Generation and maintenance of species diversity in leaf cone moths (*Caloptilia*) feeding on maples (*Acer*)

カエデ属植物を利用するハマキホソガ属蛾類の種多様性の創出と維持に関する研究

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要旨（Summary in Japanese）

植食性昆虫は地球上の記載種数の三分の一以上を構成する非常に高い種多様性を誇る。植食性昆虫がこれほど多様になった背景には、植物の化学的防御と昆虫の解毒能力の共進化が植食性昆虫による寄主植物への特殊化をもたらし、それが寄主転換による種分化を促したことが重要だったと考えられている。しかし近年の分子系統解析の結果から、植食性の進化は必ずしも多様化速度の上昇を伴わないことが明らかになっており、種分化プロセスにおける寄主転換の役割を再評価することが求められている。また、多様化速度の違いを基にした説明は、種数が時間とともに増え続けることを仮定しているが、もし種数が時間とともに飽和するならば、植食性は多様化速度そのものを上げるのではなく、種数の上限を押し上げることで多様性に貢献しているのかもしれない。つまり、種多様性の創出プロセス（種分化過程）と種多様性の維持メカニズム（種数の上限を決定する要因）の両方を理解することが植食性昆虫の種多様性を説明するためには不可欠である。

本学位論文では、カエデ属植物を寄主植物とするハマキホソガ属蛾類をモデルに、植食性昆虫の種分化における寄主転換の重要性の評価と、種数の上限に深く関わると考えられる地域的な共存種数の決定要因の解明を試みた。まず、日本の幅広い地域でのサンプリングにより、日本では未記載種 3 種を含む 14 種のハマキホソガ属がカエデ属植物を利用していることを明らかにし、その詳細な分布と寄主範囲を明らかにした。また、カエデ属以外の様々な被子植物を利用する 44 種のハマキホソガを採集し、上記の 14 種と合わせて系統解析を行ったところ、カエデ属植物を利用する 14 種のうち 13 種がウルシ属を利用する 1 種とともに一つのクレードを形成し、これらが主にカエデ属上で多様化を遂げたことを明らかになった。さらに、系統樹を使って寄主転換による種分化の重要性を調べる新しい手法を考案し、それを用いて解析を行った結果、より近縁なハマキホソガ属は利用する寄主植物が似ている傾向があった。つまり、従来の寄主転換が種分化を促進するという見方とは異なり、ハマキホソガ属

蛾類の全ての種分化が寄主転換を伴うわけではない。

一方、地域的な共存種数を決定する要因として、寄主利用様式、季節消長、寄生蜂群集の種間における違いに着目し、東京大学秩父演習林と京都大学芦生研究林において、局所的に共存するハマキホソガ属蛾類の生態を調査した。秩父演習林には7種のハマキホソガが、芦生研究林には10種のハマキホソガが共存しており、共存する種の間では寄主利用範囲が異なる傾向があることが分かった。それぞれのハマキホソガは、特定の一種、あるいは葉の化学物質（タンニン含有量やC/N比）や構造的性質（葉厚）が類似した系統的に近い数種のカエデ属植物を利用していたことから、同所的に生育するカエデ属植物の系統的多様性が高いことが、共存するハマキホソガ属蛾類の種数の多さをもたらしていると考えられる。さらに両調査地ともに、2種、または3種のハマキホソガが類似した寄主を利用しながら共存していたが、それらの間は必ずしも季節消長や寄生者群集の違いは見られなかった。

本学位論文は、植食性昆虫の高い種多様性が、従来考えられてきたように高い種分化率よるのではなく、共存種数の多さによっても説明できる可能性があるという新たな視点を示している。日本の温帯林において、カエデ属は他の木本性のどの属の植物よりも同所的に生育する種の数が多い。よって、カエデ属植物を利用するハマキホソガ属の多様性は、必ずしも高い多様化率によるものではなく、一地域におけるカエデ属植物の高い系統的多様性が、そこに共存できるハマキホソガ属の種数を高めたことでもたらされたものかもしれない。このように、植食性昆虫の種多様性をよりよく理解するためには、種多様化のプロセスだけでなく、種多様性の上限が決定される要因も考慮することが重要だと考えられる。

Summary

Herbivorous insects are remarkably species diverse and comprise over one-third of the described biodiversity on earth. The traditional explanation for the huge diversity of herbivorous insects is that high host specialization, as the result of coevolution between plant chemical defenses and insect detoxifying ability, provided opportunities for host-shift-driven speciation and thereby increased their speciation rate. However, recent phylogenetic studies found that the evolution of herbivory is not necessarily accompanied with increased diversification, prompting the need to better examine the role of host shifts during the process of speciation. An alternative view of herbivorous insect diversity is that diversity is saturated through time, and the factors that affect the upper limit on species richness, such as the number of locally coexisting species or mean range size, are the more likely determinants of current species richness. Thus, the process that *generates* diversity (i.e., speciation mode) and the factors that *maintain* diversity (i.e., determinant of limit on species richness) are both critical for our understanding of herbivorous insect diversity.

In this thesis, I focused on the relationship between leaf cone moths (*Caloptilia*) and their maple hosts (*Acer*) because, among the species of *Caloptilia*, the diversity of species using maples as hosts is notably high. By thoroughly sampling *Caloptilia* moths throughout Japan, I clarified detailed geographic distributions and host ranges of 14 maple-feeding *Caloptilia* species, including three species that are newly found. Phylogenetic analysis of 58 *Caloptilia* species collected from diverse angiosperm hosts, including the above 14 maple-feeding species, indicated that 13 of the 14 maple feeders form a clade together with a *Toxicodendron*-feeding species and thus diversified primarily on maples. Statistical test of speciation by host shift indicated that host shifts occurred more frequently in the early stage of the diversification. Thus, contrary to traditional views that host shifts promote speciation, not all *Caloptilia* speciation events were accompanied by host shifts.

I then determined the patterns of host use by locally coexisting *Caloptilia* species in the University of Tokyo Chichibu Forest and Ashiu Forest Research Station of Kyoto University. There were 7 *Caloptilia* species co-occurring at the Chichibu Forest and 10 *Caloptilia* moths co-occurring at the Ashiu Forest Research Station. Co-occurring *Caloptilia* species generally had non-overlapping host ranges, and each *Caloptilia* species used one to several phylogenetically related hosts, which shared similar leaf chemistry (tannin content and/or C/N ratio) and mechanical property (leaf thickness). This indicates that the number of co-occurring *Caloptilia* species is a function of the phylogenetic diversity of maples at each site. In addition, as many as three species with similar host ranges coexisted at both sites; however, species with similar host ranges did not necessarily have different phenology or parasitoid community.

Overall, the current thesis presents an alternative view on species diversity of herbivorous insects. In the temperate forests of Japan, the genus *Acer* is usually the largest woody plant genus. Thus, the diversity of maple-feeding *Caloptilia* may not be the result of high diversification rate, as traditionally been assumed, but a consequence of extensive local coexistence, which is brought about by the high phylogenetic diversity of locally co-occurring maple species. Therefore, to better understand the species diversity of herbivorous insects, it is important to consider not only the diversification process but the factors that determine the upper limit on species richness.

Contents

Chapter 1
General introduction

Extraordinary species diversity of herbivorous insects on the earth

Why are there so many herbivorous insects on the earth? That is one of the long-standing questions in ecology and evolution. On the earth, there are about 1,413,000 described species (Grimaldi & Engel 2005). Insects comprise almost half (925,000 species) of the world described species, and half of the insects (401,000 species) are herbivorous insects that consume plants for food (Grimaldi & Engel 2005). Colonization of angiosperms has long been recognized as the key innovation facilitating the current huge diversity of herbivorous insects, because most herbivorous insects use angiosperms as hosts (Farrell et al. 1998, Wiegmann et al. 2002, Grimaldi & Engel 2005). As a famous example, Farrell et al. (1998) showed that among the Phytophaga beetles, angiosperm feeders derived from lineages of gymnosperm feeders multiple times independently, and species richness of angiosperm feeders are much larger than that of gymnosperm feeders. The authors suggested that the diversity of angiosperms provided the opportunity for herbivorous insects to become diverse through the cycle of host plant specialization and host plant shift as they expanded onto various angiosperm lineages. The idea that host-shift-driven speciation has facilitated the diversification of herbivorous insects has long provided a major conceptual framework for understanding the extraordinary diversity of herbivorous insects.

The study of species diversification of herbivorous insects developed through a unique history compared with that of other organisms, perhaps due to their strong association with host plants. Intensive discussion of whether ecological speciation via host shift, in which specialization to novel host plant species causes reproductive isolation between species or populations using different host species (Feder et al. 1988, Groman & Pellmyr 2000, Hawthorne & Via 2001, Nosil et al. 2002, Thomas et al. 2003, Malausa et al. 2005, Ohshima 2012, Xue et al. 2014), well represents the unique situation of the study area. For example, Filchak et al. (2000) demonstrated that the apple maggot fly (*Rhagoletis pomonella*) has shifted from its original host (hawthorn; *Crataegus* spp.) onto domesticated apple (*Malus pumila*) and developed premating isolation in sympatry. Although there is potential for host shift to cause speciation, speciation by host shift inherently assumes that speciation occurs in sympatry. In contrast, study of speciation in other organisms usually assumes that geographic isolation (allopatry) is the initial cause of population divergence (Mayr 1942). Such a trend in the study of herbivorous insect speciation has gradually changed in the recent years (Imada et al. 2011, Goodman et al. 2012, Nyman et al. 2010, 2012).

Additionally, factors that determine current species diversity are not limited to the process that generate new species but also the process that maintain already diverged species (Rathcke 1976, Raboskey 2009). It is important to understand how incipient species interact with already existing, closely related species, because species may not survive over a long period of time if they cannot

stably coexist with other species, a process sometimes called “ephemeral speciation” (Stanley 1979, Rosenblum et al. 2012). Furthermore, interspecific interaction has been called upon to account for the diversification of insects into new habitats or onto new host plants (Dethier 1954, Wilson 1961, Ehrlich & Raven 1964, Greenslade 1972, Rabosky 2009) and for various observed differences between closely-related species (Hutchinson 1959, Linsley & MacSwain 1959, Heatwole & Davis 1965); such a view is the opposite of the idea of ecological speciation. Thus, the process of how closely related herbivorous insects coexist is another key factor in understanding the causality of herbivorous insect diversity. An improved understanding of herbivorous insect diversity will thus be achieved by merging the knowledge gained from studies of both generation and maintenance of species diversity.

However, most previous studies addressing herbivorous insect diversity focused either on generation or maintenance of species diversity and lacked a holistic approach. In this thesis, I study species diversity of herbivorous insects from the viewpoints of both generation and maintenance of species diversity. I focused on closely related herbivorous insects using a group of closely related hosts, because such materials can be used to study both the speciation process and the outcomes of interspecific interaction. In this study, I focused on the relationship between leaf cone moths (*Caloptilia*) and their maple hosts (*Acer*). This relationship is appropriate for examining closely related herbivores associated with closely related plants, because maple-feeding *Caloptilia* are abundant and species rich at both local and global scales (Table 1, 2). Additionally, variation in host preference among *Caloptilia* species has been reported (Kumata 1982, Kumata et al. 2013), allowing how diet shifts affect patterns of speciation and local species coexistence.

*The study system: the relationship between leaf cone moths (*Caloptilia*) and maples (*Acer*)*

The genus *Acer* is one of the most taxonomically diverse groups of trees in the Northern Hemisphere, particularly in the temperate regions of East Asia, eastern North America, and Europe (van Gelderen et al. 1994). The genus comprises 124 species in the Northern Hemisphere, 81% of which are distributed in China, Korea, and Japan (Renner et al. 2007). 28 species are distributed in Japan. In the classical interpretation of historical biogeography, *Acer* is a member of the "Arcto-Tertiary Geoflora", which supposedly was a broad-leaved deciduous forest that occupied high northern latitudes (Chaney 1938, 1959, Wolfe & Tanai 1987). In response to cooling climate, this "geoflora" migrated southward, and numerous constituent taxa, including *Acer*, became disjunct between North America and Eurasia (Wen et al. 2016).

The genus *Caloptilia* is globally distributed and includes nearly 300 described species, of which 27 feed on maples (De Prins & De Prins 2015). In Japan, 51 *Caloptilia* species are described feeding on 21 host plant families, and 11 of them use *Acer*, which is the most common host plant genus of Japanese *Caloptilia* (Kumata et al. 2013). The feeding habits of the larvae change dramatically between the early and late developmental stages. Upon hatching, larvae mine the surface

layer of the leaf (i.e., leaf miners) until the third instar, then exit the mine, and form the edge of the leaf into a roll within which they feed externally until the final instar (hence the name leaf cone moth) (Kumata et al. 2013). Each species is usually associated with a single plant genus, but detailed information on host range at plant species level is limited for most *Caloptilia* species, including those that feed on maples.

Organization of the thesis

In this thesis, I study species diversity of leaf cone moths (*Caloptilia*) associated with maples focusing on the processes of both generation and maintenance. This thesis consists of five chapters, the first of which is the introduction given here (Chapter 1). In Chapter 2, I investigated how the phylogeny and leaf traits of maples affect the community of herbivorous insects (including those other than *Caloptilia*), and found that several leaf traits associated with defense are significantly correlated with phylogeny, and that herbivore community is determined by both phylogeny and leaf traits. In Chapter 3, I examined the patterns of species diversification of leaf cone moths associated with maples in a phylogenetic context. In Section 3-1, I collected detailed host use information of 14 maple-feeding *Caloptilia* species, including three that are new, and performed a phylogenetic analysis, finding that host use changes of leaf cone moths are more concentrated toward the root of the phylogeny (i.e., early species diversification). Subsequently in Section 3-2, I showed that early species diversification of leaf cone moths may have been affected by dramatic expansion of maple distribution following Late Miocene global cooling. In Chapter 4, I examined the pattern of local species coexistence of maple-feeding *Caloptilia* in an ecological context. In Section 4-1, I quantified the patterns of host use by six locally coexisting maple-feeding *Caloptilia* species, finding that host breadth of each herbivore species is population-size-dependent and can change continuously along host plant phylogeny. This study also found multiple pairs of coexisting *Caloptilia* species that largely overlapped in their host use. In Section 4-2, I assessed the patterns of temporal and enemy niche use by nine coexisting *Caloptilia* maple feeders, and showed that both phenology and parasitoid community overlapped even among pairs of species sharing the same host plant. In Chapter 5, I synthesize the results obtained in the previous chapters and discuss the process of how leaf cone moths associated with maples attained their current species diversity. Finally, I give my perspective on what is important for a better understanding of species diversity of herbivorous insects.

Table 1 The number of leaf cone moth (*Caloptilia*) species associated with each host plant genus in Japan. The data is based on De Prins & De Prins (2015). The plant genera used by more than one leaf cone moth species are indicated.

Host plant genus	Species richeness
Acer	11
Toxicodendron	5
Alnus	4
Rhus	3
Betula	2
Castanea	2
Glochidion	2
Populus	2
Quercus	2
Rhododendron	2
Salix	2

Table 2 The number of leaf cone moth (*Caloptilia*) species associated with each host plant genus globally. The data is based on De Prins & De Prins (2015). The plant genera used by more than three leaf cone moth species are indicated.

Host plant genus	Species richeness
Acer	27
Alnus	12
Quercus	9
Betula	8
Rhus	8
Toxicodendron	7
Myrica	6
Vaccinium	6
Cajanus	5
Populus	5
Rhododendron	5
Cornus	4
Glochidion	4
Persea	4
Prunus	4

Chapter 2
Phylogeny, leaf traits, and altitudinal distribution of Japanese maples (*Acer*) and their relationship with herbivore assemblage

Introduction

The mechanisms and processes of community assembly are a central theme in ecology (Samuels & Drake 1997; Webb et al. 2002; Chase 2003). Among the various factors proposed to affect community assembly, deterministic niche-based processes are proposed to be primary drivers (Diamond 1975; Tilman 1982; Chase & Leibold 2003). In contrast, Hubbell (2001) emphasised the importance of stochastic processes of random dispersal in community assembly through his unified neutral theory of biodiversity and biogeography. Recently, ecologists have acknowledged the importance of the synergetic effects of both deterministic niche-based and stochastic processes (Adler et al. 2007; Chase 2007; Chase et al. 2009). In addition to these contemporary processes, historical and evolutionary processes also influence community assembly (Cornell & Washburn 1979; Ricklefs 1987; Sax et al. 2002; Emerson & Gillespie 2008). The introduction of phylogenetic approaches has allowed ecologists to link short-term local processes to global ones that occur over long evolutionary time scales (Losos 1996; Ackerly 2003; Ricklefs 2004; Graham & Fine 2008; Cavender-Bares et al. 2009).

Plant‐herbivore systems have been utilised to examine the effects of both contemporary and historical processes on herbivore community assemblages via evolutionary histories (i.e., phylogenies) and present statuses of host plant species (Novotny et al. 2006; Rasmann & Agrawal 2011). Recent applications of phylogenetic techniques at the community level have allowed researchers to investigate the effects of evolutionary processes on the herbivore assemblages of various plant species (Weiblen et al. 2006; Graham & Fine 2008; Cavender-Bares et al. 2009; Rasmann & Agrawal 2011). Specialisation on several host plant taxa is common in most lineages of herbivorous insects (Rasmann & Agrawal 2011). For example, Weiblen et al. (2006) showed that a large proportion of herbivore species found in the tropical rain forest of New Guinea are clade specialists at the genus to family level. Host plant specialisation at the same taxonomic level has also been found in herbivorous beetles in the tropical forests of Panama (Ødegaard et al. 2005). The phylogenetic relatedness of plants can constrain herbivore communities, a trend that can be partially attributed to plant trait similarities between close relatives (Pearse & Hipp 2009; Rasmann & Agrawal 2011). However, other studies have indicated that herbivores prefer less closely related plant species (phylogenetic overdispersion) in a community because of convergence in relevant plant defences (Becerra 1997; Kursar et al. 2009).

It is important to compare closely related plant species when examining effects of host plant relatedness on herbivore assemblages because patterns of similarity among assemblages are generated

through phylogenetic and traitbased host constraints (Weiblen et al. 2006; Novotny et al. 2010). Although several studies have investigated herbivore community assemblages of congeneric host plant species (e.g., Becerra 2007; Kursar et al. 2009), detailed phylogenetic comparisons among insect assemblages of a large number of regionally co-occurring congeners are lacking (but see Becerra 2007). The genus *Acer* (maple species) provides an excellent opportunity to explore influences of evolutionary histories of host plants on herbivore assemblages because of its diversity in Japan, its interspecific variation in life history, morphology, and physiology, and the plasticity of these traits (Lei & Lechowicz 1990, 1997; Sipe & Bazzaz 1994, 1995; Tanaka 1995; Ackerly & Donoghue 1998). A few studies have shown the specialisation of several Lepidoptera species on *Acer* plants (Kumata et al. 2013), but only a limited number have examined the herbivore assemblage of *Acer* tree species (e.g., Murakami et al. 2007; Zehnder et al. 2009).

My aim was to explore the processes generating the structure of herbivore assemblages through an understanding of the effects of phylogenetic relationships among host plant species and other factors. Other important factors potentially affecting community assemblages of herbivores include leaf traits as food resources (Pearse & Hipp 2009) and geographical position (e.g., altitude), which affects assemblages as an environmental filter and also by affecting dispersal of individuals (Beck & Khen 2007; Rominger et al. 2009). Here, the altitudinal distribution of trees was considered a proxy for current ecological process including environmental factors and dispersal. In this study, I examined leaf trait conservatism among closely related host plant species. Defensive traits were then divided into groups by evaluating representative axes of leaf traits with and without phylogenetic signals. In the field, I collected herbivorous insects from 14 *Acer* species and examined the relationships among phylogeny, leaf traits, and the altitudinal distribution of host plants and herbivore assemblages.

Materials and methods

Study sites

Field surveys were conducted in a mosaic of primary and secondary temperate mixed forest at the University of Tokyo Chichibu Forest in central Japan (35°54′N, 138°49′E). The secondary forest was dominated by *Quercus crispula*, and the primary forest was dominated by *Fagus japonica* at lower altitudes and *Tsuga diversifolia* at higher altitudes (The University of Tokyo Chichibu Forest 2012). The average annual temperature for 1996–2010 was 11.0 °C, and the average annual rainfall was 1,514.2 mm at Tochimoto (The University of Tokyo Chichibu Forest 2012).

I examined 30 species of *Acer* distributed in Japan, including species that are not found in the study area and also some subspecies. I found 23 species during the field survey in Chichibu forest (850–2,000 m in elevation). Leaf traits were measured for each *Acer* species, but because nine species were rare, only 14 were sampled for herbivore community assembly: *Acer amoenum, Acer capillipes*,

Acer carpinifolium, Acer japonicum, Acer maximowiczianum, Acer micranthum, Acer palmatum, Acer pictum subsp. *dissectum, Acer rufinerve, Acer shirasawanum, Acer sieboldianum, Acer tenuifolium, Acer tschonoskii,* and *Acer ukurunduense.* The nine rare species were *Acer argutum, Acer austral, Acer crataegifolium, Acer cissifolium, Acer diabolicum, Acer distylum, Acer nipponicum* subsp. *nipponicum, Acer pictum* subsp. *pictum,* and *Acer pictum* subsp. *savatieri*. In this study, I followed the taxonomic nomenclature of the YList (Yonekura & Kajita 2003).

Herbivore sampling and identification

Herbivorous insects were collected by hand (Novotny et al. 2002; Murakami et al. 2007) from the foliage of 14 *Acer* species over a period of 3 months (June–August 2011). Twelve individuals from 14 *Acer* species were chosen for sampling. Samples were taken from the foliage of a branch (3- to 5-cm diameter) on each tree during the day. For each species, I recorded maximum altitudinal distribution ranges within the study site (850–2,000 m). I chose trees for sampling as evenly as possible across the maximum altitudinal distribution for each *Acer* species. All sampling points were recorded using a Global Positioning System, and the altitude of each point was measured. Lepidoptera, Coleoptera, and Hymenoptera larvae were reared in the laboratory on the leaves of their host plant species at a temperature of 25 °C. All insects were identified to the morphospecies.

