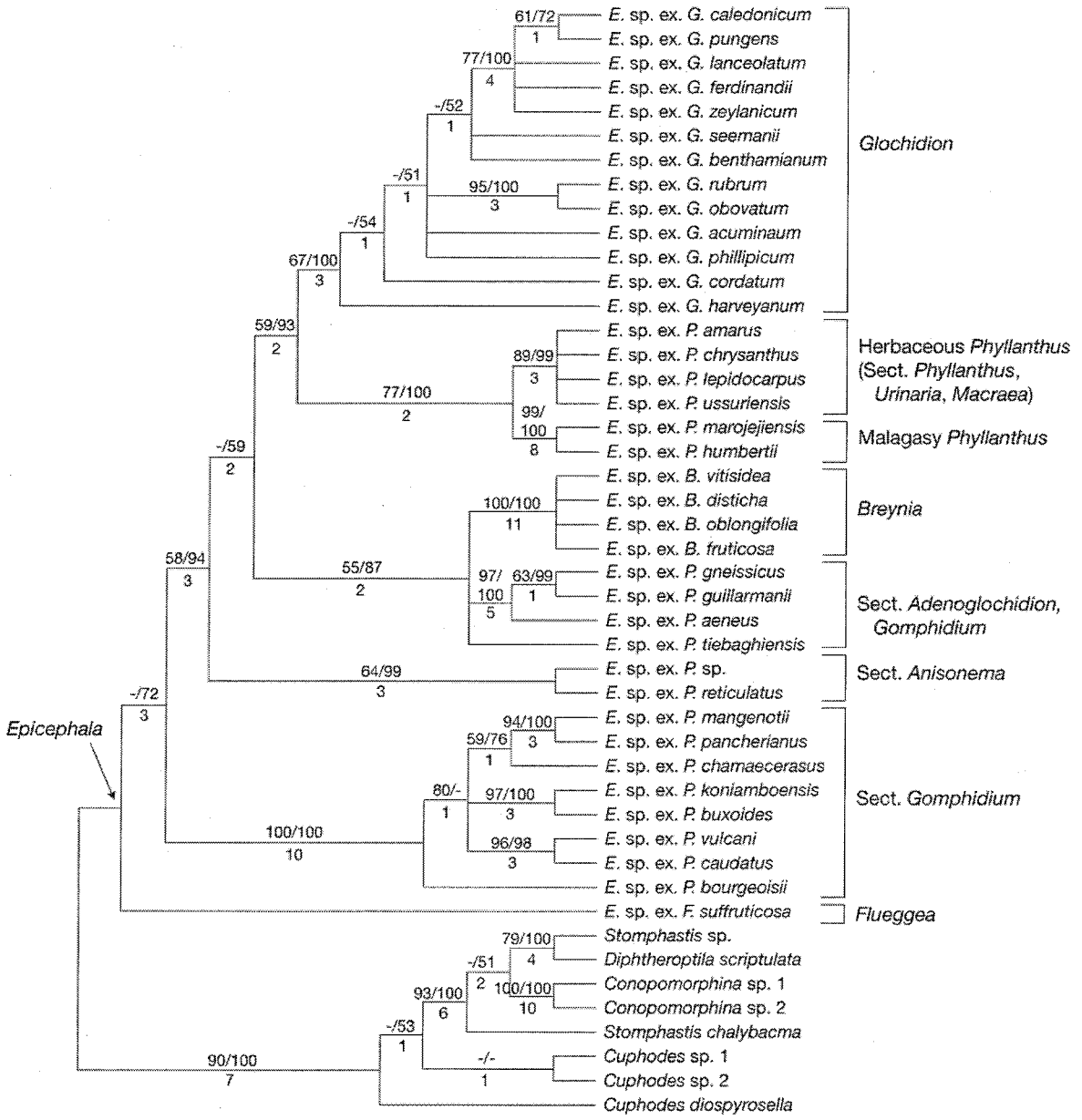


Fig. 2. Strict consensus tree of 2393 most-parsimoniuos trees (length, 536; CI = 0.44; RI = 0.73) based on ca. 550 bp of nuclear EF-1α gene. Numbers above nodes indicate parsimony bootstrap values followed by Bayesian posterior probabilities. Those below branches are decay indices.

antagonistic state in the genus.