Plant leaf traits

For 29 *Acer* species, excluding *Acer amamienns*, which I was not able to sample, I examined six leaf traits that are known to affect herbivory: leaf thickness (*T*; μm), specific leaf area (SLA; g/m^2), water content (WC; % wet weight), C/N ratio (C/N), condensed tannin (CT), and total phenolics (TPh). For each measurement, 20 leaves from five individuals were analysed. The thickness of a fresh leaf was measured with digital callipers, avoiding major leaf veins. To measure the SLA and leaf WC, four leaf disks, 17.2 mm in diameter, were punched as soon as possible after sampling. These disks were weighed to the nearest 0.01 mg and weighed again after drying for 24 h at 60 °C. The C/N ratio was measured using a CN coder (NC-220F; Sumika, Tokyo). Concentrations of total phenolics and condensed tannins were determined colorimetrically following extraction with 50 % acetone for 16 h. The procedure for phenolic measurements followed Price & Butler (1977), and the measurement of condensed tannins followed Broadhurst & Jones (1978). Although the method for phenolic quantification suggested by Price & Butler (1977) has been criticised (Appel 1993), it is still frequently used as a classic index for phenolic content (e.g., Kurokawa et al. 2010; Jackson et al. 2013). Leaf thickness is related to structural defence, and thicker leaves tend to have greater structural resistance against leaf-chewing herbivores (Onoda et al. 2011). The C/N ratio indicates nutritional quality and may act as a defence because low nutritional quality deters herbivores (Mattson 1980; Silva & Batalha 2010). N is a limiting nutrient for many terrestrial organisms, and low levels of N

increase feeding time and therefore increase exposure to natural enemies and energy expended on consuming and processing food (Lavoie & Oberhauser 2004; Craine 2009; Silva & Batalha 2010). Tannin is an organic, N-free chemical defence that binds with proteins, reducing N available to herbivores (Bergvall & Leimar 2005; Craine 2009; Silva & Batalha 2010).

Plant phylogeny

A phylogenetic analysis of 30 species of Japanese *Acer* was undertaken based on sequences of the chloroplast genes rbcL, matK, trnL-F, and rpl16. In addition, I included three sequences for three outgroups: *Dipteronia sinensis*, *Aesculus hippocastanum*, and *Koelreuteria paniculata*. Total genomic DNA was isolated from freeze-dried leaves using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The polymerase chain reactions (PCRs) followed standard protocols. Reaction products were purified with a QIAquick PCR purification kit (Qiagen), and cycle sequencing was performed with BigDye Terminator cycle sequencing kits (Applied Biosystems, Foster City, CA). The dye terminators were removed using ethanol precipitation. Purified sequencing reactions were run on an ABI 3500 automated sequencer (Applied Biosystems). The primers used to amplify the rbcL gene were 1F of Fay et al. (1997) and 1460R of Olmstead et al. (1992). For cycle sequencing, they were supplemented by the internal primers 600F and 800R (Kocyan et al. 2007). For the trnL intron and adjacent trnL-F spacer, the primers c, d, e, and f of Taberlet et al. (1991) were used. For the rpl16 intron, I used the primers 71F of Jordan et al. (1996) and 1067F of Asmussen et al. (1999), and for the matK gene, I used the primers 3F and 1R (K. J. Kim, unpublished data). Forward and reverse reads were obtained for all samples. Sequences were edited with Mega (version 5.05; Tamura et al. 2011) and aligned using mafft (version 6.901; Katoh & Toh 2008). Bayesian analysis was performed using MrBayes (version 3.2.1; Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with 500,000 generations of Markov chain Monte Carlo chains and sampling of one tree every 100 generations. Trees were rooted with *D. sinensis*, *A. hippocastanum*, and *K. paniculata* (Fig. 1). I calculated phylogenetic distances for all possible pairs among 14 targeted *Acer* species.

Statistical analyses

The phylogenetic signals were quantified using a generalization of Abouheif's test (Pavoine et al. 2008) in the R package adephylo. To summarise the multi-dimensionality of leaf traits using a defensive index (DI) (Pearse & Hipp 2012), two independent PCAs were performed, one for traits carrying a phylogenetic signal, and the other for traits not showing a phylogenetic signal. I calculated distance matrices of the DIs of all possible pairs of plant species examined here. Jaccard's dissimilarity indices were calculated for all possible pairs of herbivore assemblages among plant species (Oksanen et al. 2011). Furthermore, Euclidean distance matrices for all possible pairs of plant species were calculated for the first two axes of the PCAs for leaf traits with and without phylogenetic signals. The overlap of altitudinal distribution was also measured by Jaccard's dissimilarity indices

(van Jaarsveld et al. 1998). The proportions of intrato inter-specific leaf trait variation were compared among traits with and without phylogenetic signals using *t*-tests.

Correlations between the dissimilarity of herbivore assemblages and the following variables were tested using Mantel tests (Oksanen et al. 2011): (1) host plant phylogeny (phylogeny), (2) a PCA index of leaf defences with a phylogenetic signal (pDI1 for the first axis and pDI2 for the second axis), (3) a PCA index of leaf defences with no phylogenetic signal (nDI1 for the first axis and nDI2 for the second axis), and (4) altitudinal distribution of host plant species (altitude). Then, correlations between the dissimilarity of herbivore assemblages and the variables that showed significant correlations were tested while controlling for the effects of other factors (i.e., plant phylogeny, leaf traits, and the altitudinal distribution of host plants) using partial Mantel tests (Tuomisto & Ruokolainen 2006; Barber & Marquis 2011; Milla & Reich 2011) (Table 3). These provide a test of significance without inflating the probability of type I error caused by the indirect effect of a third factor (Barber & Marquis 2011). The correlations between phylogeny, leaf traits (pDI1, pDI2, nDI1, nDI2), and the altitudinal distribution overlap of host plants were also analysed with a partial Mantel test. All statistical tests were performed in R (R Development Core Team 2007) using the packages adephylo (Jombart et al. 2010), vegan (Oksanen et al. 2011), and prcomp.

Results

I recorded 1,859 herbivore individuals from 279 species. My data set included members of six major herbivore orders (1,005 individuals from 161 species of Lepidoptera, 627 individuals from 67 species of Hemiptera, 142 individuals from 42 species of Coleoptera, 55 individuals from five species of Orthoptera, 25 individuals from two species of Hymenoptera, and five individuals from two species of Phasmatodea).

The analysis of phylogenetic signals for each leaf trait detected significant signals for *T*, C/N ratio, and CT (Table 1, Fig. S2). No significant phylogenetic signals were detected for any other leaf traits (SLA, WC, and TPh; Table 1, Fig. S2). The PCs of leaf traits with and without phylogenetic signals were calculated to obtain summary variables (Table 2). The proportions of intra- to inter-specific variation in leaf traits did not show any explicit trend in relation to the presence of phylogenetic signals ($P = 0.520$).

Jaccard's dissimilarity among herbivore assemblages was significantly correlated with phylogenetic distance ($r = 0.245$, $P = 0.018$; Fig. 2), distances of the PCA indices of leaf defence with phylogenetic signals (pDI1, $r = 0.205$, $P = 0.032$; pDI2, $r = -0.268$, $P = 0.024$; Fig. 3), and the overlap in altitudinal distributions ($r = -0.330$, $P = 0.003$; Fig. 4), but it was not significantly correlated with distances of the PCA indices without phylogenetic signals (nDI1, $P = 0.363$; nDI2, $P = 0.256$, Fig. 3). Partial Mantel tests detected no significant correlation between assemblage dissimilarity and the distance of the index of leaf defences of host plants under the control of

phylogenetic distance (pDI1, $r = 0.152$, $P = 0.103$) or altitudinal distribution overlap (pDI1, $r = 0.156$, $P = 0.082$), but significant correlations were observed for the other combinations (Table 3). The dissimilarity of herbivore assemblages increased as the phylogenetic distance of host plants increased and as the overlap of altitudinal distribution decreased under the control of the other factors (Table 3). The dissimilarity of herbivore assemblages increased with the distance of the PCA index of leaf defences (pDI2) under the control of the other factors (Table 3). The correlations among phylogeny, the PCA index of leaf defences (pDI1), and the overlap of altitudinal distribution of host plants were also significant (Table 4, Fig. 5).

Discussion

The effect of host plant phylogeny on the herbivore assemblages of congeneric *Acer* species was clearly demonstrated in this study. Weiblen et al. (2006) reported similar effects of host plant phylogeny at the genus to family level. However, my results reveal finer level segregation of herbivore assemblages among congeneric, closely related host plant species as a result of historical processes. The decline in similarity of herbivore assemblages with increasing phylogenetic distance among host plant species reflects the phylogenetic conservatism of host plant selection: herbivores tend to feed on the same host lineages as their ancestors (Weiblen et al. 2006).

Host plant phylogeny both directly and indirectly affected the structure of herbivore assemblages. Phylogeny is an integrated measure of species traits (Pearse & Hipp 2009, Rasmann & Agrawal 2011); thus, the variation in herbivore assemblages not explained by the leaf traits examined here (i.e., the direct effect of plant phylogeny) might be caused by plant traits that were not measurable in this study (Pearse & Hipp 2009). For example, Webster et al. (2010) showed that several synthetic chemicals (i.e. characteristic blends of volatile compounds) within plants are utilised by herbivores to detect host foliage. In another study, Agrawal (2011) showed that the density of leaf trichomes also had an effect on herbivorous insects. These variations in plant traits likely affect the host plant utilisation of herbivorous insects synergistically.

In this study, only the leaf traits showing a phylogenetic signal affected the structure of herbivore assemblages (Table 1, Fig. 3, S1). Defensively effective traits, i.e., leaf thickness, C/N ratio and tannin contents, were better conserved than non-effective traits, i.e., SLA, water content and phenolic content. Agrawal et al. (2009) also showed stronger phylogenetic signals in defensive traits (cardenolides, latex, and trichomes) than in the other ones for the milkweed (*Asclepias*) species. Recently, phylogenetic signals of leaf traits were reported in a variety of plant lineages (Ackerly 2009; Rasmann & Agrawal 2011; Pearse & Hipp 2012). Agrawal & Fishbein (2006) also mentioned that defence traits appear to be convergent at lower phylogenetic levels, whereas they appear to be more conserved at higher levels. They showed that Defence traits are not congruent with phylogeny, indicating phylogenetic overdispersion, in genus *Asclepias*. However, in the present study, Defence

traits were conserved among congeneric *Acer* species, which showed slower trait changes in these. Thus, I have to seek an explanation for the different pace in trait change among plant taxa in relation to their differences in Defence strategy and their life history.

Recently, many studies have reported a significant covariation among plant defensive traits (Agrawal & Fishbein 2006; Pearse & Hipp 2012): the "plant defence syndrome" (Kursar & Coley 2003; Agrawal & Fishbein 2006). Dimensional reduction approaches (e.g., PCA) have typically been used to examine the plant defence syndrome as a defensive strategy against herbivores (Fine et al. 2006; Barber and Marquis 2011; Pearse & Hipp 2012). In this study, I confirmed the effect of the *Acer* phylogeny on the set of defensive leaf traits identified by PCA (Table 4). Although both the first and second PCA indices of leaf defence traits with phylogenetic signals (pDI1 and pDI2) showed significant correlations with the herbivore assemblage structure, the effect of the first index (pDI1) was cancelled when I included the effects of phylogeny and the altitudinal distribution of host plants (Table 3). This suggests evolutionary conservation for these leaf traits. On the other hand, the effect of the second PCA index of leaf defences (pDI2) was independent of phylogeny and the altitudinal distribution of host plants (Figs. 3, 4), indicating an independent effect of these leaf traits (pDI2) on herbivore assemblages. However, the distance of pDI2 showed a negative correlation with the dissimilarity of the herbivore assemblages (Table 3), which is more complicated to interpret. One possible explanation for this phenomenon is competitive exclusion among ecologically similar herbivore species (Cavender-Bares et al. 2009). If closely related herbivore species select similar leaf traits when selecting food resources, they may not be able to coexist due to competition (Cavender-Bares et al. 2009).

An altitudinal gradient in the composition of herbivore assemblages (Fig. 4) may be caused by an environmental gradient following altitude. The montane zone contains gradients of a variety of factors, e.g., temperature, soil fertility, risk of photodamage from ultraviolet‐B radiation, and precipitation (Preszler & Boecklen 1996; Darrow & Bowers 1997). Simultaneously, the difference in the altitudinal distribution of individual trees implies a spatial gap among herbivore populations, resulting in dispersal limitations. Because host plants are distributed patchily within a forest, the limited dispersal ability of herbivores might also affect the magnitude of the beta diversity of herbivore assemblages (Hanski 1999; Novotny & Weiblen 2005).

In this study, I investigated the complicated effects of phylogeny, leaf traits, and the altitudinal distribution of host plants on the structure of herbivore assemblages among congeneric maple tree species (Fig. 5). Both historical (phylogeny) and current (e.g., the spatial distribution or environmental filters) ecological processes may have created the existing herbivore assemblage structure. Pearse & Hipp (2012) examined the influence of phylogeny and geographical distribution on leaf traits, and demonstrated that plant defences track the abiotic environment slowly over macroevolutionary time. In my study, I found evidence that these variations in leaf traits following

plant phylogeny leave a signature on the herbivore assemblages that feed on them. Specifically, leaf traits with a phylogenetic signal affected herbivore assemblages. However, this was only identified in local processes (i.e., with narrow environmental gradients and geographical ranges). We must further examine the interactions among herbivorous insects, leaf traits, phylogeny, and the geographical distribution of host plants in a larger-scale study and over evolutionary time to understand how herbivore assemblages are constructed, and why herbivorous insects have become one of the most diverse groups in nature.

Table 1 Phylogenetic signal (Abouheif/Moran's test) of plant leaf traits (Pavoine et al. 2008).

Leaf trait	Moran's I	*P* values
Thickness	0.194	**0.025**
Specific leaf area	−0.002	0.364
C/N ratio	0.209	**0.027**
Water	−0.161	0.898
Condensed tannin	0.339	**0.002**
Total phenolics	0.035	0.226

Bold letters indicate the significance in Phylogenetic signals

Table 2 Loading of leaf trait variables on the first and second principal components (*PC*s) for each trait group

Leaf traits with phylogenetic signal	PC1	PC2	Leaf traits with no phylogenetic signal	PC1	PC2
Thickness	0.711	0.288	Specific leaf area	1.000	0.007
C/N ratio	−0.018	−0.315	Water content	0.007	−0.315
Condensed tannin	0.703	−0.904	Total phenolics	−0.005	0.949
Total variance explained (%)	46.6	37.1		75.0	17.4

Table 3 Results of partial Mantel tests of the correlations between community dissimilarity and phylogenetic distance, the distance of a PCA index of leaf defences, and altitudinal overlap, including the third variable as a covariable

	Partial Mantel *r*	*P* values
Phylogeny|Leaf traits (pDI1)	0.204	**0.048**
Phylogeny|Leaf traits (pDI2)	0.212	**0.035**
Phylogeny|Altitude	0.296	**0.010**
Leaf traits (pDI1)|Phylogeny	0.152	0.103
Leaf traits (pDI1)|Altitude	0.156	0.082
Leaf traits (pDI2)|Phylogeny	−0.238	**0.043**
Leaf traits (pDI2)|Altitude	−0.316	**0.013**
Altitude|Phylogeny	−0.368	**0.001**
Altitude|Leaf trait (pDI1)	−0.305	**0.003**
Altitude|Leaf trait (pDI2)	−0.369	**0.001**

Bold letters indicate the significant effect of phylogeny or leaf trait on community dissimilarity of herbivores

For example, A|B indicates the correlation between A and community dissimilarity with the effect of B

Table 4 Results of partial Mantel tests of the correlations among phylogenetic distance, leaf trait distance, and altitudinal distribution overlap

	Partial Mantel *r*	*P* values
Phylogeny-Leaf traits (pDI1)|Altitude	0.280	**0.029**
Phylogeny-Leaf traits (pDI2) |Altitude	−0.156	0.103
Altitude-Leaf traits (pDI1)|Phylogeny	−0.215	**0.034**
Altitude-Leaf traits (pDI2)|Phylogeny	−0.074	0.255

Bold letters indicate the significant effect of factors examined

For example, A-B|C indicates the portion of B that is explained by A with the effect of C as a co-variable

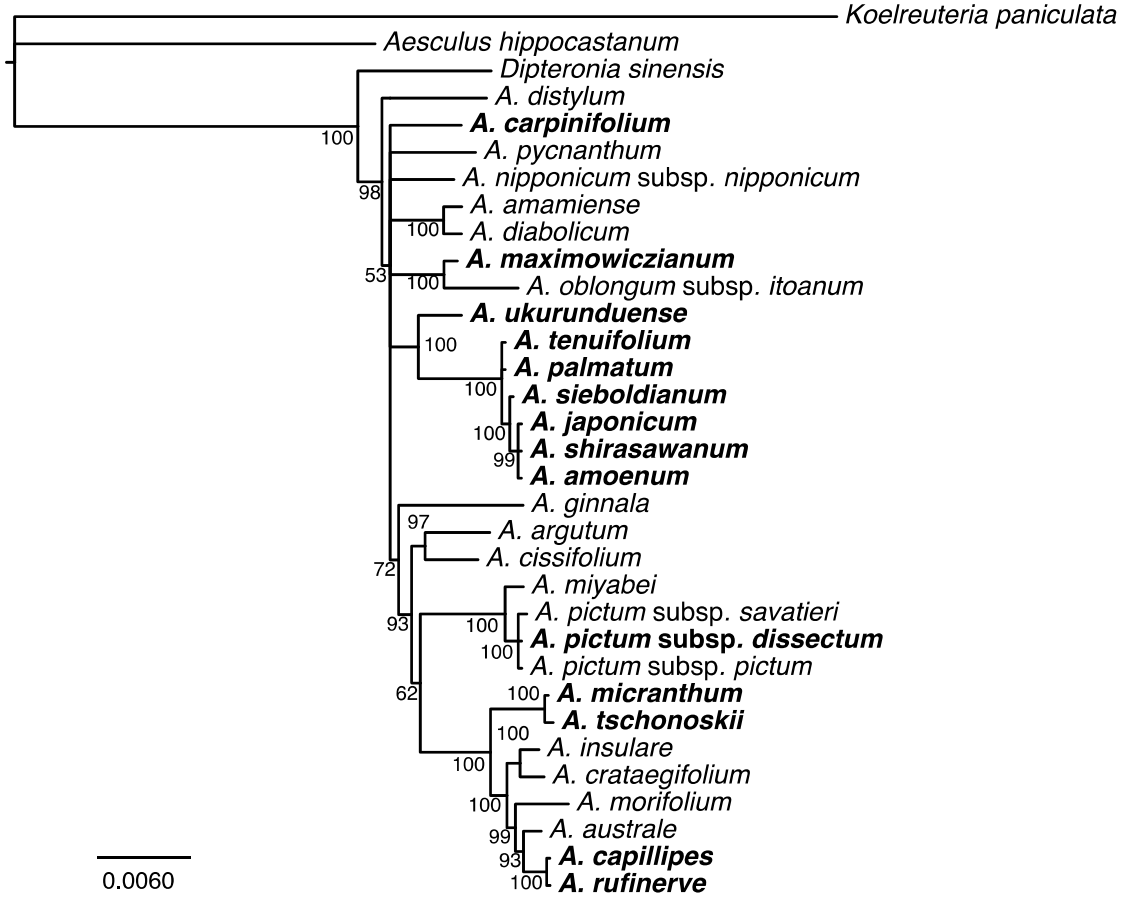

Figure 1 Bayesian phylogram of 30 species of Japanese *Acer* (plus three outgroups) based on 3,694 nucleotides, excluding gaps from four chloroplast DNA loci. Values *above* the nodes indicate Bayesian posterior probabilities >50 %. The 14 species sampled in this study are shown in *bold*

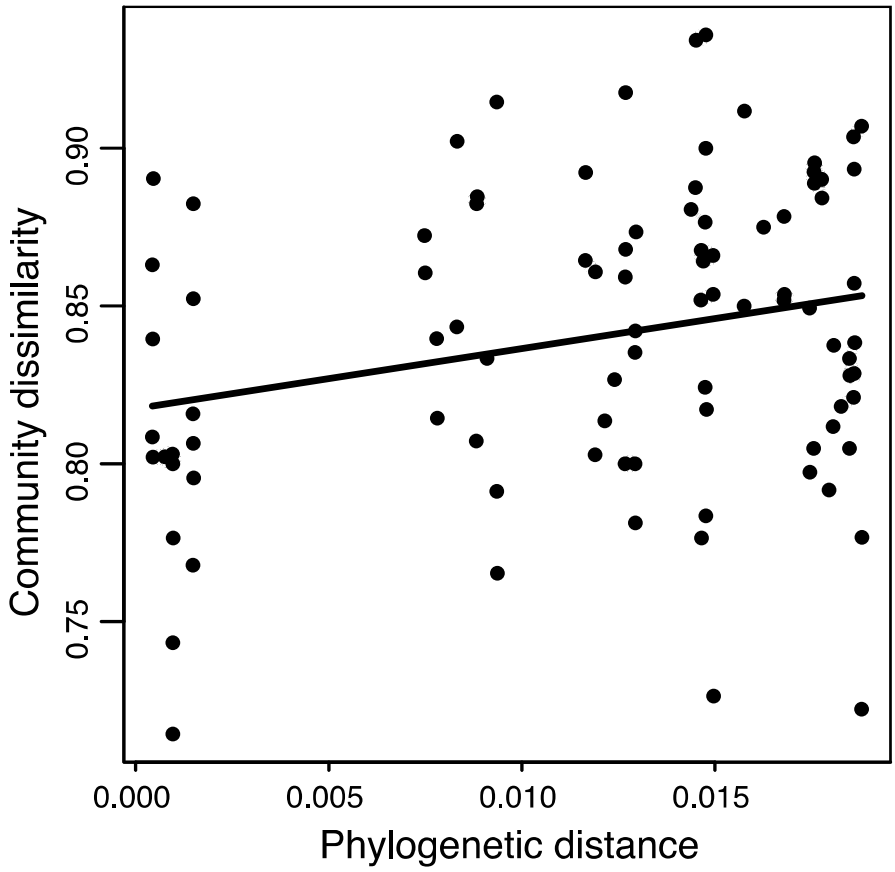

Figure 2 The relationship between phylogenetic distance and community dissimilarity (Pearson $r = 0.245$, $P = 0.018$; Mantel test)

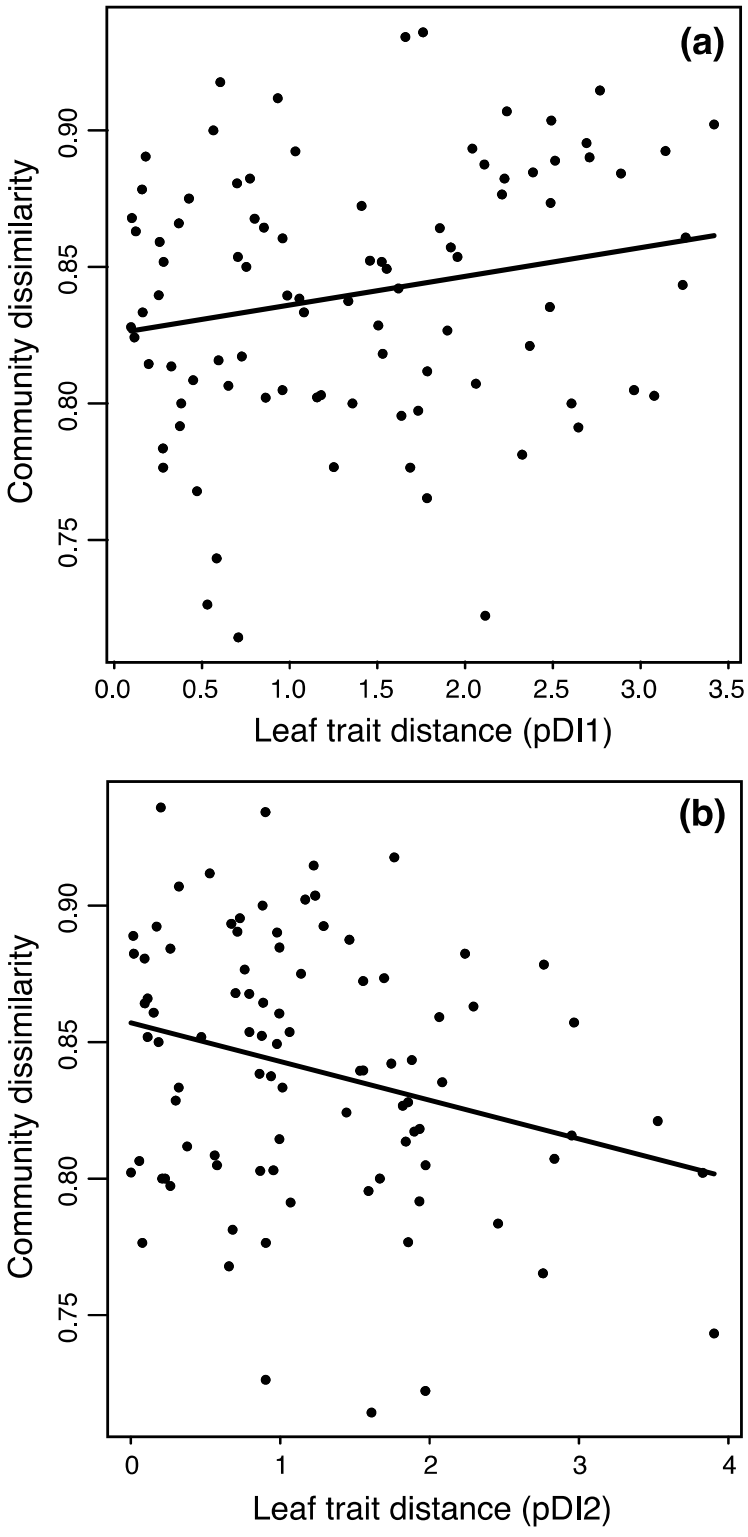

Figure 3 Relationships between community dissimilarity and the distances of the indices of leaf defences with a phylogenetic signal for a the first principal component (PC) axis, pDI1, and b the second PC axis, pDI2 (a Pearson $r = 0.205$, $P = 0.032$; b Pearson $r = -0.268$, $P = 0.024$, Mantel tests).

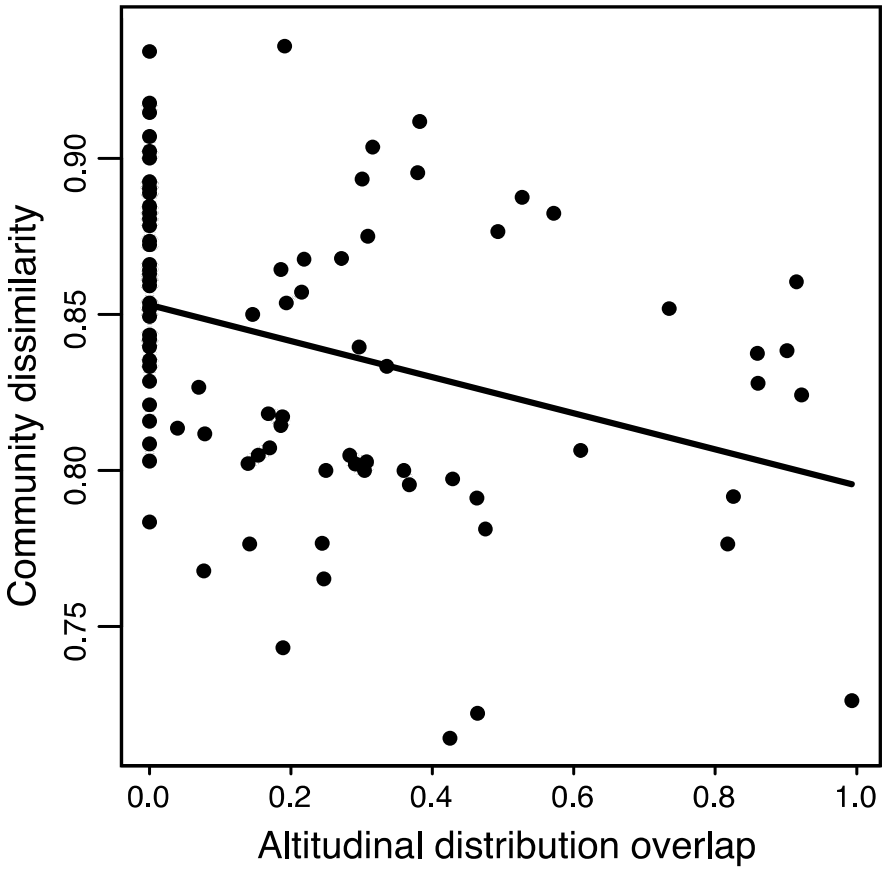

Figure 4 Relationships between the altitudinal distribution overlap and community dissimilarity (Pearson $r = -0.330$, $P = 0.003$; Mantel test).

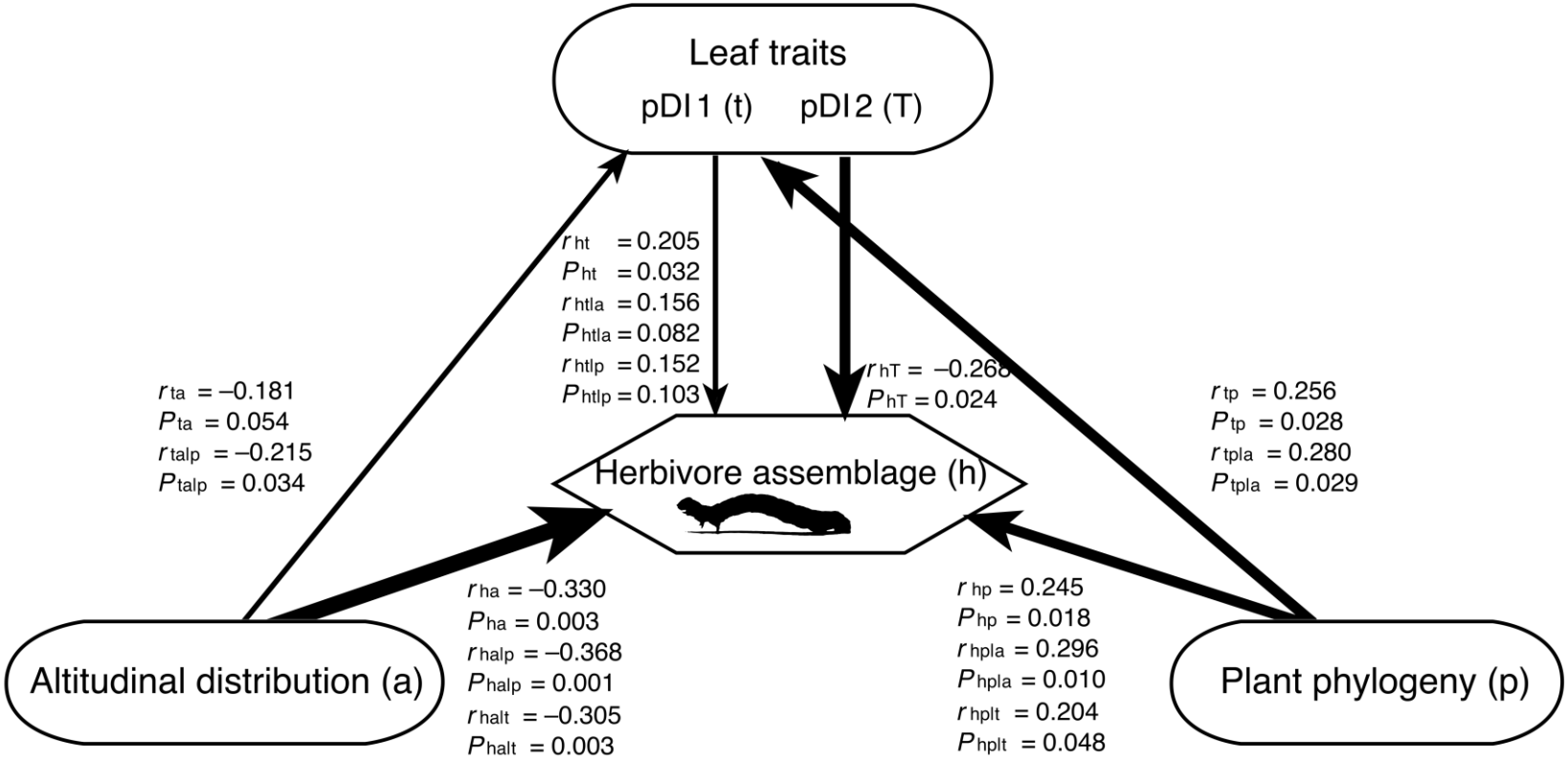

Figure 5 A scheme to explain the community assembly of herbivores on congeneric tree species. Relative importance of plant phylogeny, altitudinal distribution, and leaf traits are shown. The simple correlations (r_{yx}, P_{yx} where y is the response and x is the predictor) and partial correlations ($r_{yx}|w$, $P_{yx}|w$ where y is the response, x is the predictor, and w is a potential covariate whose effect on both y and x is accounted for before assessing the correlation) of each path, were estimated. The *r*- and *P*-values of the correlations shown are averaged over bootstrap replicates. The *width* of each path arrow is proportional to the strength of the relationship

Chapter 3 Phylogenetic test of speciation by host shift in leaf cone moths (*Caloptilia*) feeding on maples (*Acer*)

Introduction

Herbivorous insects comprise one of the major components of earth's biodiversity. Because the diversity of herbivorous insects is often correlated with host plant diversity (Lawton & Schroeder1977; Wiegmann et al. 2002; Janz et al. 2006; Joy & Crespi 2012; Ferrer-Paris & Sánchez-Mercado 2013; Isaka & Sato 2015; Lin et al. 2015), the cycle of host plant adaptation and host-plant shift is commonly invoked as the major process generating high diversity (Mitter & Brooks 1983; Craig et al. 2001; Wheat et al. 2007; Futuyma & Agrawal 2009; Bennett & O'Grady 2012). For example, a classical study by Farrell (1998) showed that herbivorous insects using angiosperms as hosts are more species-rich than those using gymnosperms among the Phytophaga beetles, suggesting that the diversity of angiosperms has facilitated speciation by host shift in the beetles that feed on them. Studies of host races in herbivorous insects showed that specialization to a novel host plant sometimes results in reproductive isolation between insects using different hosts (Feder et al. 1988; Groman and Pellmyr 2000; Hawthorne & Via 2001; Nosil et al. 2002; Thomas et al. 2003; Malausa et al. 2005; Ohshima 2012; Xue et al. 2014), providing a mechanistic explanation of how host shifts may promote speciation. Understanding the role of host-plant shifts in generating diversity is thus a current focus in the study of herbivorous insect diversification (Marvaldi et al. 2002; Stireman et al. 2005; Wheat et al. 2007; Winkler et al. 2009; Fordyce 2010; Funk 2010; Matsubayashi et al. 2010; Nyman 2010; Soria-Carrasco et al. 2014).

However, phylogenetic analyses of herbivorous insect radiation have often demonstrated conservatism in host plant use by herbivorous insects (Crespi et al. 1998; Lopez-Vaamonde et al. 2003; Wahlberg 2007; Winkler and Mitter 2008; Nyman et al. 2010; Jousselin et al. 2013; Doorenweerd et al. 2015). For example, Nyman et al. (2010) showed that only 20% of the speciation events in nematine sawflies were accompanied by shifts between host plant families, and Doorenweerd et al. (2015) showed that host use was generally conserved at the plant family level, with biogeographic processes playing a greater role in the recent speciation of nepticulid moths. Extreme cases of host plant conservatism are found in gall wasps feeding on oaks (Stone et al. 2009) or micropterigid moths that have radiated on a single liverwort species (Imada et al. 2011). However, many phylogenetic studies that tested for host conservatism defined host plants at the plant family or genus level (Lopez-Vaamonde et al. 2003; Wahlberg 2007; Nyman et al. 2010; Jousselin et al. 2013; Doorenweerd et al. 2015). The relative importance of host shifts in herbivorous insect speciation should ideally be assessed using species-level phylogenies with data on all known host associations.

Two major obstacles hamper analysis at the species level. First, because most radiations of herbivorous insect groups occur at the continental scale, it is usually difficult to achieve complete taxon sampling while having host association data for each species. It is therefore not surprising that

some of the best-sampled phylogenies are those for less mobile herbivorous insect groups (e.g., Imada et al. 2011). Second, an appropriate method of analyzing host-plant shifts along phylogenies has been lacking. Coding host plant associations at the family or genus level would simplify analysis because methods such as ancestral character state reconstructions are then applicable. However, many herbivorous insects use several closely related plant species (i.e., polyphagy) with varying levels of preference (Smiley 1978; Roininen & Tahvanainen 1989; Thompson 1998; Scheirs et al. 2000; D'Costa et al. 2013; Nakadai & Murakami 2015), which complicates analysis of the ancestral state regarding host use. In addition, individual host plant species cannot be considered as discrete character states because they are phylogenetically non-independent (Pearse & Altermatt 2013). Ideally, the dissimilarity of host use between a pair of herbivorous insect species should be weighed by the phylogenetic disparity of the host plants.

In this study, I assess the importance of host shifts in the speciation process of herbivorous insects by developing a new method that overcomes these issues. This method focuses on whether host-plant shifts are concentrated towards the roots or the tips of the insect phylogenetic tree, while taking into account host plant phylogeny in the calculation of host use dissimilarity between a pair of herbivorous insect species. If most speciation events are associated with host shifts, the level of disparity in host use between a pair of herbivorous insect species will on average be greater for phylogenetically more closely related pairs (Fig. 2a). Alternatively, if most host shifting events occurred during the initial stage of the radiation and more recent speciation events were independent of host shifts, the level of difference in host use would be larger toward the root of the phylogenetic tree (Fig. 2b). I focused on the interaction between a group of leaf cone moths (*Caloptilia*, Gracillariidae) and their maple hosts (*Acer*, Sapindaceae). The *Caloptilia–Acer* interaction is appropriate for testing host-shift-driven speciation at fine taxonomic scales because a previous study demonstrated large variation in the pattern of host use among *Caloptilia* species (Nakadai & Murakami 2015). The genus *Acer* is one of the most taxonomically diverse groups of trees in the northern hemisphere, particularly in the temperate regions of East Asia, eastern North America, and Europe (van Gelderen et al. 1994). The genus comprises 124 species in the northern hemisphere, 81% of which are distributed in China, Korea, and Japan (Renner et al. 2007). A previous taxonomic study of *Caloptilia* identified 11 species associated with *Acer* in Japan alone, which have high morphological affinity to each other (Kumata 1982). Based on extensive geographic sampling, I establish full host plant records for these 11 species and three newly found ones, and analyze them using the above method to assess the relative importance of host shift in the speciation of *Caloptilia* moths feeding on *Acer* trees.

Materials and Methods

Study material

The genus *Caloptilia* is globally distributed and includes nearly 300 described species, of which 27 feed on maples (De Prins & De Prins 2015). In Japan, 51 species are described feeding on 21 host plant families, and 11 of them use *Acer*, which is the most common host plant genus of Japanese *Caloptilia* (Kumata et al. 2013). The feeding habits of the larvae change dramatically between the early and late developmental stages. Upon hatching, larvae mine the surface layer of the leaf (i.e., leaf-miners) until the third instar, then exit the mine, and form the edge of the leaf into a roll within which they feed externally until the final instar (hence the name leaf cone moth) (Kumata et al. 2013). Some species are leaf-gallers or blotch-miners at the final instar and do not roll leaves. Each species is usually associated with a single plant genus.

Sampling, DNA sequencing, and phylogenetic analyses

I sampled *Caloptilia* moths that use *Acer* trees at 73 sites covering a wide geographic range in Japan (Fig. 1, S2) during May–October of 2011–2015. Moths were sampled by searching for larvae in leaf rolls (fourth or fifth instar) or pupae on maple leaves. In total, 254 specimens were obtained, used to delimit species, and to establish the host range for each species. Delimitation of species was based on sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene; major divergences in COI sequences clearly corresponded with differences in wing pattern and genital morphology. Species were morphologically identified following Kumata (1982). To further determine whether the *Caloptilia* species feeding on maples resulted from a single radiation, I additionally sampled 44 *Caloptilia* species that use non-maple hosts and six species in closely related genera (*Gracillaria*, *Calybites*, and *Eucalybites*; for details see Table S1) and reconstructed a species-level phylogeny of *Caloptilia*. For the species-level phylogeny, one representative specimen of each *Caloptilia* species feeding on maple was included in the analysis. All moth specimens were kept in ethanol prior to DNA extraction.

I extracted genomic DNA using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). The head capsule of the larva or the head, wings, and abdomen of the adult were stored as vouchers. The COI gene was sequenced for all of the 254 moths collected from maples. For the species-level phylogenetic analysis, I sequenced four genomic regions: COI and the nuclear arginine kinase (ArgK), carbamoyl-phosphate synthetase 2 (CAD), and elongation factor 1-alpha (EF-1α) genes. I designed new primer sets for ArgK, CAD, and EF-1α (Table S3) based on sequences available for other species of Gracillariidae in the database. The information on existing primer sets for CO1 and EF-1α is also provided in Table S3. Polymerase chain reaction (PCR) amplifications were carried out under the following conditions: initial denaturation step at 94°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. Products were sequenced on an ABI 3100 automated sequencer using BigDye chain termination chemistry (Applied Biosystems, Foster City, CA, USA), and obvious sequence errors were manually corrected

using MEGA 6.06 (Tamura et al. 2013). Obtained sequences were aligned using Mafft ver. 6.901 (Katoh & Toh 2008) under the default settings. The resulting dataset contained 658, 573, 614, and 541 base pairs of COI, ArgK, CAD, and EF-1α, respectively. Species-level phylogenetic trees were constructed using two datasets: (a) an all-genes dataset (COI + ArgK + CAD + EF-1α) and (b) a nuclear-only dataset (ArgK + CAD + EF-1α). The latter was created because a previous phylogenetic study of Gracillariidae suggested that nuclear genes provide strong phylogenetic signals at the genus and species levels (Kawahara et al. 2011). I reconstructed phylogenetic trees by maximum likelihood and Bayesian methods for each dataset. The maximum likelihood analysis was performed using RAxML ver. 8.0 (Stamatakis 2014). I conducted 100 replicates of shotgun search for the likelihood ratchet, and assessed nodal support using bootstrap analyses with 1000 replications. I also conducted Bayesian phylogenetic analysis using MrBayes5D (Tanabe 2008), a modified version of MrBayes3.1.2 (Ronquist & Huelsenbeck 2003). I used the following settings for the Bayesian analysis: number of Markov chain Monte Carlo generations, five million; sampling frequency, 100; and burn-in, 5001. The burn-in size was determined by checking the convergence of log likelihood (ln L) plotted against generation time. In both methods, I used Kakusan4 (Tanabe 2011) to determine appropriate models of sequence evolution under the BIC4 criterion.

Hypothesis and randomization tests for validation

To test the relative importance of host shift in the speciation process from phylogeny, I assumed two contrasting scenarios (Fig. 2). If most speciation events are associated with host shifts, the dissimilarity in host use will on average be larger for phylogenetically more closely related pairs of *Caloptilia* moths (Fig. 2a). Conversely, if most speciation events occur during the initial stage of the radiation and more recent speciation events are independent of host shifts, host use dissimilarity will be larger for phylogenetically more distantly related pairs of *Caloptilia* moths (Fig. 2b). A similar framework was proposed by Nyman et al. (2010), but their method cannot be applied to species-level analysis. Following Barraclough et al. (1999), I used randomizations to compare the observed pattern of host use to that expected under a null model of no association with cladogenesis. My null model hypothesized that changes occurred at random and independently across the tree. The statistic used to test the association between phylogenetic distance and the degree of difference in host use is expressed as the sum across all nodes of phylogenetic distance X_i multiplied by the degree of host use dissimilarity H_i (see the next section for detailed calculation of dissimilarity),

$\sum_{i=l}^{i=m} XiHi.$

If differences in host use are greater between closely related species, the above statistic is expected to be smaller than that under the null model, and *vice versa*. Thus, I tested for a significant concentration of changes toward either the tips or the root of the tree. A positive sign indicates the concentration of changes toward the tips, whereas a negative sign indicates that more changes occurred toward the root.

The null distribution was obtained by randomly shuffling observed changes among branches of the tree and calculating the above statistic in each null trial. The two-tailed probability of the observed value was calculated based on 10,000 randomizations. A similar randomization method was used by Barraclough et al. (1999) and Sauer & Hausdorf (2009) to study adaptive character evolution in tiger beetles and land snails, respectively.

In addition, I calculated the standardized effect size (SES) as the observed test statistic minus the mean of the null distribution, divided by the standard deviation of the null distribution. This null model approach is commonly used for expressing biological differences regardless of the units of the indices (McCabe et al. 2012).