Analysis of divergence times indicated that early divergences within the tribe Phyllantheae date back to early Tertiary (Fig. 3), which corresponds to the period of warm climate when high latitudes harbored tropical rainforest vegetations as those inhabited by recent members of Phyllanthaceae (Morley 2000). The MRCA of *Epicpehala*-pollinated plants (Node 7 in Fig. 3) was estimated to be 49.6 mya (range: 44.4–60.7 mya; Table 3). In contrast, the origin of active pollination in *Epicephala* (Node 15 in Fig. 3) is estimated to have occurred 23.5 mya (16.1–29.4 mya; Table 3), which significantly postdates initial divergence of *Epicephala*-pollinated plants. When host stem-group ages are used to constrain *Epicepahla* phylogeny, the associated age largely overlaps that of host divergence, but the optimal estimate is still younger (43.3 mya; Table 3). Considering that the use of stem-group ages could overestimate *Epicephala* divergence times, these results are consistent with the hypothesis that *Epicephala* colonized Phyllantheae that has already partly diversified in the absence of obligate mutualisms.

DISCUSSION

The result of the phylogenetic analysis indicates that the five Phyllantheae lineages with *Epicephala* mutualism are not related to each other (Figs. 1, 3), suggesting that there have been multiple occurrences of transition between specialized and generalized pollination systems in Phyllantheae. *Epicephala*

Table 2. Ancestral states for the presence/absence of *Epicephala*-pollination in Phyllanthaceae or active pollination in *Epicephala* as inferred from maximum likelihood. Proportional likelihood values are given for the two alternative states.

Node	Present	Absent
Phyllanthaceae		
Node 1	0.30	0.70
Node 2*	0.06	0.94
Node 3*	0.03	0.97
Node 4*	0.07	0.93
Node 5	0.12	0.88
Node 6	0.15	0.85
Node 7	0.18	0.82
Node 8	0.15	0.85
Node 9*	0.06	0.94
Node 10	0.40	0.60
Epicephala		
Node 11*	0.99	0.01
Node 12*	0.94	0.06
Node 13*	0.99	0.01
Node 14*	0.94	0.06
Node 15	0.82	0.18

Asterisks indicate reconstructions judged as best as determined by a decline of at least two units between states (i.e., the threshold value).

pollination could have evolved once in the MRCA of *Epicephala* pollinated plants (Node 7 in Fig. 3) and lost repeatedly, or arose multiple times in independent lineages. The present results are consistent with the latter possibility, with mutualisms evolving at least three times and as much as five times within the tribe. These results indicate that the similar specialized floral morphologies found among these plants, such as fused styles and imbricate male calyx, are the result of convergence in response to similar ecological requirements. On the other hand, the pollination habit arose only once in *Epicephala* (Fig. 3), indicating that this behavior has been of critical importance to the evolution and maintenance of the mutualism between Phyllanthaceae plants and *Epicephala* moths.