Indices of dissimilarity in host use

I used both Jaccard (Jaccard 1912; Koleff et al. 2003) and Unifrac (Lozupone & Knight 2005) indices to quantify the degree of difference in host use between a pair of *Caloptilia* moths feeding on *Acer* trees. Both indices are commonly used in community ecology for assessing the degree of dissimilarity between two communities (Cavender-Bares et al. 2009). The Unifrac index is analogous to the Jaccard dissimilarity index, but takes into account phylogenetic information (Lozupone & Knight 2005), which in the present case is the plant phylogeny. The Unifrac index has an advantage over the Jaccard index especially when there is missing information on host association; the latter index assumes an equal weight for all host plant species, whereas the former weighs host plants according to their phylogenetic relatedness and is thus less sensitive to missing data. In this study, I used the phylogeny of 30 Japanese *Acer* species published by Nakadai et al. (2014). In addition, both Jaccard and Unifrac indices can be partitioned into two components of dissimilarity: turnover and nestedness (Baselga 2010; Leprieur et al. 2012). In community ecology, the turnover of a species assemblage refers to the replacement of some species by others as a consequence of historical events, such as geographical barrier formation or environmental sorting (Baselga 2010). In contrast, the nestedness of a species assemblage occurs when the species composition of sites with a smaller number of the species is a subset of that of species-rich sites, which reflects a spatial pattern of species loss resulting from dispersal limitation or environmental filtering (Hirao et al. 2015). In my study, the turnover component indicates the degree of non-overlapping host use, and the nestedness component represents the difference in the degree of specialization between insect species with shared host plants (Fig. 3). All indices were calculated using the "betapart" package (Baselga & Orme 2012) in R ver. 3.2.2 (R Core Team 2015).

Results

Extensive sampling of *Caloptilia* moths throughout Japan identified 14 species feeding on maples (Fig. 4, S1), of which three were newly discovered in this study. This represents ca. 40% of the *Caloptilia* species known to feed on maples (De Prins & De Prins 2015). Most species were widely

distributed throughout the range, although some were only found at a limited number of sites (Fig. S2). Some species were apparently specialists on single *Acer* species (e.g., *C. hidakensis*, *C. kurokoi*), whereas others were collected from many hosts. Overall, each species uses 1–11 *Acer* species, with a mean of 3.0 ± 3.0 (Fig. 5).

Species-level phylogenetic analyses based on 2386 bp of the combined COI, ArgK, CAD, and EF-1α dataset produced a well-resolved phylogeny (Fig. 4). All of the *Caloptilia* species feeding on *Acer* were closely related, although they were not monophyletic. One species, *C. gloriosa*, was positioned outside of the clade consisting mainly of *Acer*-feeding *Caloptilia* (Fig. 4), and another species, *C. aurifasciata*, feeding on *Toxicodendron* (Anacardiaceae), was embedded within this clade (Fig. 4). I thus focused on the clade containing *C. aurifasciata* and the 13 species feeding on *Acer* for the analysis of host shifts. I conducted randomization tests separately for datasets with and without *C. aurifasciata*. Because information on the phylogenetic distance between *Acer* and *Toxicodendron* (the host of *C. aurifasciata*) was not available, I assumed the maximum turnover (1) and minimum nestedness (0) for the calculation of dissimilarity indices between *C. aurifasciata* and *Acer*-feeding *Caloptilia*.

The results of randomization tests indicated that the turnover components and the combined turnover and nestedness components of both Jaccard and Unifrac indices are greater between distantly related species than expected under the null model (positive signs in Table 1), although the trend was not significant for the Jaccard index except for the turnover component of the all-genes dataset. The nestedness component showed negative signs but was not statistically significant (Table 1). These results support the hypothesis of phylogenetic conservatism in host use (Fig. 2b). Inclusion of *C. aurifasciata*, which feeds on *Toxicodendron*, did not change the overall pattern but slightly strengthened the trend, with tests using both Jaccard and Unifrac indices becoming significant (Table S4).

The SES values provide a quantitative measure of the strength of association between host use dissimilarity and phylogenetic distance (Table 1). Overall, the values for the turnover component and the combined turnover + nestedness component were greater when host plant phylogeny was taken into account (Unifrac index) than when it was not (Jaccard index).

Discussion

Application of randomization test in the study of herbivorous insect speciation

In this article, I describe a new method for testing the role of host shift in herbivorous insect speciation. I identified three beneficial features of this method. First, it is less sensitive to incomplete species sampling. It is usually difficult to sample every species for the entire radiation (Lopez-Vaamonde et al. 2003; Nyman et al. 2006; Agrawal & Fishbein 2008; Stone et al. 2009; Doorenweerd et al. 2015), and conventional methods of analyzing the effects of host shifts on phylogeny (e.g., ancestral character state reconstruction) are sensitive to species sampling. However,

because my analysis focuses on whether host use changes are concentrated toward either the root or the tips of the phylogenetic tree, complete sampling is not required as long as species sampling is not biased (e.g., toward species feeding only on a particular species of host).

Second, the method permits analysis of speciation by host shift at a broader geographic scale. In many cases, herbivorous insect species have broader distributions than individual host plant species, so sister herbivore species occurring in allopatry should always use different hosts, even if diet shift was not the major cause of speciation. The use of a dissimilarity index controlling for host phylogeny partly remedies this problem (Pearse & Altermatt 2013; Pearse et al. 2013) because related plant species are generally similar in their traits associated with susceptibility to herbivores (Rasmann & Agrawal 2011; D'Costa et al. 2014; Nakadai & Murakami 2015), and thus host use dissimilarity will consistently be low if no major diet shift has occurred during speciation. Caution is needed in cases where the group of herbivores being studied has extremely high or low host specificity because, in both cases, the method may overestimate host use conservatism.

Finally, calculation of SES allows comparison of trends among different studies (McCabe et al. 2012) because SES is independent of differences in the number of herbivore species included in the dataset. Previous phylogenetic studies assessed the percentage of host shifts between host plant families in each taxonomic group (Lopez-Vaamonde et al. 2003; Nyman et al. 2010; Doorenweerd et al. 2015), but quantitative comparisons among studies were difficult due to the lack of a standardized measure for comparison.

I note that my method has a link to those developed previously to test the degree of cospeciation between a pair of host and parasite. However, because they are designed to test for cospeciation, they either assume that each parasite is associated with only one host at any given time (Page 1994; Ronquist 1995; Charleston & Robertson 2002; Merkle & Middendorf 2005; Conow et al. 2010) or that host and parasite speciation events are simultaneous in time (Legendre et al. 2002), which are not realistic for many plant–herbivore associations. Recently, Rafferty & Ives (2013) and Hadfield et al. (2014) developed methods that do not require such assumptions and uses GLMM to test for interaction effect of two phylogenies, but the methods are not designed to test the polarity of trait divergence occurring either toward the tips or the root of the phylogeny as in my method.

One weakness of my analysis is that I treated host association based on presence/absence, but in reality, preference levels are not equal for all of the host plant species observed. I could not quantify host preference in this study because it is necessary to standardize both sampling effort and host abundance to obtain a comparative measure of host preference, which was difficult to accomplish at all sampling sites. However, the above-described method can easily incorporate host preference when such data are available, as dissimilarity measures (Unifrac and Jaccard indices) are also designed for quantitative data. The newly developed method is presently intended for testing host-shift-driven speciation in herbivorous insects, but the overall framework is applicable, in

principle, to studies of other types of ecological speciation. The source code for running the analysis in R is provided as supplementary material.

Alternative hypothesis on the speciation process of leaf cone moths feeding on maples

Application of the present method to the 13 species of maple-feeding leaf cone moths suggested that major dietary changes are concentrated toward the root of the herbivore phylogenetic tree (Table 1). Because the Unifrac index takes into account plant phylogeny whereas the Jaccard index does not, significant positive sign for the Unifrac index and lack of significance for the Jaccard index indicates that the trend exists only when host plant phylogeny is taken into account in the calculation of dissimilarity. Thus, the results indicate that major dietary shifts play a minor role in recent speciation events, but shifts between very closely related hosts may have took place during recent *Caloptilia* speciation. The addition of *C. aurifasciata* generally strengthened the trend for both Jaccard and Unifrac indices because *C. aurifasciata* diverged from all other species toward the root of the tree and has a completely different diet. The Jaccard test, which was only marginally insignificant in the absence of *C. aurifasciata*, became significant after the inclusion of this species (Table S4).

Although my test indicated that speciation assisted by host shift may be relatively minor in this group, I do not deny the importance of major dietary changes as such events occur in some of the earliest speciation events. Nevertheless host-shift-driven speciation may not be as important as commonly thought in generating the current diversity of *Caloptilia*. Because my analysis only tests for patterns, the alternative process that drives speciation in *Caloptilia* cannot be inferred from my data. However, previous studies proposed several possible processes by which herbivorous insects speciate without changing their diet (Imada et al. 2011; Bennett & O'Grady 2012; Yamamoto & Sota 2012; Hamm & Fordyce 2015). For some phytophagous insect groups, allopatric speciation without host shift may be a major factor causing radiation (Nyman et al. 2010; Imada et al. 2011), but in the case of Japanese leaf cone moths, the pattern is unclear based on visual inspection of the current geographical distribution (Fig. S1). Ecological shift within a host plant is also a significant process (Condon & Steck 1997; Cook et al. 2002; Joy & Crespi 2007; Althoff 2014; Mishima et al. 2014). For example, Zhang et al. (2015) demonstrated divergence induced by host plant ages in sympatric sister beetles (*Pyrrhalta maculicollis* and *P. aenescens*) feeding on elm. There is clearly a need to sample from a broader geographic area and to collect additional information on micro-niche divergence among leaf cone moths to fully understand the process underlying their diversification. Adding timeline to the divergence events of both herbivores and host plants should also facilitate the understanding of the role of host shift in herbivore radiation.

Revealing the role of host shifts in herbivorous insect diversification

my study proposed a method for assessing the relative importance of host shifts in herbivorous insect speciation. This method allows quantitative analysis at a fine taxonomic scale, but because I only

applied it to one herbivorous insect group, the application of this method to various herbivorous insect groups will facilitate a more general discussion on herbivorous insect diversification. If host-shift-driven speciation turns out to be relatively minor in recent speciation, there may be another role for host shifts in promoting herbivorous insect diversification rather than facilitating speciation per se, such as facilitating the entry into novel niche spaces (Janzen 1968) and the coexistence of already diverged species (Rabosky 2009). Information on the phylogenetic pattern of host use is clearly increasing rapidly, and a standardized method would link studies using different systems and facilitate my understanding of the effects of host shift on herbivorous insect diversity.

Table 1 Relationships between differences of host use and phylogenetic distance between *Caloptilia* species feeding on *Acer* according to randomization tests. Positive signs of differences in host use with phylogenetic distance suggest that changes are concentrated toward the root and negative signs suggest that changes occur near the tips. Significance level: n.s., $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$.

		Turnover + Nestedness			Turnover			Nestedness		
Dataset		Sign	SES		Sign	SES		Sign	SES	
All-genes dataset	Jaccard index	+	1.66	n.s.	+	1.95	*	−	−1.26	n.s.
	Unifrac index	+	2.17	*	+	2.16	*	−	−0.85	n.s.
Nuclear-only dataset	Jaccard index	+	1.90	n.s.	+	1.95	n.s.	−	−1.10	n.s.
	Unifrac index	+	2.72	**	+	2.40	*	−	−0.60	n.s.

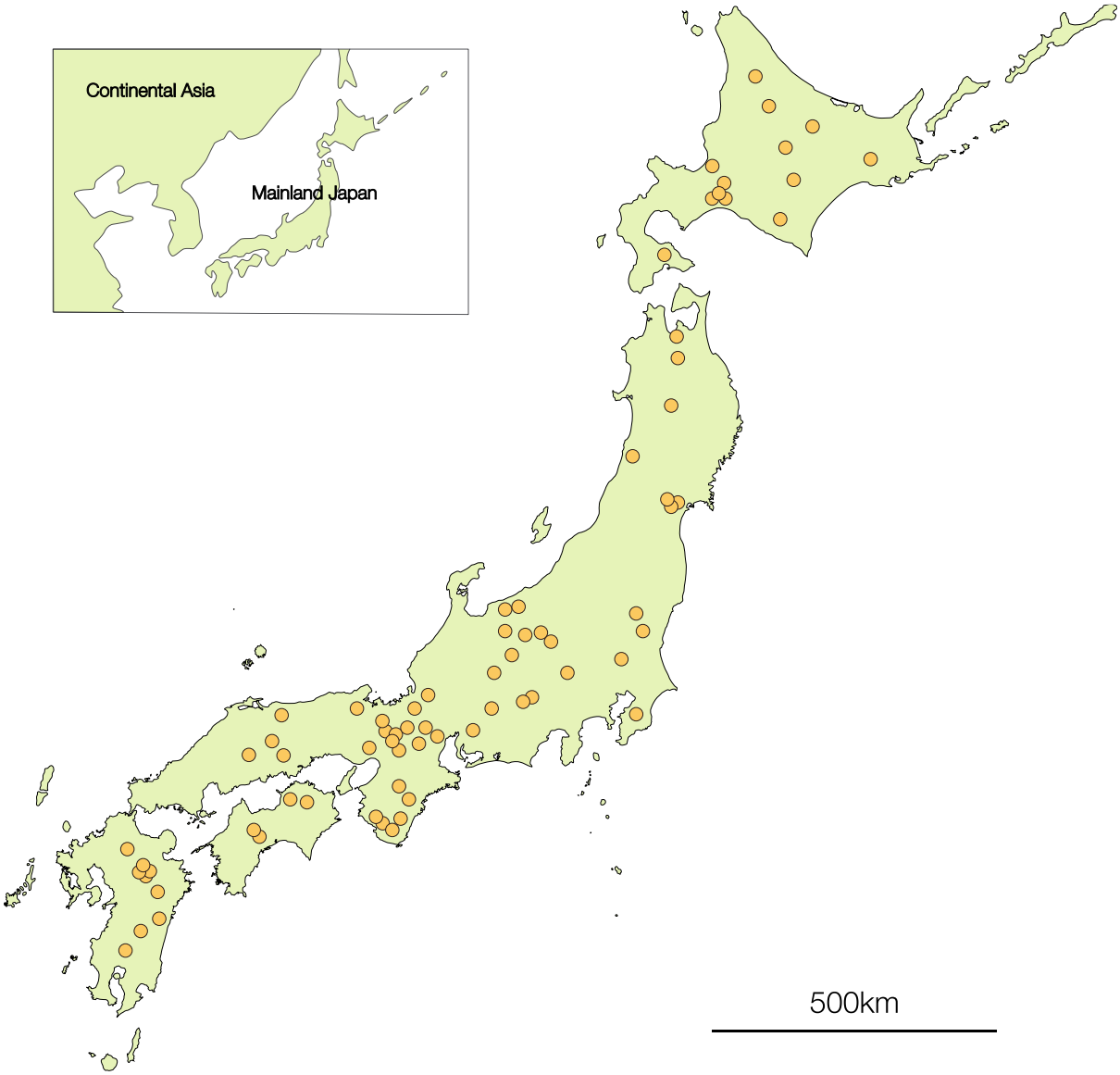

Figure 1 Sampling localities of *Caloptilia* moths collected from *Acer* trees in Japan. Sampling information for each species shown in Figure S2.

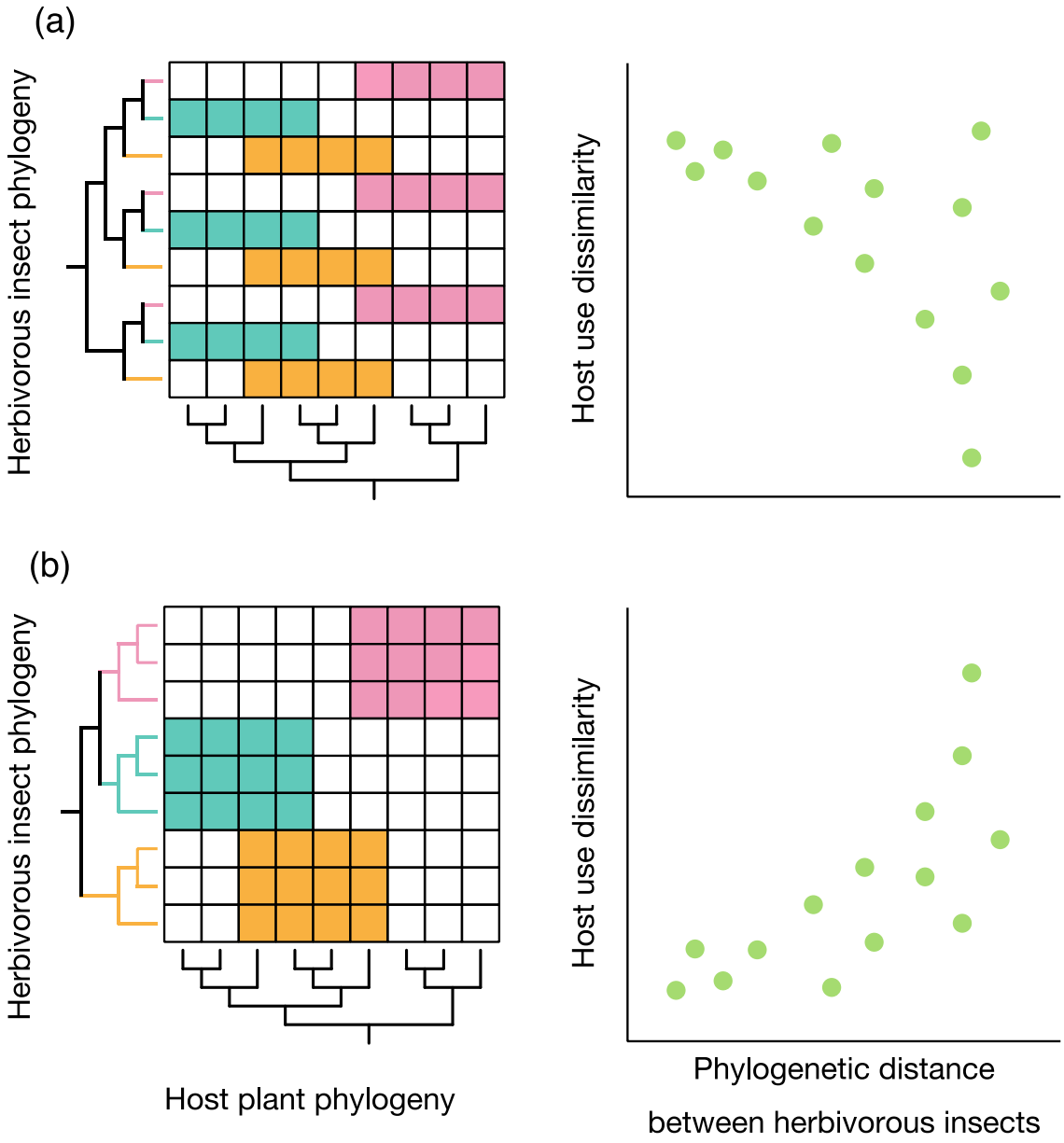

Figure 2 Phylogenetic distributions of host use arising from different speciation modes in herbivorous insects. (a) Distribution of host use on the phylogeny of a hypothetical insect group in which speciation is mainly associated with host shifts. (b) Distribution of host taxa when speciation mainly involves other processes without host shifts.

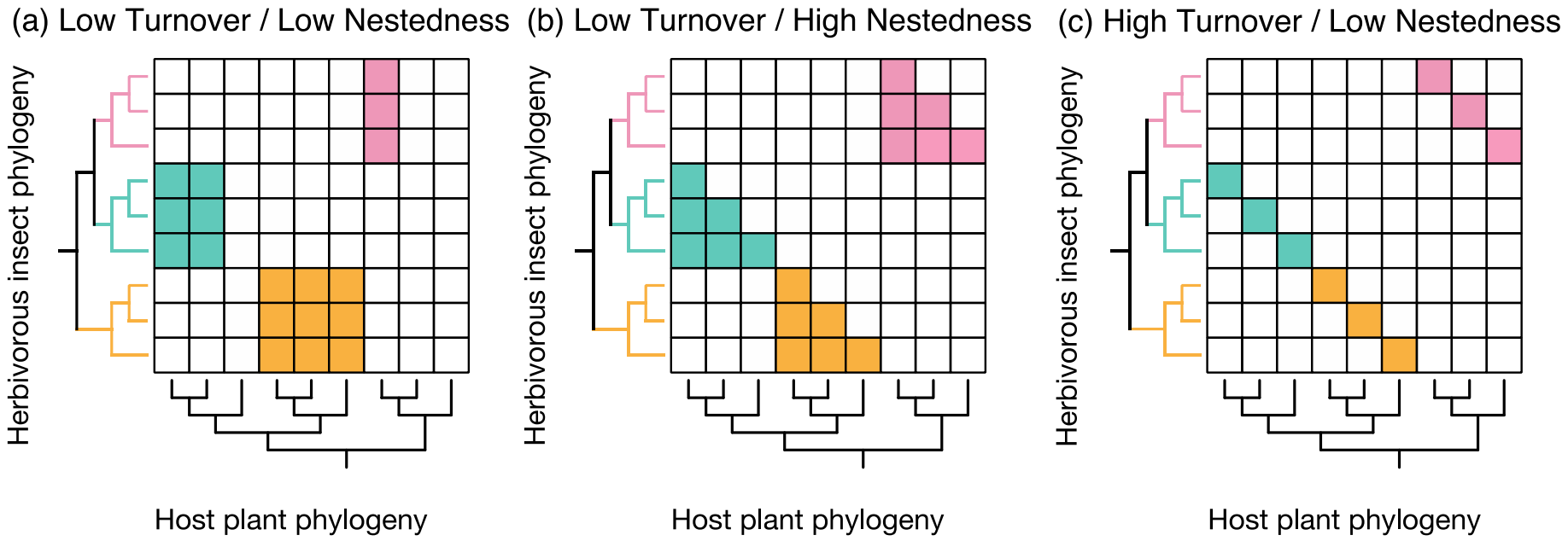

Figure 3 Possible patterns of plant–herbivore association. (a) Low turnover / low nestedness, (b) low turnover / high nestedness and (c) high turnover/ low nestedness. Both Jaccard and Unifrac indices perform similarly in (a) and (b), whereas in (c), the nestedness component of the Unifrac index between a pair of closely related herbivores will be lower than that of the Jaccard index. This is because host use is similar when host phylogeny is taken into account but maximally dissimilar in the absence of host phylogenetic information.

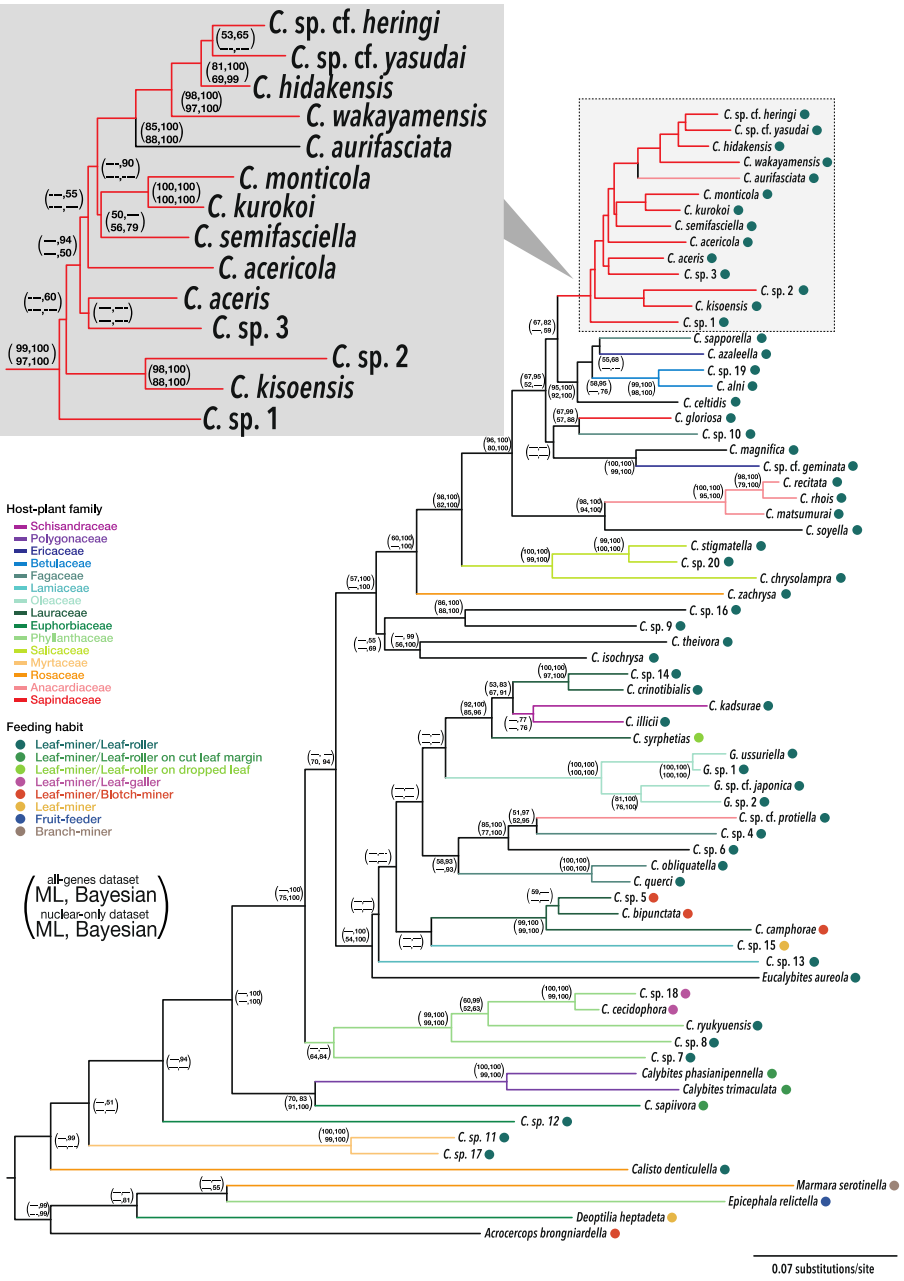

Figure 4 Phylogeny of *Caloptilia* moths and their related groups. The phylogeny was constructed by maximum likelihood method using four genomic regions (COI, ArgK, CAD and EF-1α) of 71 species.

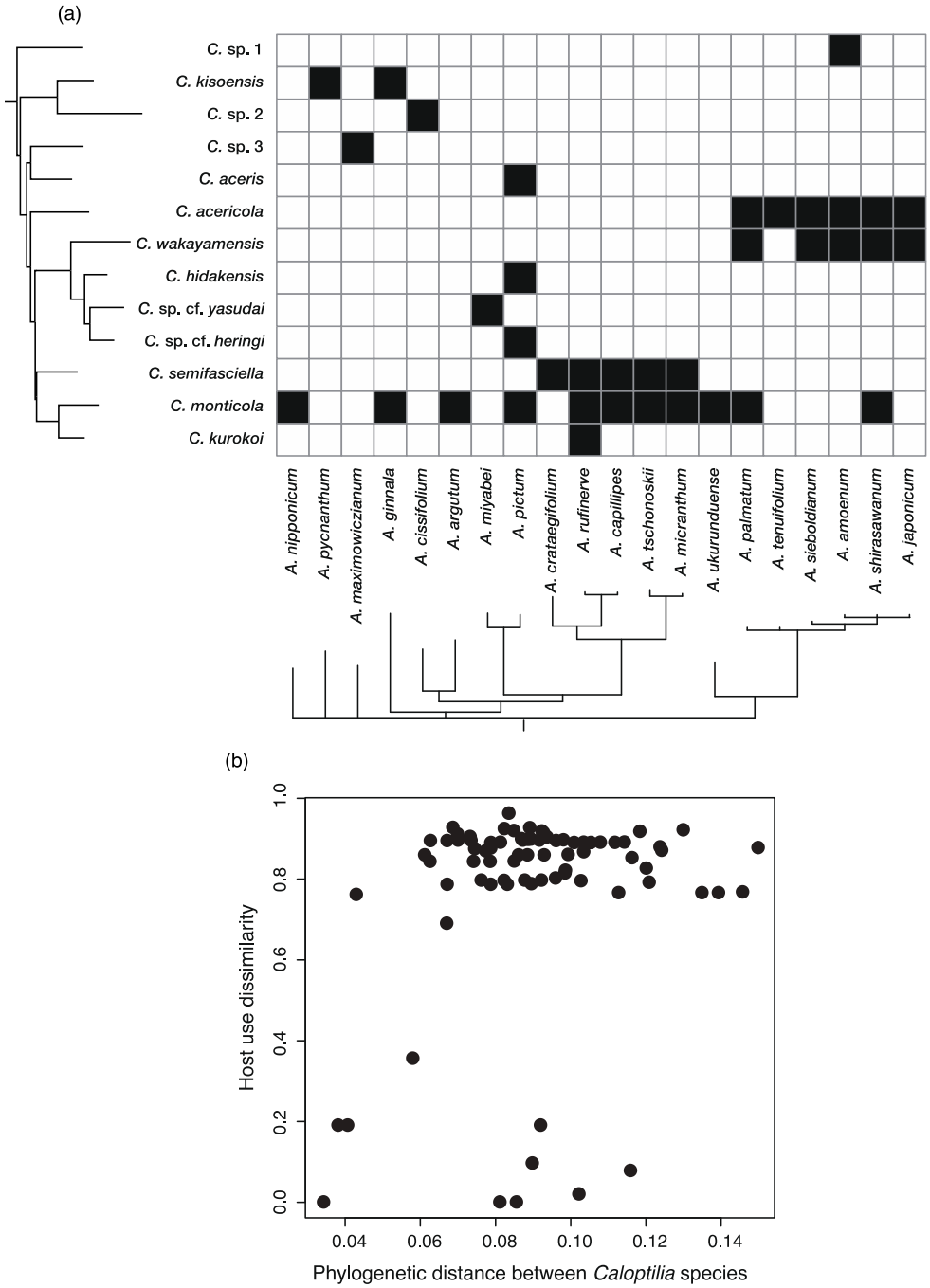

Figure 5 The results of *Acer–Caloptilia* interactions obtained from wide range sampling in Japan. (a) Phylogram of 13 species of *Caloptilia* pruned from a phylogeny of this genus and related groups (Table S3) and a phylogram of 20 species of *Acer* trees pruned from a phylogeny of this genus in Japan (Nakadai et al. 2014). The complete phylogeny of *Acer* trees was the 50% majority-rule consensus of trees sampled from the stationary distribution of a Bayesian analysis of four chloroplast DNA loci sampled from 30 species, including some varieties. (b) The plot of phylogenetic distance between *Caloptilia* moths (all-genes dataset) versus host use dissimilarity (turnover and nestedness components of the Unifrac index)

Chapter 4
Maintenance of local species diversity of leaf cone moths (*Caloptilia*) feeding on maples (*Acer*)
Section 4-1
Patterns of host use by herbivore assemblages of the genus *Caloptilia* (Lepidoptera; Gracillariidae) on congeneric maple tree (*Acer*) species

Introduction

Terrestrial plants and herbivorous insects participate in some of the most diverse ecological and evolutionary interactions on the planet (Novotny et al. 2010). Numerous studies have confirmed associations between defensive plant traits and host use by herbivorous insects (Agrawal & Fishbein 2006; Pearse & Hipp 2009). For example, Rasmann & Agrawal (2011) showed that the distribution of defensive cardenolides among *Asclepias* species explains host use by the milkweed beetle (*Tetraopes tetraophthalmus*). However, plant defensive traits are not the only factor that determines host specificity. Webster et al. (2010) demonstrated that herbivores use several chemicals (i.e., characteristic blends of volatile compounds) to detect host plant species, indicating that numerous plant traits can mediate plant–herbivore interactions.

Plant phylogeny can act as an integrating measure of plant traits among species (Pearse & Hipp 2009; Rasmann & Agrawal 2011; D'Costa et al. 2013), causing covariance between host plant phylogeny and herbivore assemblages (Ødegaard et al. 2005; Weiblen et al. 2006; Novotny et al. 2010; Watanabe et al. 2014). However, plant phylogeny is useful only when there is phylogenetic signal in the plant traits that determine herbivore use of host plants (Pearse et al. 2013). If key plant traits diverge among close relatives or there has been evolutionary convergence, phylogenetic relatedness will not correspond to plant trait similarity (Becerra 1997; Pearse et al. 2013).

The pattern of host use by herbivorous insects can be described as a multi-filtering model that includes several sequential processes, each of which filters out host plant species (see Pearse et al. 2013). The first step is oviposition preference (Fig. 1a), in which an insect must identify a host plant from a distance by detecting a blend of chemical compounds (Nishida & Fukami 1989). Oviposition preference can be tested by measuring the correlation between the number of eggs laid and the phylogenetic distance from the most suitable host plant (Rasmann & Agrawal 2011). The second step is larval feeding and growth (Fig. 1b). Larval performance should directly depend on foliage quality of the host plant in relation to host phylogeny

(Rasmann & Agrawal 2011). Nishida & Fukami (1989) and Murata et al. (2011) showed that both oviposition and feeding preference are determined by the mixture of chemical compounds, and preference decreases as the number of chemical compounds in common with the natural host decreases. The third step is escape from natural enemies (Fig. 1c), and several studies have suggested that use of novel plant hosts increases the probability of survival (i.e., enemy-free space; Singer & Stireman 2005). For example, a race of galling fly, *Eurosta solidaginis*, exhibited lower levels of parasitism on the derivative host *Solidago gigantea* than did the presumed ancestral sympatric race on *Solidago altissima* (Brown et al. 1995).

In this study, I focused on the interaction between congeneric *Acer* (Sapindales, Sapindaceae) species and congeneric *Caloptilia* (Lepidoptera, Gracillariidae, subgenus Caloptilia) caterpillars. The *Acer* genus is one of the most taxonomically diverse groups of trees in the northern hemisphere, particularly in the temperate regions of East Asia, eastern North America, and Europe (van Gelderen et al. 1994). The genus comprises 124 species in the northern hemisphere, with most of them distributed in China, Korea, and Japan (81% of total species; Renner et al. 2007). The *Caloptilia* genus is globally distributed and is one of the largest groups in the Gracillariidae moth family (Kumata 1982). *Caloptilia* species that feed on the genus *Acer* are most diverse in Japan (11 of 51 total species in Japan, Kumata et al. 2013). All *Caloptilia* species that feed on *Acer* are specialists on the taxon. Several recent studies have examined plant–herbivore interactions of Gracillariidae (Ohshima 2012; D'Costa et al. 2013; Hembry et al. 2013; Okamoto et al. 2013). For example, D'Costa et al. (2013) confirmed a gradient in oviposition preference and larval performance for *Cameraria ohridella* among congeneric *Aesculus* tree species. The interaction between *Acer* and *Caloptilia* offers an ideal opportunity to examine interactions between congeneric specialist herbivores and congeneric host plants.

Although many previous studies have examined interactions between plants and herbivorous insects, host specificity at fine taxonomic scales such as genera is rarely examined. I proposed two hypotheses related to the multi-filtering model of host use and community assembly among closely related host plants (Fig. 1). First, I hypothesised that there is a negative correlation between phylogenetic distance from the most suitable host plant species and the abundance of emerging adult herbivorous insects on a host. Phylogenetically controlled correlations between plant traits and larval abundance are necessary to clarify the relationship between traits and larval performance independent of phylogenetic distance (Rasmann & Agrawal 2011). Secondly, I used Mantel and partial Mantel tests to examine the hypothesis that the assemblage dissimilarity of *Caloptilia* insects among host plant species increases with

increasing distance of plant phylogeny and traits.

Materials and methods

Study sites

I conducted field surveys in a mosaic of primary and secondary temperate mixed forest at the University of Tokyo's Chichibu Forest in central Japan (35°54′N, 138°49′E). The secondary forest was dominated by *Quercus crispula*, and the primary forest was dominated by *Fagus japonica* at lower altitudes and *Tsuga diversifolia* at higher altitudes (The University of Tokyo Chichibu Forest, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 2012). The average annual temperature between 1996 and 2010 was 11.0 °C, and the average annual rainfall was 1514.2mm (The University of Tokyo Chichibu Forest, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 2012).

Acer species

I selected 14 *Acer* species for the survey: *A. amoenum*, *A. capillipes*, *A. carpinifolium*, *A. japonicum*, *A. maximowiczianum*, *A. micranthum*, *A. palmatum*, *A. pictum* ssp. *dissectum*, *A. rufinerve*, *A. shirasawanum*, *A. sieboldianum*, *A. tenuifolium*, *A. tschonoskii*, and *A. ukurunduense*, all of which commonly occur at the study site. Although nine other *Acer* species (i.e., *A. argutum*, *A. austral*, *A. crataegifolium*, *A. cissifolium*, *A. diabolicum*, *A. distylum*, *A. nipponicum* ssp. *nipponicum*, *A. pictum* ssp. *pictum*, *A. pictum* ssp. *savatieri*) are present in the forest, they were too rare for a detailed survey. I followed the taxonomic nomenclature of the Y-list (Yonekura & Kajita 2003).

Herbivore sampling and identification

I collected *Caloptilia* species by hand (Novotny et al. 2002; Murakami et al. 2007) from the foliage of 14 Acer species over a period of 3 months for 2 years (June–August, 2011 and 2012). I collected *Caloptilia* species from 12 individuals of each *Acer* species in 2011 and from 10 individuals of each species in 2012 from a branch of diameter 3–5 cm. *Caloptilia* larvae and pupa were reared in the laboratory on the leaves of their host plant species at 25 °C, and all individuals were identified to morphospecies.

Statistical analyses

Among the 29 Japanese *Acer* species, there are significant phylogenetic signals for three leaf traits – thickness, C:N ratio, and condensed tannins – whereas none of phylogenetic signals

were detected on the other leaf traits: specific leaf area, water content, and total phenolics (Nakadai et al. 2014). I performed two independent principal component analyses (PCAs), with and without phylogenetic signals, to summarise leaf quality as an index of leaf defences (DI, Pearse & Hipp 2012). I calculated Jaccard's dissimilarity indices for all possible pairs of herbivore assemblages among plant species (Oksanen et al. 2011). For this analysis, the data on two *Acer* species (*A. amoenum* and *A. carpinifolium*), on which no *Caloptilia* were found, were excluded. I also utilized Euclidean distance matrices for all possible pairs of plant species for the first two axes of the PCAs for leaf traits with and without phylogenetic signals (Nakadai et al. 2014). Phylogenetic distances from the phylogeny of 30 *Acer* species in Japan (Nakadai et al. 2014) were utilized.

To test the first hypothesis, I examined correlations between herbivore abundance and host phylogentic distance for *Caloptilia* species that were observed on more than three *Acer* species. In the present study, the most suitable hosts were assumed to be the species with the greatest number of emerging adult herbivores. I also tested all the correlations by excluding the zero point of phylogenetic distance to eliminate the effect of the most suitable host (Rasmann & Agrawal 2011).

To test the second hypothesis, I used Mantel tests (Oksanen et al. 2011) to examine the effect of the following plant variables on the composition of herbivore assemblages: (i) host plant phylogeny (phylogeny); (ii) a PCA index of leaf defences with phylogenetic signal (pDI1 for the first axis and pDI2 for the second axis); and (iii) a PCA index of leaf defences with no phylogenetic signal (nDI1 for the first axis and nDI2 for the second axis). Additionally, I compared the relative importance of each independent variable with a significant ($P<0.05$) effect in the tests of dissimilarity of herbivore assemblages by controlling the effect of phylogeny and leaf traits using partial Mantel tests (Tuomisto & Ruokolainen 2006; Barber & Marquis 2011; Reich & Milla 2011). These provide a test of significance without inflating the probability of type I error caused by the indirect effect of a third factor (Barber & Marquis, 2011). The correlations between phylogeny and PCA indices of leaf defences (pDI1, 2, nDI1, 2) of host plants were also analysed by a Mantel test. All statistical tests were performed in r using the packages adephylo (Jombart et al. 2010), vegan (Oksanen et al. 2011), and prcomp (R Development Core Team, 2007).

Results

A total of 154 individual larvae of six *Caloptilia* species (*C. aceris*, *C. acericola*, *C. hidakensis*, *C.* sp., *C. monticola*, *C. semifasciella*) were reared on 12 *Acer* species (Fig. 2). The abundance

of emerging adults decreased with increasing phylogenetic distance from the most suitable host for all four dominant *Caloptilia* species (*C. monticola*, P<0.001; *C. acericola*, P=0.022; *C.* sp., P=0.052; *C. semifasciella*, P<0.001; excluding zero point P=0.002, P=0.031, P=0.102, P<0.001; Fig. 3).

Jaccard's measure of dissimilarity between herbivore assemblages showed a significant positive correlation with phylogenetic distance (r =0.509, P=0.002; Fig. 4). The correlation between the first PCA index of leaf defences and phylogenetic signal also showed a significant positive trend (pDI1, r =0.279 P=0.036; Fig. 4). None of the other PCA indices for leaf defences showed significant relationships by Jaccard's measure of dissimilarity (pDI2, r=−0.123, P=0.215; nDI1, r=−0.101, P=0.272; nDI2, r =0.046, P=0.339). Partial Mantel tests detected significant positive correlation between assemblage dissimilarity and phylogenetic distance under the control of the distance of the PCA index of leaf defences (r =0.465, P=0.004), but there were no significant correlations between assemblage dissimilarity and distance of the PCA index of leaf defences under the control of phylogenetic distance (pDI1, r =0.149, P=0.155). The positive correlation of the PCA index of leaf defences with phylogenetic signal (pDI1) and phylogeny of host plants (r =0.239, P=0.040) and the other PCA indices of leaf defences showed no significant relationship to host plant phylogeny (pDI2, r=−0.150, P=0.114; nDI1, r =0.017, P=0.406; nDI2, r=−0.118, P=0.171).

Discussion

This study showed the process of the community assembly of congeneric herbivore species on congeneric host plant species. Both of my hypotheses about the multi-filtering model of host plant use of herbivore assemblage (Fig. 1) were confirmed. Host plant use followed a unimodal pattern around the most suitable host plant, and the overlap of these unimodal patterns described the assembly of *Caloptilia* assemblages.

Caloptilia abundance followed a unimodal distribution of host suitability in relation to host plant phylogeny (Fig. 2), similar to the pattern found by Rasmann & Agrawal (2011). This pattern was observed for all of the *Caloptilia* species in this study (Figure S1). The same pattern was also reported by Kumata (1982) for *C. acericola* and *C. aceris* on five *Acer* species. Nishida & Fukami (1989) and Murata et al. (2011) revealed that host suitability for herbivores is explained by the combination of chemical compounds most similar to the most suitable host plant. They also observed that oviposition frequency decreased with a decrease in the number of chemical compounds associated with the most suitable combination.

I also found increasing dissimilarity of herbivore assemblages with increasing distance

of plant phylogeny and leaf traits, in accordance with my second hypothesis (Fig. 4). The application of phylogenetic techniques at the community level is a powerful tool for describing the composition of insect assemblages among host plant species (Rasmann & Agrawal, 2011). A correlation between host plant phylogeny and the composition of herbivore assemblages has been observed in many previous studies (Ødegaard et al. 2005; Weiblen et al. 2006; Novotny et al. 2010). For example, Weiblen et al. (2006) showed that a large proportion of herbivore species in tropical rainforests of New Guinea are clade specialists at the genus or family level. However, the mechanism creating this pattern at the population level had rarely been examined with appropriate statistical methods prior to Rasmann and Agrawal (2011). I found that the effect of phylogeny combined with leaf traits (pDI1) was clearer than the effect of leaf traits alone for *Caloptilia* assemblages (Fig. 4). Host plant phylogeny was a good predictor of plant traits even among congeneric plant species (see also Pearse & Hipp, 2009; Nakadai et al. 2014).

The host range of each herbivore species should be determined by the abundance of the species as a result of sampling effects. I suggest that species-specific fluctuations in population size among each herbivore species should change the observed host ranges of herbivorous insects. For example, the observed frequency of host use on less suitable hosts should increase with increasing population size of herbivorous insects.

I observed multiple peaks of herbivore abundance that were shared by several *Caloptilia* species (Fig. 2). This suggests that the role of interspecific competition was less important than phylogenetic relatedness of host plants and that *Caloptilia* assemblages may not be determined by a single absolute trait among host plant species. Furthermore, the peaks in abundance (i.e., the most suitable host) were evenly distributed along the host plant phylogeny (Figs 2 and 3). Nyman (2010) proposed that the probability of speciation is determined by the balance between the probability of colonisation and the likelihood of disruptive selection. Speciation probabilities are maximized when new hosts are distributed at an intermediate distance in resource space between the original host and the host of co-occurring larval species. Consequently, the sequence of allopatric speciation without host shifts and sympatric speciation with host shifts could cause the observed pattern of herbivore assemblages.

Although I demonstrated the role of host plant phylogeny and traits on the assembly of *Caloptilia* assemblages, it is still unknown what factors determine the most suitable host species and the unimodal patterns of fitness. Additionally, I only investigated local plant–herbivore interactions. A definitive understanding of the assembly of herbivore assemblages would require examination of geographical and historical effects on plant–herbivore interactions. The multi-filtering model would help to reveal the process of constructing current assemblages of

herbivorous insects on host plants.