Previous studies in other obligate pollination mutualisms or less-specialized plant–seed parasite interactions have identified various ecological conditions that are necessary for the evolution of obligate mutualisms, such as host specificity, limited seed consumption, absence of copollinators, and high pollinating ability (Pellmyr and Thompson 1992; Thompson and Pellmyr 1992; Thompson 1994, 2005; Pellmyr et al. 1996; Westerbergh and Westerbergh 2001; Westerbergh 2004; Kephart et al. 2006). These requirements are not commonly met in most plant–insect interactions, but in lineages where these preconditions are present, mutualisms can arise in a relatively few evolutionary steps, ocassionally resulting in repeated evolution of similar mutualisms within a single association (Thompson 1994, 2005; Pellmyr et al. 1996). The results of this study provide novel empirical example suggesting that repeated evolution of mutualisms can occur as a predicted outcome in coevolutionary interaction with traits that promote mutualisms. Although ecological data are still insufficient to allow trait-by-trait analysis of the factors promoting mutualisms in the Phyllanthaceae–*Epicephala* association, available evidence indicate that many of the predicted conditions are common to this system. Host specificity is a general attribute of the mostly leaf-mining gracillariid moths, and the pollinating ability has obviously evolved in *Epicephala* as the direct result of coevolution with the hosts. Limited seed consumption can not be inferred for the *Epicephala* ancestor from available data, but this condition is not a prerequisite for the evolution of the mutualism, as larvae of *Epicephala* moths that pollinate some species of *Gomphodium* and *Breynia* consume all the seeds within single fruits (Kawakita and Kato 2004a,b). Ecological factors that limit seed destruction in the *Epicephala* system is not fully understood, but the phylogenetic framework provided here allows for a future assessment of their importance in promoting the evolution of mutualisms. Ancestral condition for the presence of co-pollinators is also unknown, but the loss of pollinating behavior in *Epicephala* species infesting herbaceous *Phyllanthus* suggests that Phyllantheae plants may have been commonly pollen-limited. This is because herbaceous species occupy weed-like habitats and commonly achieve nearly full fruit set (Chapter 6; A. Kawakita, personal observations), thus active pollination by *Epicephala* is redundant to efficient pollination provided by the co-pollinators. Future ecological studies would clarify factors that limit fruit production in non-*Epicephala* pollinated species, which provides important insights into ecological conditions that facilitate the evolution of mutualisms.

Table. 3. Estimated ages for the origin of obligate mutualism in the Phyllanthaceae-*Epicephala* association. Node numbers correspond to those in Fig. 3.

Node	Constraint	Min	Optimal	Max
Node 7	fossil	44.42	49.63	60.69
Node15	host crown-group age	16.12	23.49	29.36
	host stem-group age	29.73	43.31	54.14