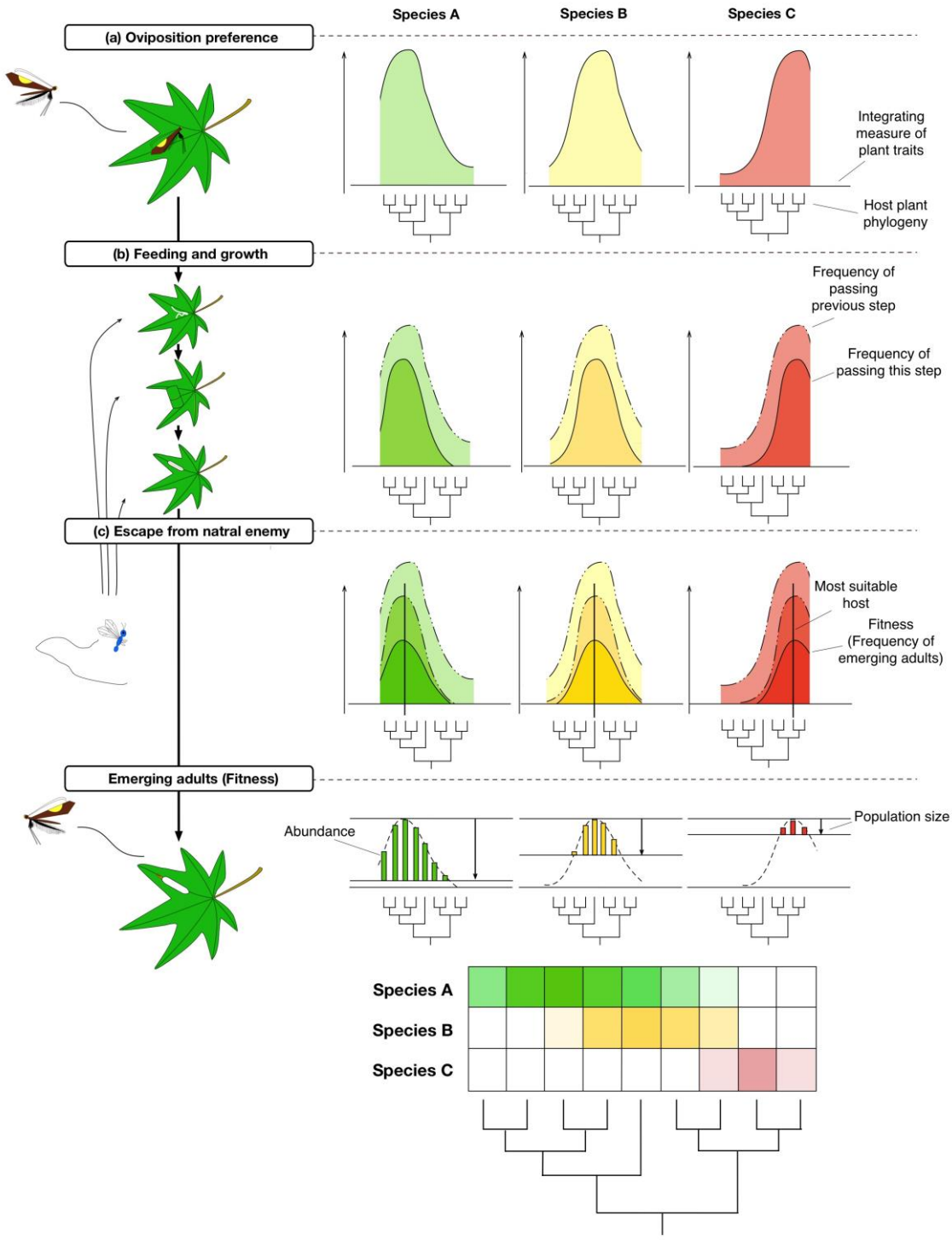

Figure 1 The multi-filtering model of host utilisation among closely related host plants. The frequency of host utilisation is determined by filtering at each step of colonisation, and the overlap of multiple patterns of gradual host utilisation determines the community structure of herbivorous insects at a local scale.

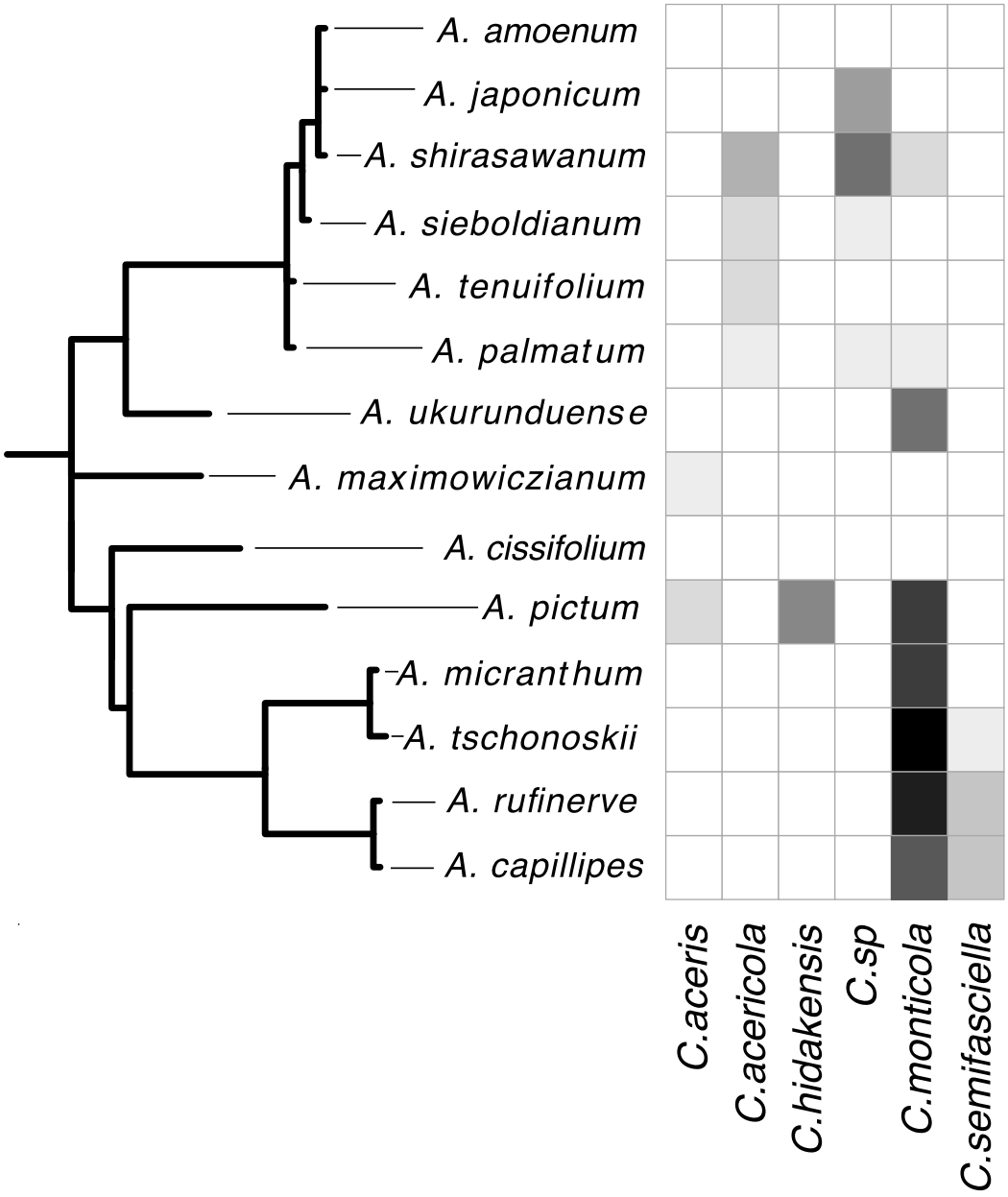

Figure 2 Phylogram of 14 species of *Acer* pruned from a phylogeny of the *Acer* genus in Japan (Nakadai *et al.*, 2014). The result of *Acer*–*Caloptilia* interactions was obtained from sampling of herbivorous insects. The complete phylogeny was the 50% majority-rule consensus of trees sampled from the stationary distribution of a Bayesian analysis of four chloroplast DNA loci sampled from 30 species, including some varieties. The network image was developed using the 'visweb' function in the bipartite library in the R environment.

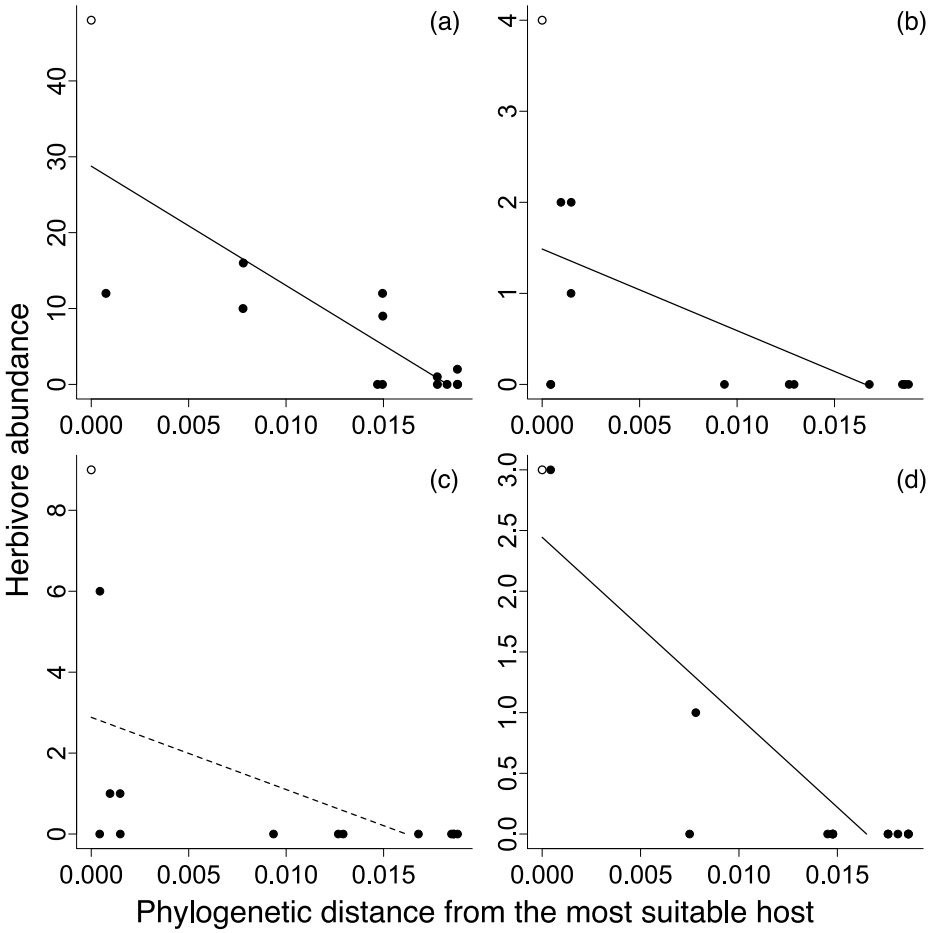

Figure 3 The number of emerging adults of four *Caloptilia* species predicted by phylogenetic distance from the most suitable host of 13 *Acer* species (filled dots). The most suitable host is represented by zero phylogenetic distance (open dot). Three of four relationships [lines, panel (a) *C. monticola* Pearson, n=14, r=−0.799, P<0.001; (b) *C. acericola* Pearson, n=14, r=−0.604, P=0.022; (d) *C. semifasciella* Pearson, n=14, r=−0.889, P<0.001] remained significantwhen the zero pointwas removed [(a) Pearson, n=13, r=−0.764, P=0.002; (b) Pearson, n=13, r=−0.597, P=0.031; (d) Pearson, n=13, r=−0.829, P<0.001]. The other relationship [dashed line, panel (c) *C.* sp, Pearson, n=14, r=−0.528, P=0.052] was marginally significant, but showed no significant correlation when the zero point was removed [(c) Pearson, n=13, r=−0.474, P=0.102].

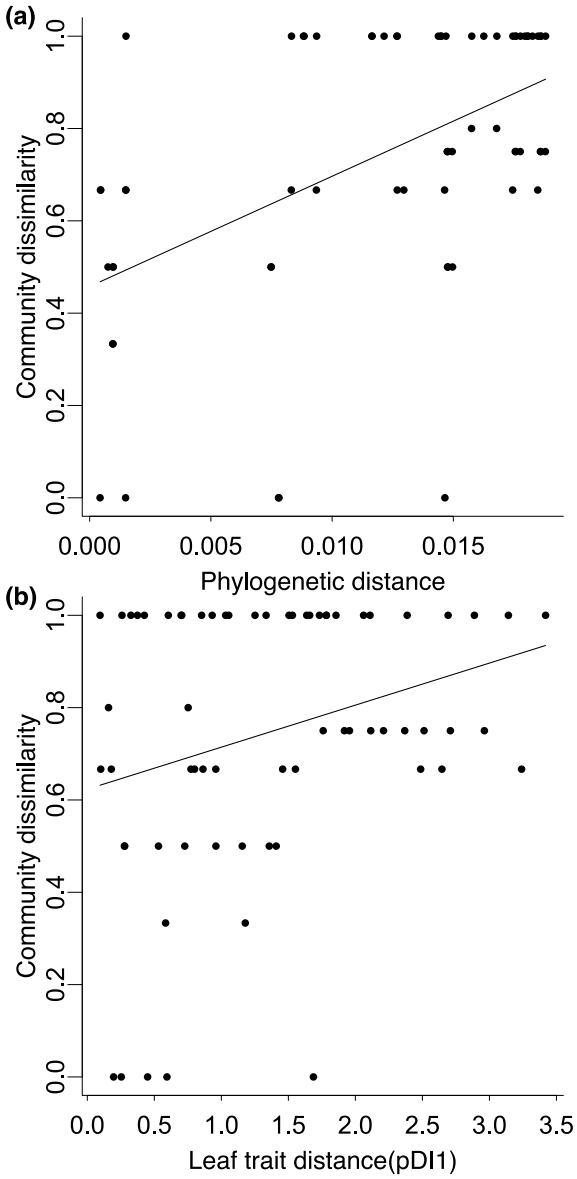

Figure 4 Relationships between assemblage dissimilarity and phylogenetic distance (a) and the distance of the leaf defence index of pDI1 (b). Both relationships were significant according to a Mantel test [(a) Pearson, r =0.509, P=0.002; (b) Pearson, r =0.279, P=0.036].

Section 4-2
Patterns of temporal and enemy niche use by a community of leaf cone moths (*Caloptilia*) coexisting on maples (*Acer*) as revealed by metabarcoding

Introduction

Host plant diversity is arguably the primary factor that drives the diversity of herbivorous insects on earth (Novotny et al. 2006). Because herbivore species are usually specialized to a narrow taxonomic group of plants, ecological speciation as the result of a shift to a new host (host-shift-driven speciation) is often considered a major driver of herbivorous insect diversification (Feder et al. 1988; Hawthorne & Via 2001; Nosil et al. 2002; Malausa et al. 2005). For example, a classic study by Farrell (1998) showed that among the Phytophaga beetles, lineages that use angiosperms as hosts are more species rich than are those that use gymnosperms, suggesting that the diversity of angiosperms has facilitated host-shift-driven diversification of the beetles that feed on them. However, different views about the effects of host plants in generating herbivorous insect diversity have arisen (Rabosky 2009; Kisel et al. 2011; Nyman et al. 2012) as studies increasingly document examples where closely related herbivores share the same host plants (Nyman et al. 2010; Imada et al. 2011; Nakadai & Kawakita 2016). One hypothesis is that host plants facilitate the coexistence of species that have already diverged, and a shift to a new host is not necessary at the time of speciation (Rabosky 2009; Nakadai & Kawakita 2016); however, the exact role of host plants in facilitating local coexistence has not been well studied. Studying the mechanisms that permit local coexistence of closely related herbivores is important, as both the number of locally coexisting species and the mean geographical range size are significant estimators of global species diversity (Storch et al. 2012)

Correlation between host plant diversity and herbivorous insect diversity is often confirmed at the local community level (Siemann et al. 1998; Borer et al. 2012). This indicates that the use of different host plants is important for niche partitioning and species coexistence (MacArthur & Levins 1967; Benson 1978). However, there are many examples where closely related herbivores overlap in their use of host plants. Herbivores that share hosts sometimes partition resources by using different parts of the same plant or leaves of different ages (Benson 1978; Bailey et al. 2009; Condon et al. 2014). For example, Benson (1978) confirmed niche partitioning among *Heliconiini* butterflies along three different niche axes (plant species, plant habitat, and plant part). However, in many instances, closely related herbivores co-occur on the

same host without any apparent means of resource partitioning (Strong et al. 1982), indicating that there are other factors besides resource partitioning that facilitate coexistence of species sharing the same host plant.

One mechanism that allows coexistence of species with similar resource use is phenological partitioning. For example, the geometrid winter moth *Inurois punctigera* has two allochronic races that coexist stably without partitioning resources; allochrony is even postulated as the direct cause of divergence in this case (Yamamoto & Sota 2009). Alternatively, species that share the same food resources can have different natural enemies and thereby occupy non-overlapping niches. Condon et al. (2014) demonstrated that species-specific parasitoids increase the niche diversity of *Blepharoneura* flies co-existing on the same-sex flowers of curcurbit host plants. Also, the more than 20 *Andricus* gall wasp species that coexist on shared oak hosts display remarkable diversity of gall forms; because gall morphology is a major determinant of parasitoid community structure, differences in natural enemies also provide a comprehensive explanation for the coexistence of multiple gall wasp species on oaks (Bailey et al. 2009). However, analysis of parasitoid communities among closely related herbivores is still limited, and our understanding of the role of natural enemies will increase with additional data.

In this study, I examined whether differences in phenology or natural enemies explain the coexistence of closely related herbivorous insects on shared host plants. I focused on interactions between a group of leaf cone moths (*Caloptilia*, Gracillariidae) and their maple hosts (*Acer*, Sapindaceae) because previous studies have identified multiple pairs of species that occur sympartrically with a great deal of overlap in host use (Kumata 1982; Nakadai & Murakami 2015). With 124 species, the genus *Acer* is one of the most species-rich groups of trees in the northern hemisphere, particularly in the temperate regions of East Asia, eastern North America, and Europe (van Gelderen et al. 1994). In temperate Japan, as many as 20 *Acer* species can occur in a single location (Nakadai et al. 2014), which may host to up to 10 sympatric *Caloptilia* species, as predicted from the geographic distribution of leaf cone moths (Nakadai & Kawakita 2016). Twenty-eight *Acer* species occur in Japan (Nakadai et al. 2014), and a previous study confirmed 14 *Acer*-feeding *Caloptilia* species; 13 of the 14 *Caloptilia* species formed a monophyletic group, together with a *Toxicodendron*-feeding *Caloptilia*, in the global *Caloptilia* phylogeny and thus are very closely related (Nakadai & Kawakita 2016). I investigated the phenology (i.e., temporal niche) and parasitoid community (i.e., enemy niche) of locally co-occurring, maple-feeding *Caloptilia* species by sampling *Acer* leaves containing *Caloptilia* larvae every 2–3 weeks for a total of 13 sampling events, yielding 274 moth larvae.

Species identification of moth larvae and detection of internal parasitoids were based on a simultaneous barcoding (metabarcoding) approach using high-throughput sequencing with the aid of *Caloptilia*-specific blocking primers that effectively reduced the number of redundant moth reads.

Materials and Methods

Study materials

The genus *Caloptilia* is globally distributed and includes nearly 300 described species, of which 27 feed on maples (De Prins & De Prins 2015; Kawahara et al. 2016). In Japan, there are 51 described *Caloptilia* species feeding on 21 host plant families (Kumata et al. 2013). Eleven of these species are known to use *Acer*, which is the most common host plant genus for Japanese *Caloptilia* (Fig. 1) (Kumata et al. 2013). Three additional *Caloptilia* species were newly found feeding on *Acer* in recent years. Most of the Japanese *Caloptilia* moths are multivoltine (Kumata et al. 2013). The feeding habits of the larvae change dramatically between the early and late developmental stages. Upon hatching, larvae mine the surface layer of the leaf, until the third instar. They then exit the mine and roll the edge of the leaf to form a cone, within which they feed externally until the final instar (Kumata et al. 2013). Some species are leaf-gallers or blotch-miners at the final instar and do not roll leaves (Nakadai & Kawakita 2016). Previous phylogenetic analysis of *Caloptilia* moths showed that the Japanese species of *Caloptilia* moths that feed on maples are closely related (Fig. S1) (Nakadai & Kawakita 2016).

Study sites

I conducted field surveys in a natural temperate forest at Ashiu Forest Research Station of Kyoto University (35°18' N, 135°43' E). The forest is dominated by *Fagus crenata* and *Quercus crispula* above 600 m elevation and *Q. serrata*, *Q. salicina*, and *Ilex pedunculosa* below 600 m (Ashiu Forest Research Station 2015). The average annual temperature for 1981–2010 was 12.1°C, and the average annual rainfall for 1981–2010 was 2,257 mm (Ashiu Forest Research Station 2015).

Acer species

Fourteen *Acer* species have been confirmed in the Ashiu forest (Yasuda & Nagamasu 1995), but because four of these species are rare (*A. cissifolium*, *A. diabolicum*, *A. palmatum*, and *A. tenuifolium)*, I targeted the following ten species: *Acer amoenum*, *A. carpinifolium*, *A.*

crataegifolium, *A. japonicum*, *A. maximowiczianum*, *A. micranthum*, *A. nipponicum* subsp. *nipponicum*, *A. pictum*, *A. rufinerve*, and *A. sieboldianum*.

Sampling and species identification of leaf cone moths and the search for internal parasitoid wasps

Caloptilia moths feeding on *Acer* trees were sampled every 2–3 weeks by searching for active larvae in leaf rolls (i.e., fourth or fifth instar) on the foliage of 10 *Acer* species from mid-May to mid-November of 2015 (Fig. 3). I sampled only larvae in leaf rolls because some leaf-mining larvae die early due to inconsistency between maternal oviposition and larval performance, and host use cannot be assessed precisely in such cases. This also enabled us to avoid sampling artifacts caused by the difficulty of conducting an exhaustive search for leaf miners. To standardize sampling effort, I sampled *Caloptilia* moths from branches with a diameter of 2.1 ± 4 mm from five individuals of each tree species. After sampling, moths were preserved in 99.5% ethanol and stored at –20°C.

Delimitation of species was based on sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene for all samples. I extracted genomic DNA using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). To simultaneously perform species identification of larvae and an exhaustive search for internal parasitoid wasps by high-throughput sequencing, I amplified the mitochondrial cytochrome oxidase I gene using the primers mlCOIlintF (Leray et al. 2013), 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3', and HCO2198 (Folmer et al. 1994), 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', which produced fragments with a standard sequence length of 313 base pairs. The COI region has been adopted as the standard 'taxon barcode' for most animal groups (Hebert et al. 2003) and is by far the most represented in public reference libraries. This primer set has performed well in previous studies that exhaustively searched for animal phyla (Leray et al. 2013; Brandon-Mong et al. 2015). I employed a two-step tailed PCR approach to conduct massively parallel paired-end sequencing (2 × 250 bp) on the MiSeq platform (Illumina, San Diego, CA, USA) (FASMAC Co., Ltd., Kanagawa, Japan).

The first PCR was carried out in a total volume of 10 μl including 0.5 ng of DNA, 5 μl of Kapa HiFi Hotstart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), and 0.3 μM each of forward and reverse primers. I also added the blocking primer (Vestheim & Jarman 2008) for *Caloptilia* moths at eight times the concentration of versatile primers (see the next section for details about the blocking primer). The protocol for the first PCR was 2 min at 95°C, followed by 35 cycles of 20 s at 98°C, 15 s at 67°C, 30 s at 52°C, and 30 s at 72°C, with a final extension

at 72°C for 1 min. Purification of the first PCR products was done with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). The second PCR was then carried out in a total volume of 10 μl including 1 μl of the template DNA amplified in the first PCR, 5 μl of Kapa HiFi Hotstart ReadyMix, and 0.3 μM each of forward and reverse primers for the second PCR. The protocol for the second PCR was 2 min at 95°C, followed by 12 cycles of 20 s at 98°C, 30 s at 60°C, and 30 s at 72°C, with a final extension at 72°C for 1 min. Purification of products of the second PCR was also done with Agencourt AMPure XP. PCR products were normalized and pooled. I normalized PCR products after quantifying them with a Nano Drop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Design of Caloptilia moth-specific annealing blocking primer

The bodies of *Caloptilia* moths include both their own tissues and, occasionally, those of internal parasitoids; the ratio of parasitoid tissue to moth tissue is very small. Thus, the amplification of *Caloptilia* moth COI sequences must be suppressed to allow detection of the sequences of internal parasitoids. I used the blocking primer approach for this purpose (Vestheim & Jarman 2008). A blocking primer is a modified primer that overlaps with one of the binding sites of the versatile primer. Blocking primers are usually designed for only one species (Leray et al. 2015), so it is difficult to apply them to multiple closely related species, as in this study, where I was unable to identify the larvae morphologically. I designed the blocking primer 5'-CCCCCCHCTTTCATCWAAYATYGCHCATRGWGGWAGATC-3' to block sequences of *Caloptilia* moths feeding on *Acer* based on known information about the COI sequences of the moths and already-confirmed parasitoids (Table S4). The blocking primer overlaps six bases with mlCOIlintF. The blocking primer was modified at the 3'-end with a Spacer C3 CPG (three hydrocarbons) to prevent elongation without affecting its annealing properties (Vestheim & Jarman 2008). The performance of the blocking primer for *Caloptilia* moths feeding on *Acer* was also tested (Supplemental files 2).

Analysis of sequencing data

I extracted the reads, which fully contain both primer sequences, from the output FastaQ files of Miseq using FastX Toolkit (ver. 0.0.13.2; Gordon & Hannon 2010). The remaining adapter, primer region, and the last nucleotide were trimmed from the reads using FastX Toolkit. Additionally, the reads were quality filtered using Sickle (ver. 1.33; Joshi & Fass 2011) with a minimum Sanger quality of 20 and a minimum length of 100. Paired reads were assembled using FLASH (ver. 1.2.10; Magoč & Salzberg 2011) with a minimum overlap of 20, and then

transformed into Fasta format using FastX Toolkit. A de novo chimera removal was performed using the UCHIME algorithm (Edgar et al. 2011) of USEARCH (ver. 8.0.1623_i86linux64; Edgar 2010). Duplicate and singleton reads were removed using USEARCH. I used the UPARSE-OTU algorithm (Edgar 2013) of USEARCH for clustering OTUs with an identity threshold of 97%. Thereafter, taxonomic assignment of individual OTUs was performed by BLAST+ (ver. 2.2.29; Camacho et al. 2009).

Subsequently, non-target OTUs (organisms other than *Caloptilia* moths and Hymenoptera) were removed, and OTUs were filtered with a sequence length of 313 ± 15 base pairs. I found some artifacts in the region overlapping with the blocking primer, so after excluding that region, I clustered OTUs manually with an identity threshold of 97% using MEGA (ver. 6.06; Tamura et al. 2013) once again. Additionally, rare OTUs, whose total read number in the whole sample was under 100, were also removed. The most abundant OTU of *Caloptilia* moth in each sample was used for the identification of *Caloptilia* moths, and the OTUs of internal parasitoid wasp over 10 reads in each sample were defined as states of presence.

Statistical analysis

To assess niche use trends (i.e., niche partitioning or overlap), I calculated the degree of niche overlap using the Pianka (Pianka 1973) and Czekanowski (Feinsinger et al. 1981) indices, which are common measures of niche overlap, and compared the observed values with the expectations of null models. Null model-based analyses are one of the most general approaches for assessing niche use trends (Gotelli 2001; Albrecht & Gotelli 2001). As a well-known example, Lawlor (1980) tested the patterns of niche use among 10 North American lizards using four types of null model (the algorithms RA1–4) and confirmed significantly low overlap in resource use, suggesting that interspecific competition plays an important role in constructing community structure. I used the R package EcoSimR (Gotelli et al. 2013) for the enemy niche and the program TimeOverlap, which is based on the algorithm ROSARIO (Castro-Arellano et al. 2010), for the temporal niche. Because of the sequential and continuous nature of time, a different kind of randomization model is required for the temporal niche (Castro-Arellano et al. 2010). Samples that did not host parasitoid wasps were excluded from the analysis of enemy niche. In all tests, the two-tailed probability of the observed value was calculated based on 10,000 randomizations. I employed Lawlor's (1980) algorithm RA3 for constructing null models. Additionally, I calculated the standardized effect size (SES) as the observed test statistic minus the mean of the null distribution, divided by the standard deviation of the null distribution (Nakadai & Kawakita 2016). This null model approach is commonly used for expressing

biological differences regardless of the units of the indices (McCabe et al. 2012). Moreover, to reveal the relationships among the niches of *Caloptilia* moths, I also tested the correlations between overlaps of three niches (resource, temporal, and enemy) and phylogenetic distances between *Caloptilia* moths using Mantel tests. Phylogenetic distances among *Caloptilia* moths were calculated from the phylogeny of Nakadai and Kawakita (2016).

Results

A total of 274 *Caloptilia* larvae were sampled from nine *Acer* species (all target species except *A. carpinifolium*) in 13 seasonal sampling events. Through high-throughput sequencing, I obtained 5,423,301 reads and 152 OTUs after bioinformatics preprocessing, and 10 OTUs of *Caloptilia* moths and 13 OTUs of internal parasitoid wasps after manual filtering (Table S1). The OTUs include 10 *Caloptilia* species (*C. acericola*, *C. aceris*, *C. gloriosa*, *C. heringi*, *C. hidakensis*, *C. monticola*, *C. semifasciella*, *C.* sp. 1, and *C.* sp. 3) and 13 internal parasitoid wasps (Braconidae, Eulophidae, Icheumonidae, and Trichogrammatidae) (Figs. 3, 4, and Table S1). The names of *Caloptilia* moths were matched with those found by Nakadai and Kawakita (2016). Each *Caloptilia* species uses 1–3 *Acer* species, with an average of 1.7 ± 0.9; I visually confirmed three sets of *Calptilia* moth species with largely overlapping host use (Fig. 2). The average parasitism rate throughout the year was 46.4%. The parasitism rate for each species is described in Table S3. Six of 13 parasitoid wasps were previously confirmed to have emerged from *Caloptilia* larvae; they provided the reference sequences that were used for constructing *Caloptilia*-blocking primers.

The results of the null model analysis indicated that both temporal and enemy niches showed significantly more overlap among species than the expected random distribution given by both indices (temporal, Pianka $SES = 4.29$, $P = 0.003$, Czechanowski $SES = 4.77$, $P = 0.001$; enemy, Pianka $SES = 4.77$, $P = 0.001$, Czechanowski $SES = 5.73$, $P = 0.000$; Table 1). T This indicates that phenology is significantly overlapping among *Caloptilia* species, and parasitoid wasps are widely shared among *Caloptilia* species. In Mantel tests, only the relationship between temporal and enemy niches, as assessed by the Pianka index, showed a significant correlation ($r = 0.41$, $P = 0.046$; Fig. 5, Table 2), and the Czechanowski index indicated a similar, but not significant, trend ($r = 0.35$, $P = 0.063$; Table 2). Mantel tests showed no significant correlations between other factors (Table 2). This indicates that species with overlapping phenology tend to share common parasitoid wasps.

Discussion

Role of phenology and natural enemies in facilitating species coexistence

The present study found three sets of *Caloptilia* moth species, each consisting of species with largely overlapping host ranges. Although the species that share hosts are not monophyletic, they are very closely related in the *Caloptilia* phylogeny (except for *C. gloriosa*, which belongs to a different clade than the rest of the maple-feeding *Caloptilia*) and have almost identical larval feeding modes (Fig. S1: Nakadai & Kawakita 2016). Additionally, I found large overlaps in both phenology and parasitoid community among species sharing the same host and among the community of maple-feeding *Caloptilia* as a whole (Table 1). These findings suggest that niche partitioning might not be necessary for closely related herbivores to coexist on shared hosts.

An obvious shortcoming of the above conclusion is that factors not accounted for in my analysis may be critical for niche partitioning among *Caloptilia* species. For example, although there is no apparent difference in the age of leaves used by the larvae or larval feeding mode among the species studied (Fig. 1), there may be a fine-scale difference that I did not detect. Also, because I used larvae at the leaf-rolling stage for my analysis of internal parasitoids, the role of parasitoids at the egg or leaf-mining stage was left uninvestigated. Condon et al. (2014) showed that parasitoids often attack the larvae of unusual hosts but do not successfully emerge as adults in such occasions. Because I only searched for parasitoids using the larvae of prey herbivores, such lethal interactions may have been included in the data, obscuring differences in parasitoid communities. Examining every aspect of *Caloptilia* life history may thus reveal an unexpected mechanism that facilitates coexistence of species with overlapping host use.

Alternatively, niche partitioning may genuinely be absent, and species coexistence may be facilitated by other mechanisms. For example, shared natural enemies enhance species coexistence, either if random predation eases interspecific competition among herbivores (Strong et al. 1982) or if negative frequency-dependent predation decreases the population of the more abundant species (Ishii & Shimada 2012). Strong (1982) found resource partitioning to be virtually absent among hispine beetles (Chrysomelidae), which commonly coexist as adults in the rolled leaves of *Heliconia* plants, suggesting that pressure from predators and parasites has a stronger influence on community structure in this species than does interspecific competition. Additionally, Ishii and Shimada (2012) showed that frequency-dependent predation by the pteromalid wasp *Anisopteromalus calandrae* enhanced the coexistence of two bruchid beetles, *Callosobruchus chinensis* and *C. maculatus*. Exploring how parasitoids and other predators (e.g., birds, bats, spiders) control the dynamics of *Caloptilia* populations will be useful in determining whether closely related herbivores can coexist without niche partitioning.

Data on phenological partitioning among closely related herbivores is still sparse (e.g., Yamamoto & Sota 2009), so the generality of temporal niche overlap as observed among the *Caloptilia* species is still unknown. The seasonal dynamics of their food source (maple leaves) may be a straightforward explanation for the observed synchronization of *Caloptilia* phenology, although other abiotic factors, such as temperature or precipitation, may be responsible. In any case, my results strongly indicate that partitioning of phenology is unlikely to be important in facilitating the coexistence of closely related herbivores on shared host plants.

Assessment of the enemy niche using matabarcoding

Recently, metabarcoding has increasingly been used as a tool for discerning less-visible patterns in food webs (Pompanon et al. 2012; Andrew et al. 2013; Kartzinel et al. 2015; Leray et al. 2015). Most studies that employ metabarcoding investigate diet using stomach contents (Kartzinel et al. 2015; Leray et al. 2015), but such an approach has rarely been used to evaluate parasitoid–prey interactions. Internal parasitoids are usually searched by developing specific primers for each parasitoid taxon (Rougerie et al. 2011; Condon et al. 2014; Wirta et al. 2014). However, metabarcoding allows detection of parasitoid taxa not targeted by specific primers. This approach is particularly useful when the parasitoid community includes taxonomically diverse or unknown species, or when analyzing a large number of prey samples, as in the present study, which involved 274 *Caloptilia* larvae. I also pioneered the use of the blocking primer approach for multiple closely related herbivorous insects by constructing a blocking primer that targets the region in which the sequences are shared only within *Caloptilia*. This method allows metabarcoding to be employed in cases where it is difficult to distinguish between closely related species based on morphology alone. As discussed above, assessment of paraitoid communities solely based on barcoding of herbivore larvae potentially overestimates the breadth of the enemy niche because lethal parasitoid–prey interactions are not omitted from the results. Thus, a combined approach incorporating both barcoding and laboratory rearing will allow a more precise assessment of the enemy niche.

The link between local species coexistence and global species diversity

Recently, ecologists and evolutionary biologists have recognized that local species coexistence (e.g., current ecological processes) has a major effect on global species diversity (e.g., macroevolutionary outcome) (Rabosky 2009; Tobias et al. 2013; Storch et al. 2012; Germain et al. 2016; Prinzing et al. 2016). For example, Prinzing et al. (2016) found that angiosperm clades with a greater extent of local co-occurrence are more species rich. To clarify these findings, it is

necessary to document examples in other organisms, including herbivorous insects. The results of the present study show that the number of locally co-occurring *Acer*-feeding *Caloptilia* species is a function of both host plant diversity and abundance of species coexisting on the same hosts. Although coexistence of the 10 *Caloptilia* species found in this study is not yet fully explained, improved knowledge of the mechanisms that enable such coexistence is ultimately necessary to our understanding of the processes that generate diversity in herbivorous insects.

Table 1 The results of comparison with the null model based on 10,000 randomizations. These tests employed Lawlor's (1980) algorithm RA3, using the R package EcoSimR (Gotelli et al. 2013) for enemy niche and the program TimeOverlap, based on the algorithm ROSARIO (Castro-Arellano et al. 2010), for temporal niche.

	Pianka index				Czechanowski index			
	Observed	Trend	SES	*P*-values	Observed	Trend	SES	*P*-values
Temporal	0.36	overlap	4.29	**0.003**	0.79	overlap	4.77	**0.001**
Enemy	0.47	overlap	4.77	**0.001**	0.39	overlap	5.73	**0.000**

Bold letters indicate significant results in two-tailed randomization tests

Table 2 Results of Mantel tests of the correlations among resource niche overlap, temporal niche overlap, enemy niche overlap, and phylogenetic distance between *Caloptilia* moths.

	Pianka index		Czechanowski index	
	Mantel *r*	*P*-values	Mantel *r*	*P*-values
Resource-Temporal	0.00	0.973	-0.06	0.690
Resource-Enemy	-0.08	0.701	-0.07	0.736
Resource-Phylogeny	0.14	0.244	0.19	0.154
Temporal-Enemy	0.41	**0.046**	0.35	0.063
Temporal-Phylogeny	0.01	0.974	0.07	0.844
Enemy-Phylogeny	-0.19	0.288	-0.14	0.430

Bold letters indicate significant results in Mantel tests

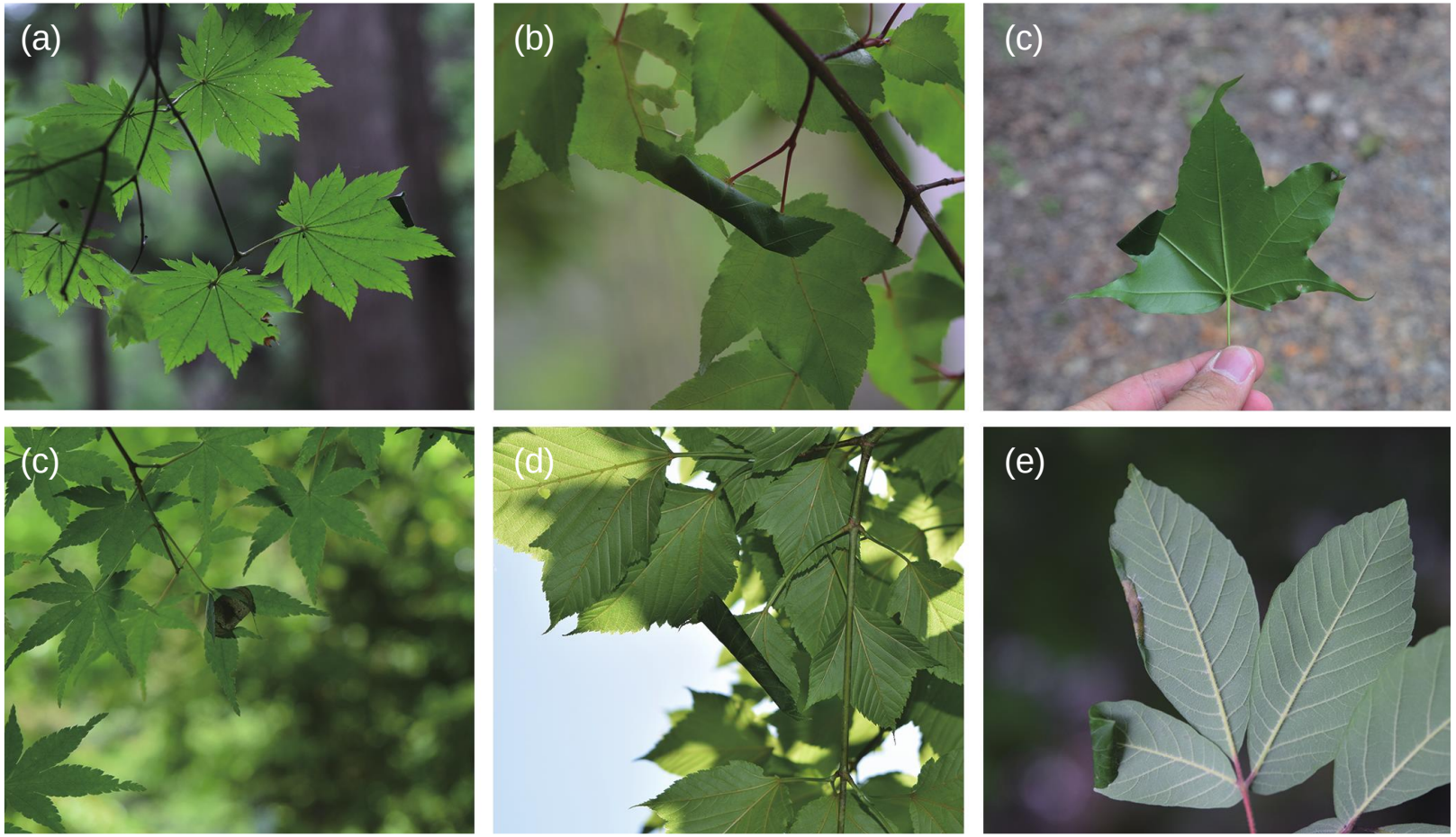

Figure 1 Leaves rolled by leaf cone moths on Japanese maples; moth species are very difficult to identify based solely on the morphology of rolled leaves and larvae. (a) *Acer japonicum*, (b) *A. crataegifolium*, (c) *A. pictum*, (d) *A. palmatum*, (e) *A. rufinerve*, (f) *A. maximowiczianum*.

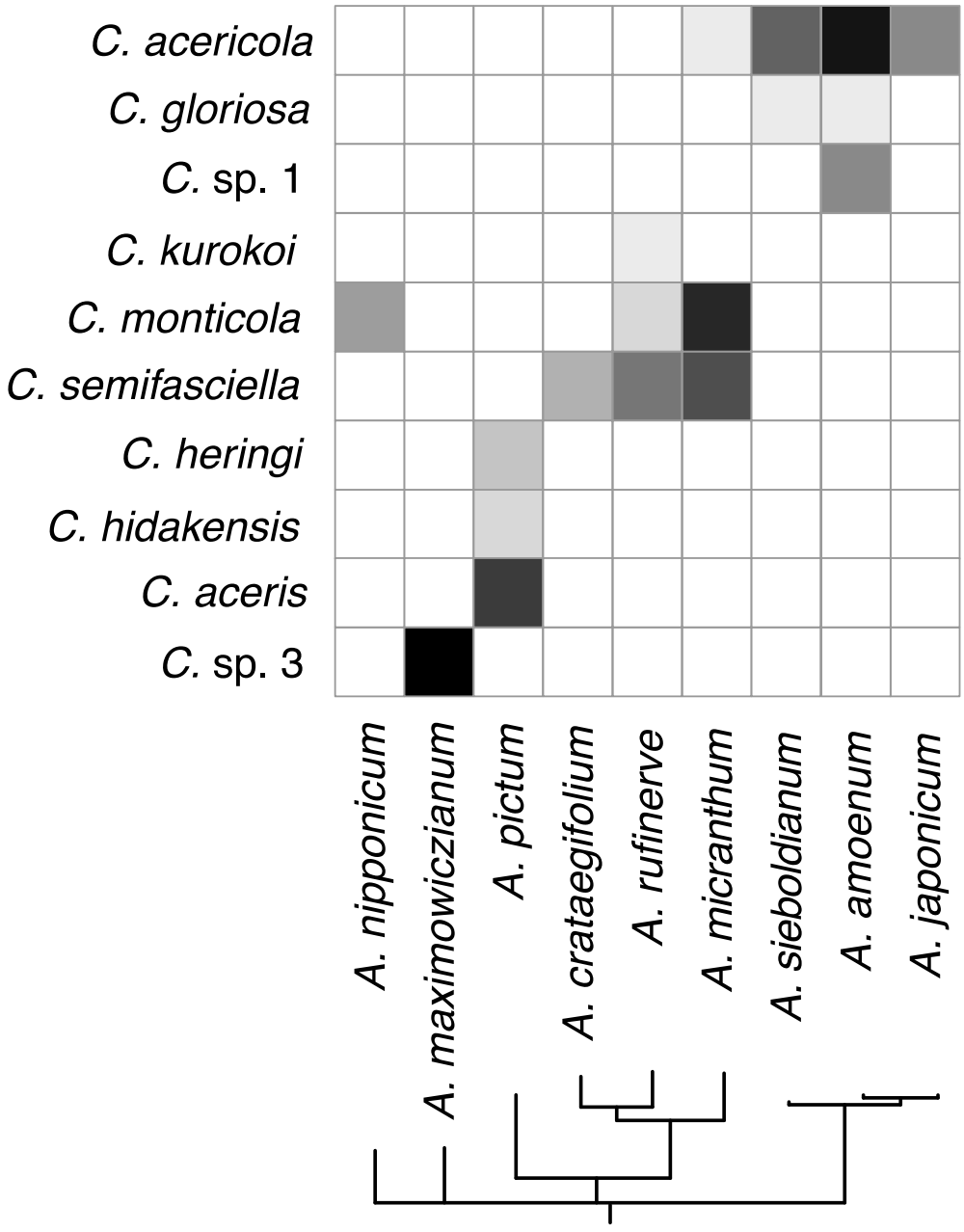

Figure 2 The results of *Acer–Caloptilia* interactions.