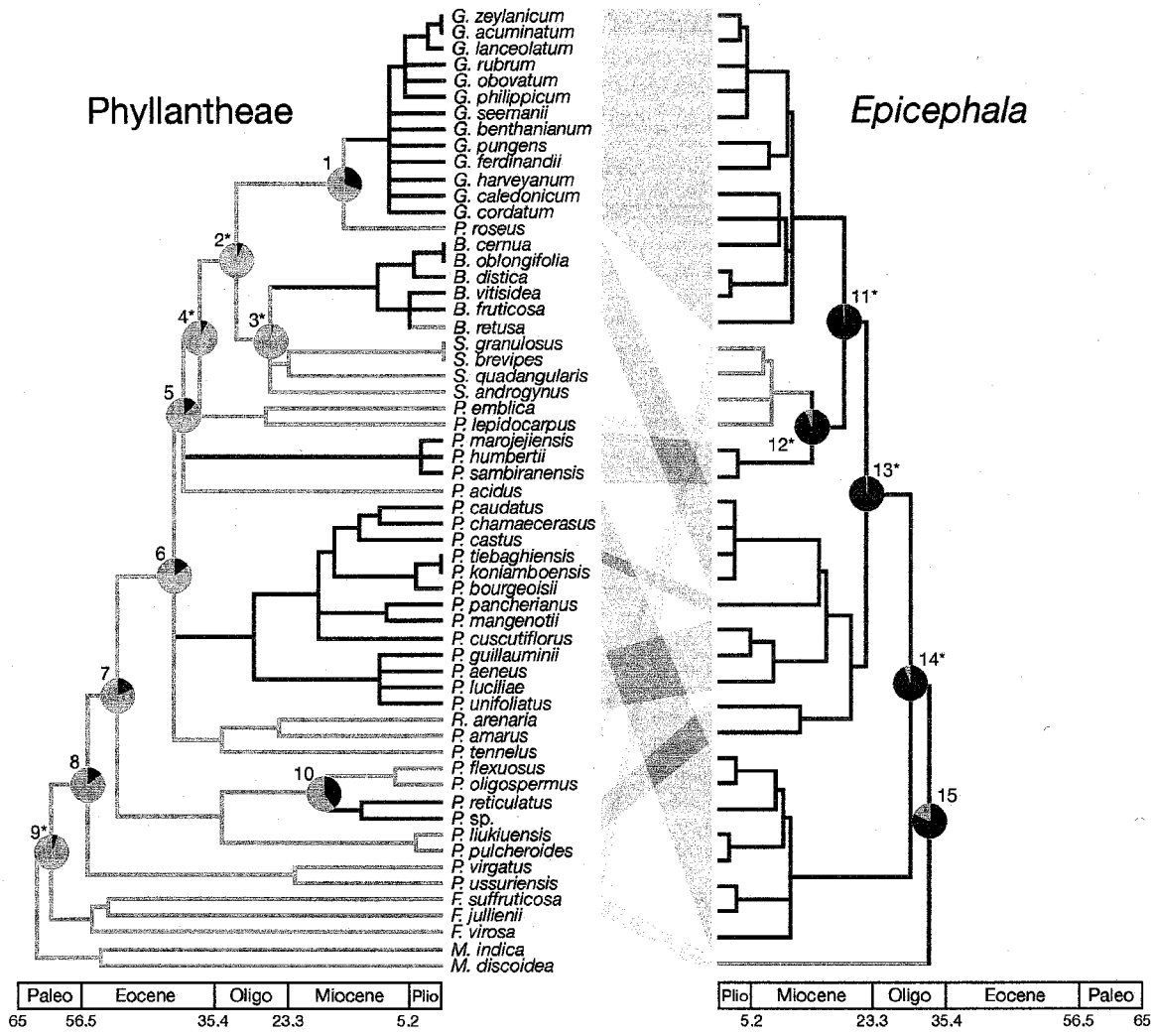


Fig. 3. Character state reconstructions and divergence time estimates for Phyllantheae and *Epicephala*. Orange indicates non-*Epicephala* pollinated taxa in the plants, and non-pollinating seed parasites in *Epicephala*; green indicates *Epicephala*-pollinated plants and actively pollinating *Epicephala*. Pie grams are given for selected nodes to indicate proportional likelihood scores for the two alternative states. Nodes with asterisk indicate significant difference in likelihood score between the two states. Branch lengths are proportional to time. Ecological links are given between species or species groups; orange and green links donate antagonism and mutualism, respectively.

Overall, the present results indicate that the genus *Epicephala* has gone through extensive diversification since the initial origin the mutualism. The currently described members of the genus merely comprise ca. 40 species with mostly unknown larval habit (de Prins and de Prins 2005), but considering the high species specificity and overwhelming diversity of potential hosts in *Glochidion* (>300 spp.), *Gomphidium* (>150 spp.), or *Breynia* (ca. 35 spp.), the genus is probably a giant group that has co-diversified with their host Phyllantheae plants. The evolution of active pollination behavior is therefore considered an evolutionary key innovation, in a sense that it is strongly linked to rapid radiation (Schluter 2000). Furthermore, repeated colonization of distant host lineages and reqruitment of new mutualists provided multiple independent opportunities for reciprocal diversification with their hosts. The historical role of coevolution in promoting diversification has been previously difficult to investigate (Becerra 1997; Farrell 1998), but the Phyllanthaceae–*Epicephala* association provides a promising candidate system for such analysis, because repeated origins of the mutualism allow for multiple independent comparisons of patterns and processes of reciprocal diversification. The phylogenetic framework presented here provides a robust baseline for testing such predictions, which will be possible once improved classification and estimates of species abundance becomes available for various lineages of Phyllantheae.

Chapter 8

General discussion

DIVERSITY OF POLLINATION SYSTEMS AND ECOLOGICAL ASSOCIATIONS WITH *EPICEPHALA* MOTHS IN PHYLLANTHACEAE

The striking discovery of the intriguing obligate pollination mutualism in *Glochidion* and *Epicephala* (Kato et al. 2003) was the strong motive of the present thesis. Before this study has taken place, there was almost no information on pollination system of plants in Phyllanthaceae or larval feeding habit of *Epicephala*. A series of field works conducted during this study has revealed the diversity of pollination systems in Phyllanthaceae and highly variable associations with *Epicephala* moths. Pollination system ranged from diurnal bee and fly pollination to ant pollination and nocturnal *Epicephala* pollination. The association with *Epicephala* varied from truly mutualistic associations to putatively plesiomorphic antagonism and secondarily evolved parasitism. Even within the mutualistic associations, there was a great degree of variation in traits that likely affect ecological outcome of the interaction, such as the proportion of seeds destroyed per larva, mode of egg deposition, and local faunal composition of *Epicephala* pollinators. The extent of variation in life history traits found in this association is comparable to, or even greater than those found in the two classic cases of obligate pollination mutualism, namely the fig–fig wasp and yucca–yucca moth associations. The great diversity of species involved, coupled with observed variation in life history attributes, provides wealth of opportunities for studying various ecological and evolutionary outcomes of mutualism, and makes this association a promising new model system in ecology and evolutionary biology.

ASYMMETRIES IN PATTERNS OF SPECIFICITY AND DIVERSIFICATION IN OBLIGATE POLLINATION MUTUALISMS

The present study found that the association between partners in obligate pollination mutualisms is largely asymmetric at various taxonomic levels. At the level of species within a range of geographic distribution, *Epicephala* species are locally specific to a single host species, although a single species can have associations with more than one host across different populations. In contrast, some host species had more than one pollinator species within a single population, or even within a single tree (Kawakita and Kato 2006). At the supraspecific level, patterns of speciation in the two partners are correlated but not completely parallel, and drastic shifts between distant host species have apparently occurred repeatedly in these interactions (Kawakita et al. 2004). At the macroevolutionary level, the asymmetry was even more prominent, with the association gained and/or lost multiple times within the plant lineage, although major moth clades were largely restricted to major host lineages.

Recent studies in other obligate mutualisms have also found similar asymmetries of association, even among interactions that have been long believed to have diversified in parallel (Aanen et al. 2002; Currie et al. 2003; Molbo et al. 2003; Villesen et al. 2004; Mikiheyev et al. 2006). For example, the interaction between figs and fig wasps has long provided textbook examples of faithful associations, but recent molecular techniques have revealed cryptic and complex associations as those found in *Glochidion* and *Epicephala* (Molbo et al. 2003). In a coevolutionary interaction between leaf cutter ants and their cultivated fungi, Mikheyev et al. (2006) found genetic evidence that fungal cultivars are commonly transmitted horizontally, and the pairwise specificity is often broken down, despite long held assumption of strict clonality and vertical transmission of the cultivars in these associations. Improved understandings of asymmetries in various coevolutionary interactions would yield specific predictions that can be further analyzed. For example, how does mutualisms remain stable in face of co-occurrence of multiple partners that can potentially disrupt the mutualism? How do asymmetries of association affect genetic structure of the partners and how does this contribute to speciation and diversification? How do global human introductions of mutualist lineages affect local ecological dynamics and evolutionary outcomes? Appreciation of widespread asymmetries in coevolving interactions provide important framework for these analyses, which are central to our understandings of coevolutionary process, mutualilsm stability, and maintenance of biodiversity.

MULTIPLE ORIGINS OF OBLIGATE POLLINATION MUTUALISMS

The present study showed that obligate pollination mutualism arose independently in several lineages of Phyllanthaceae. In constrast, there was only a single origin of active pollination behavior in *Epicephala*, indicating that this trait has been critically important in the evolution and maintenance of this intriguing association (Fig. 1). The analysis of divergence times indicated that *Epicephala* moths colonized Phyllantheae plants after they have partly diversified, and subsequently spread the mutualism among multiple plant lineages. These results illuminate the importance of evolutionary innovations

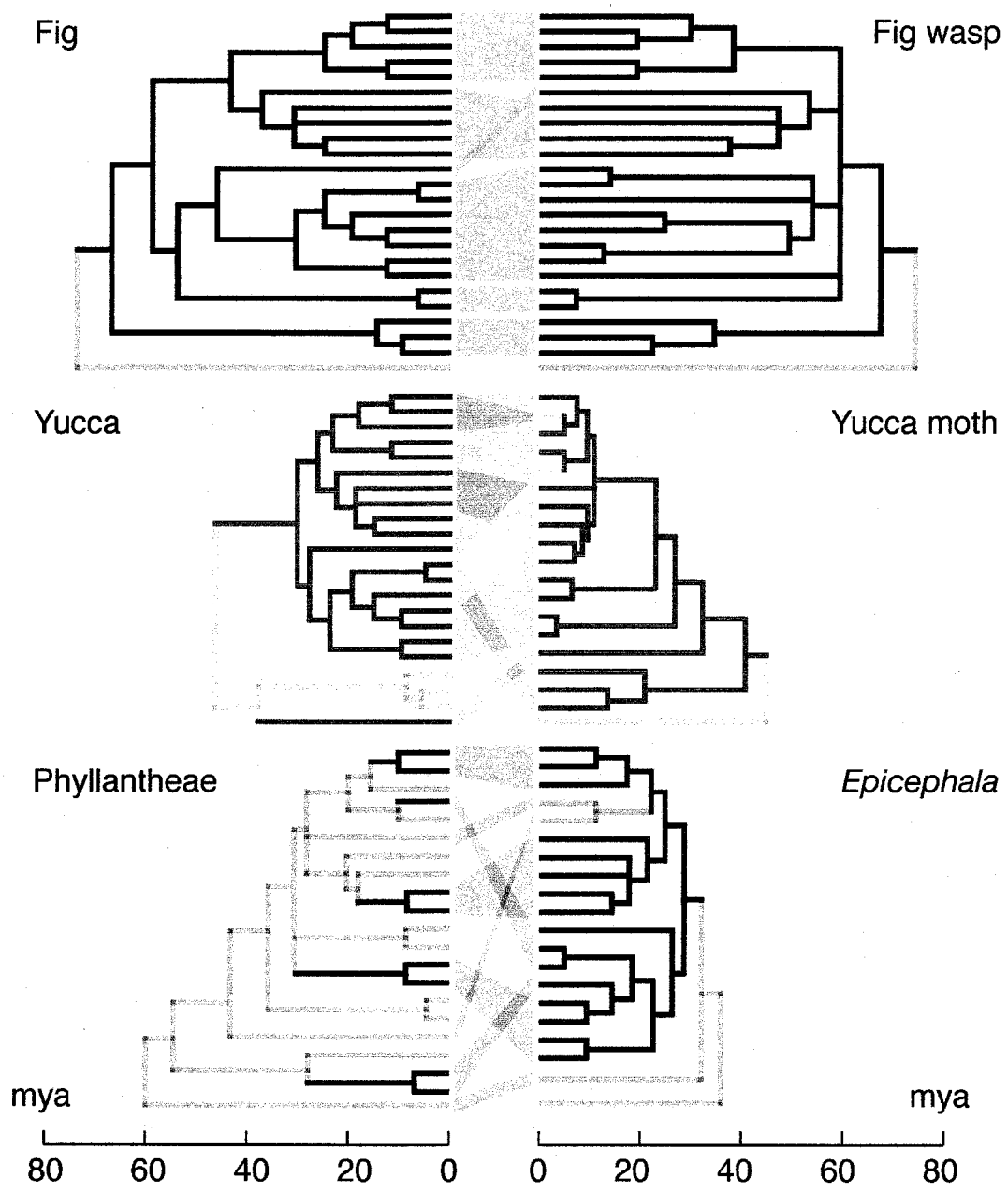


Fig. 1. Schematic illustration of phylogenetic relationships and divergence times in three systems of obligate seed-parasitic pollination. Thick lines indicate lineages involved in the mutualistic association, and thin lines indicate those that are not. The fig–fig wasp mutualism is estimated to have originated ca. 75 mya in the Cretaceous, whereas the yucca–yucca moth mutualism is suggested to have originated ca. 40 mya in the Eocene. The Phyllantheae–*Epicephala* mutualism is the youngest of the three systems. Pollination habit has evolved only once in each of the three systems and subsequently lost several times. Evolution of mutualism in the plant lineage has occurred once in the figs, twice in the yuccas, and as much as five times in Phyllantheae.

in promoting the origin of mutualisms and enormous impacts they can have on evolutionary consequences of the partner. Also, these findings highlight the importance of ecological preconditions that facilitate multiple occurrences of similar mutualistic associations (Thompson 1994, 2005; Pellmyr et al. 1996). Particularly important traits include active pollinating habit, high host-plant specificty, and pollen limitation of fruit set.

Studies in the yucca–yucca moth system has also found that active pollination behavior had been the key innovation in this mutualism (Pellmyr and Thompson 1992). Ecological conditions that have promoted the evolution of mutualisms were common in their ancestors, and these preadaptations have likely facilitated independent evolution of obligate pollination mutualisms between related yucca moths and saxifragaceous plants (Pellmyr and Thompson 1992; Pellmyr et al. 1996). The importance of ecological conditions in promoting mutualisms is also noticed in obligate protective ant–plant mutualisms such as those between pseudomyrmecines and acacias. Janzen (1966) identified ten life-history traits in the ants and 14

plant traits as critical to the mutualism, but most of the traits were already present in their ancestors or slightly modified at most. Consequently, there are at least five independent origins of obligate mutualisms in this association (Janzen 1996; Ward 1991) with production of food bodies by the plants and pruning behavior of encroaching vegetation by ants being truly novel innovations.

Mutualisms are major components of most ecosystems, and thus understanding the evolutionary process of these important interactions are critical to improving our knowledge on their roles in promoting biodiversity. A robust phylogenetic framework and details of life histories as provided in this study allow for future detailed analyses of the factors that facilitate the evolution of mutualisms. The association between Phyllanthaceae and *Epicephala* is particularly useful because the mutualisms have independent origins, and thus allow multiple comparisons within a single coevolving interaction (Fig. 1).

OBLIGATE POLLINATION MUTUALISM AS DRIVER OF RECIPROCAL DIVERSIFICATION

The obligate pollination mutualisms between Phyllanthaceae plants and *Epicephala* moths are evolutionarily successful interactions, as each of them has diversified into at least more than 500 species in total. Although only 40 species have been currently described as *Epicephala* (de Prins and de Prins 2005), the actual diversity of this genus is perhaps, with the exception of a few hyper-diverse genera (e.g., *Phyllonorycter*), one of the highest of all genera in Gracillariidae. Similarly, the tribe Phyllantheae includes more than 1200 species, being by far the largest tribe within the family (Hoffmann et al. 2006). Although identifying the historical role of coevolution in shaping diversification requires studies from various approaches, the present system provides a promising candidate for such analyses. Independent origins of mutualisms allow macroevolutionary analysis of the patterns of diversification in lineages with and without *Epicephala* pollinators, which will be possible once robust classification and estimate of species abundance for each lineage become available. Meanwhile, ecological analyses should disentangle various factors that are extrinsic to the interaction and identify truly coevolutionary traits that actually function to promote reciprocal diversification. Coevolution is one of the major processes promoting diversification and speciation (Thompson 1994; 1999), and thus insights obtained from these model systems would surely provide templates for general understandings of the coevolutionary processes shaping other, more complex plant–pollinator mutualisms.

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