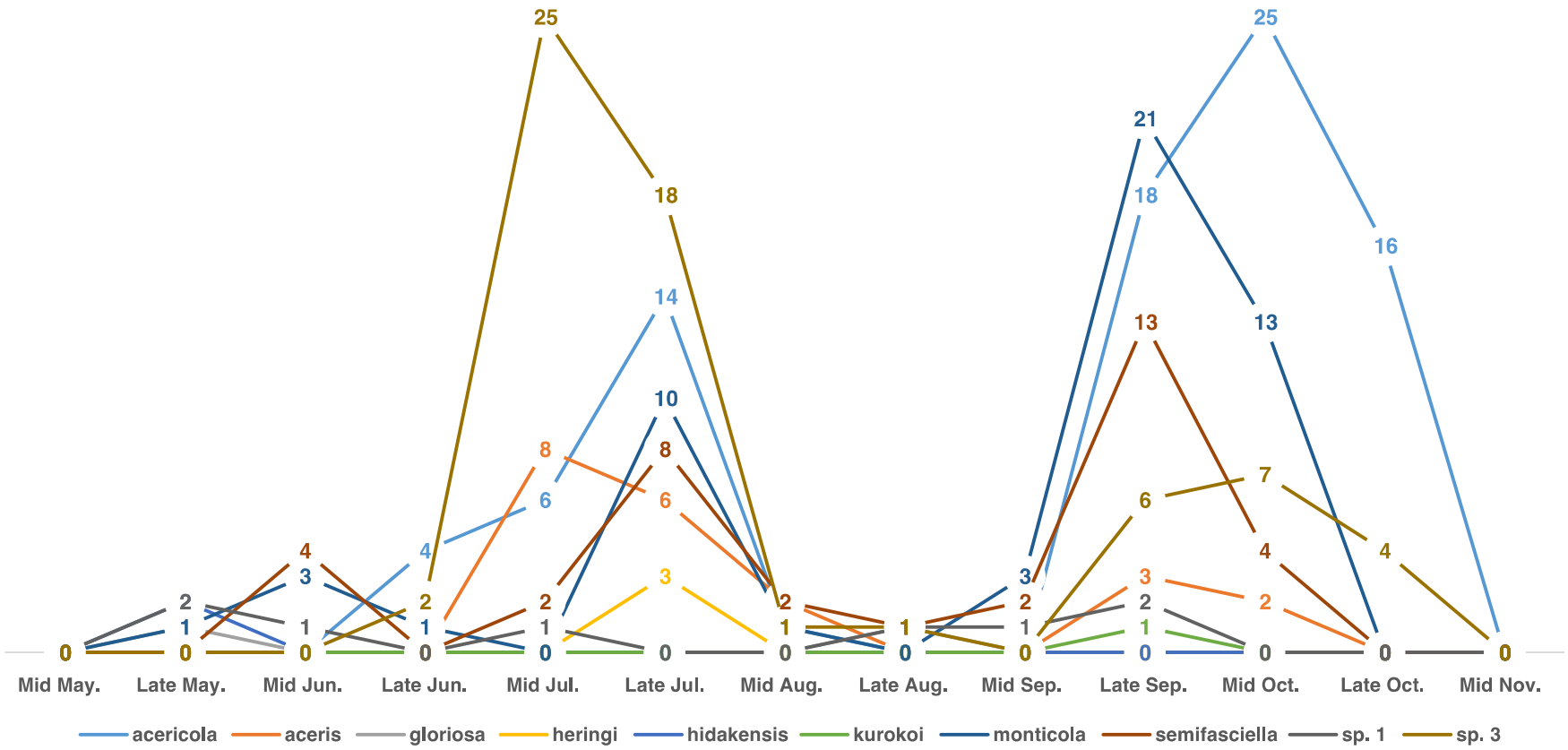

Figure 3 Phenology of 10 *Caloptilia* moths feeding on maples in this study area.

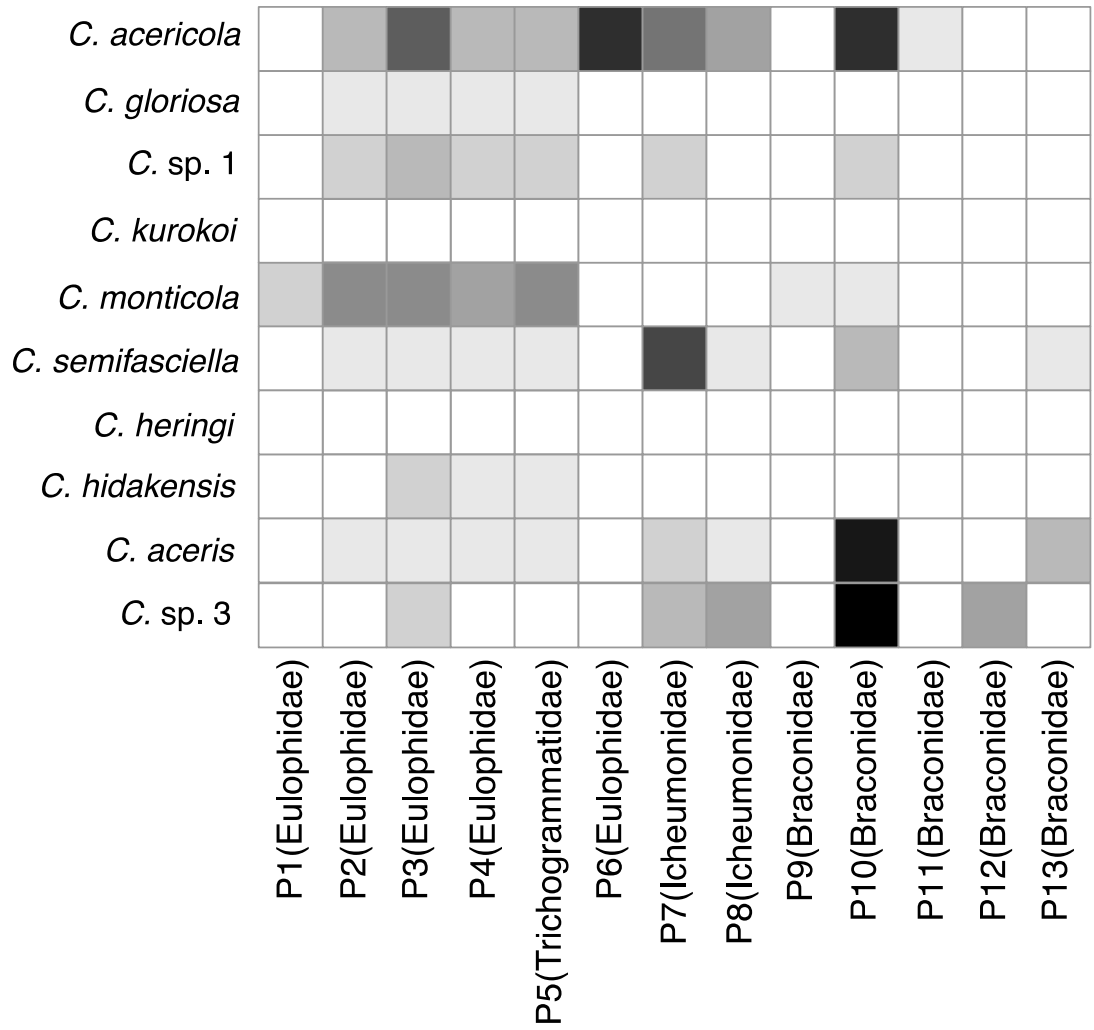

Figure 4 The results of parasitoid wasp–*Caloptilia* moth interactions.

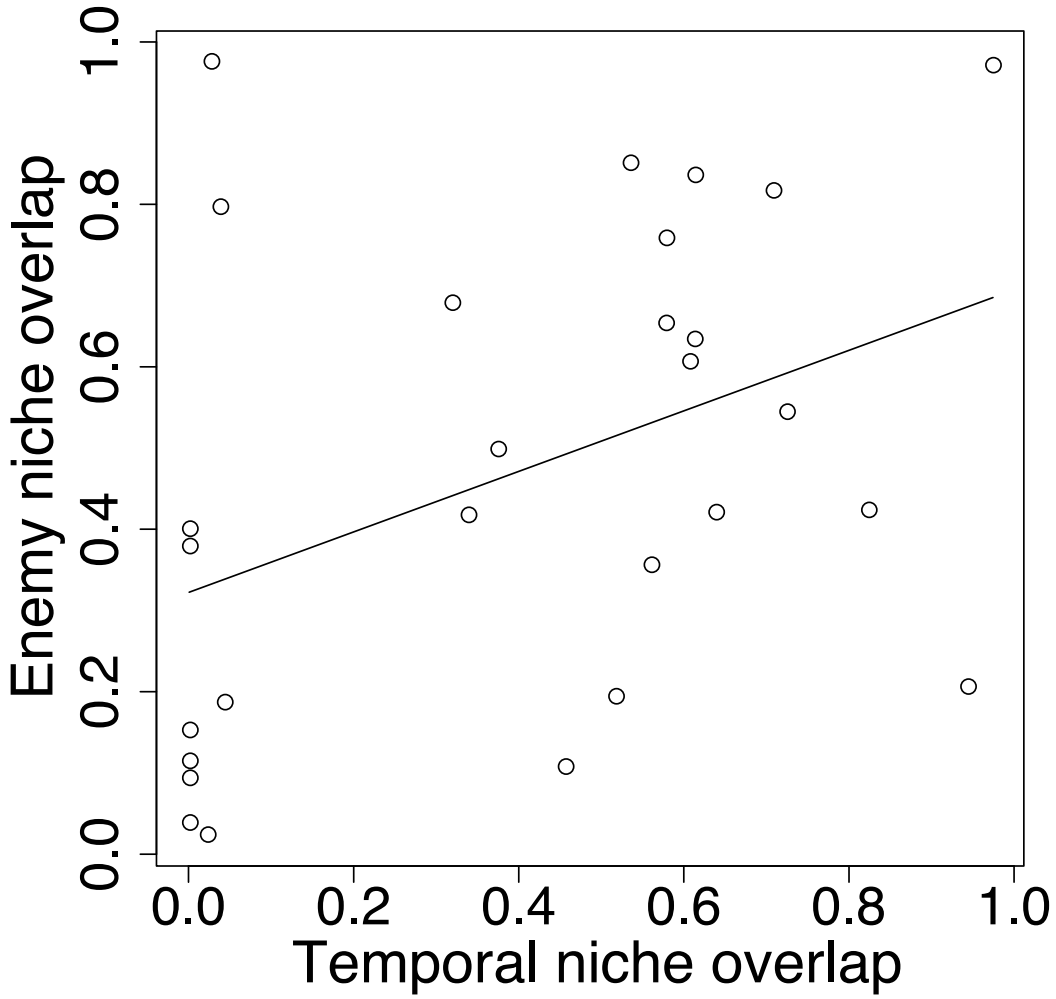

Figure 5 The relationship between the overlaps of temporal and enemy niches in the Pianka index (Mantel $r = 0.41$, $P = 0.046$).

Chapter 5
General discussion

In the present thesis, I investigated various aspects of leaf cone moths (*Caloptilia*) associated with maples (*Acer*) to determine the processes that generate and maintain species diversity. I found significant association between several leaf traits associated with defense and the phylogeny of maples in Chapter 2. In Chapter 3, I showed that major shifts in host use occurred primarily during the initial radiation of maple-feeding *Caloptilia*, and that the initial diversification occurred during Late Miocene–Early Pliocene, which coincided with the period of global cooling. In Chapter 4, I proposed an alternative view of host range of herbivorous insects, which assumes population size dependence, showed that species with overlapping host use coexist without clear difference in ecological characteristics. In this Chapter 5, I will review the results of the previous chapters, discussing the likely causality of species diversity of leaf cone moths associated with maples and highlighting future challenges in this study area.

Insights gained through this thesis about generation and maintenance of species diversity of herbivorous insects

As I mentioned in the general introduction (Chapter 1), the traditional idea in the study of herbivorous insects is that the cycle of specialization to specific host plants and shifts among hosts provides great opportunities for new species to arise, and this view of host-shift-driven diversification has dominated the literature of herbivorous insect diversification (e.g., Farrell et al. 1998). In contrast, this thesis provided three novel insights compared with the traditional views. Firstly, I showed that host use changes occurred more frequently in the period of early species diversification among closely related herbivorous insects as a general trend (Section 3-1), and that species diversification were concentrated at the period corresponding to global cooling (Section 3-2). If speciation is solely based on host shifts, the frequency of both host shifts and speciation events should be constant through time, but, in contrast to the traditional view (Chapter 3), this study shows that there is large variation in the rate at which host shifts or speciation events occur. Second, I showed that host breadths of herbivorous insects are not fixed variables but can change according to population size (Section 4-1) and that host use is constrained by plant phylogeny due to phylogenetic constraint of plant traits (Chapter 2). Third, closely related herbivores can overlap in host use without clear partitioning in other niches (Chapter 4).

In the traditional view, host switches are necessary for new species to arise, and resource partitioning as the result of host shift is necessary for closely related herbivores to coexist. On the other hand, this thesis showed that speciation is not always accompanied by host switches, and the frequency at which speciation events and host switches occur is inconstant through time. Because

closely related herbivores easily share host plants, resource partitioning is not necessary for species to coexist. Herbivorous insect diversity is a function of host species diversity, but the ways in which host plants contribute to the overall herbivore diversity is largely different.

Generation and maintenance of species diversity in leaf cone moths associated with maples

Above all, leaf cone moths associated with maples are exceptional in the light of high species diversity at both local and global scales (Fig. 1, Chapter 1), especially in East Asia (Nakadai & Murakami 2015, Section 4-2, De Prins & De Prins 2015, Section 3-2). Why are the leaf cone moths associated with maples more species rich than leaf cone moths associated with other plants? In this section, I address this question by focusing on the two processes that affect diversity: generation and maintenance of species diversity.

In Chapter 4, I suggested that the dramatic range expansion of maples acted as a driver of species diversification of leaf cone moths through increase in their abundance as the result of increased resource availability and expanded range size. However, this geographical factor is not unique to maples and thus does not explain why maple-feeding leaf cone moths are more species rich than others. One possibility is that the genus *Acer* is more species rich than other tree genera in the Northern Hemisphere, allowing more leaf cone moth lineages to arise as the result of host shifts during the initial stage of diversification. However, *Acer* (124 spp.) is not especially diverse as compared other tree genera, such as *Quercus* (ca. 600 spp.), *Salix* (400 spp.), and *Rhododendron* (>1,000 spp.), which are also major host plants of leaf cone moths. Thus, maples are unlikely to facilitate the speciation process itself of leaf cone moths than do other plant groups.

I suggest that the effect of local species diversity of maples is the most powerful factor determining the current species diversity of associate leaf cone moths, especially in East Asia. Maples are one of the representative tree species of deciduous broad-leaved forests in Japan, and various kinds of species can be seen along altitudinal gradient in a single area. Interestingly, local species richness of maples is much higher than that of other woody plant genera occurring in Japan (e.g., *Rhododendron*, *Rubus*) (Table 1, 2), which is in major contrast with global diversity as mentioned above. For example, there are 20 maple species in the University of Tokyo Chichibu Forest (Table 1) and 14 maple species in the Ashiu Forest Research Station of Kyoto University (Table 2). In both forests, *Acer* is the most species rich genus. Also, the locally co-occurring maple species include high phylogenetic diversity (Fig. 2). Both species richness and phylogenetic diversity of host plants well explain species richness and composition of herbivorous insects (Whitfeld et al. 2012, Pellissier et al. 2013). Thus, I suggest that local coexistence of a large number of maple species allows more species of leaf cone moths to be maintained, leading to an overall higher species richness. Surely, local species richness of maples is not uniform across the Northern Hemisphere, so in regions where maple species richness has declined historically (e.g., Western USA, which has only 3 maple species), the

diversity of maple-feeding *Caloptilia* is also very low (there is only one maple-feeding *Caloptilia* in Western USA).

If local species richness of maples is in fact an important determinant of *Caloptilia* species richness, all other herbivorous insects associated with maples should also be species diverse. Although information is still lacking, it is necessary to determine whether leaf cone moths are special as compared to other herbivorous insects feeding on maples to gain a better understanding of the determinants of species diversity of herbivorous insects.

Conclusion

In the present thesis, I studied species diversity of leaf cone moths (*Caloptilia*) associated with maples focusing on the processes of both generation and maintenance. The results demonstrated that the patterns observed in maple-feeding leaf cone moths do not necessarily match those expected from classical views. Therefore, to fully understand species diversity of herbivorous insects, it is important to correctly access how both processes of species generation and maintenance contribute to overall species richness. By doing so, we will finally achieve our goal, which is to answer the classical question, “Why are there so many herbivorous insects on the earth?”

Table 1 The number of species coexisting in the University of Tokyo Chichibu Forest for each plant genus. The data is based on Igarashi & Yoshida (2013). The genera with more than four species are indicated.

Genus	Species richeness
Acer	20
Rhododendron	11
Rubus	10
Betula	8
Enonymus	8
Cerasus	7
Viburnum	7
Hydrangea	6
Fraxinus	5
Lindera	5

Table 2 The number of species coexisting in the Ashiu Forest Research Station of Kyoto University for each plant genus. The data ise based on Yasuda & Nagamasu (1995). The genera with more than three species are indicated.

Genus	Species richeness
Acer	14
Rubus	10
Rhododendron	7
Viburnum	7
Enonymus	6
Ilex	6
Lindera	6
Salix	5
Carpinus	4
Hydrangea	4
Prunus	4
Quercus	4
Sorbus	4
Vaccinium	4

Legends

Figure 1 The adult leaf cone moths associated with maples. (a) *C. gloriosa*, (b) *C. semifasciella*, (c) *C. kurokoi*, (d) *C. kisoensis*, (e) *C. aceris*, (f) *C. monticola*, (g) *C.* sp. 3
(h) *C. rufipennella*, (i) *C. negundella*.

Figure 2 The phylogeny of Japanese maples. Blue asterisks indicate the species confirmed in the University of Tokyo Chichibu Forest, and green asterisks indicate the species confirmed in the Ashiu Forest Research Station of Kyoto University.

Figure 1

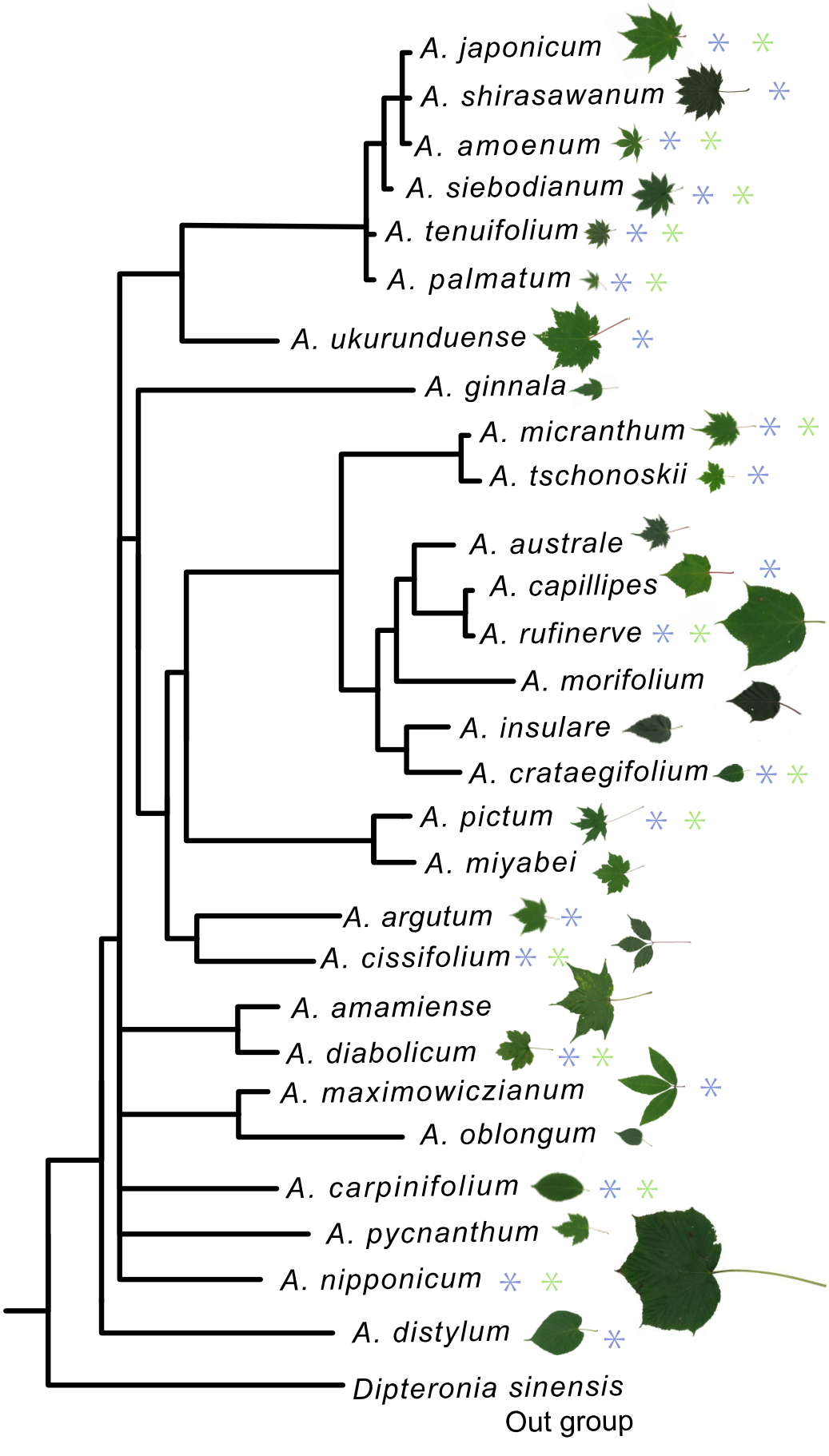
A. japonicum
A. shirasawanum
A. amoenum
A. siebodianum
A. tenuifolium
A. palmatum
A. ukurunduense
A. ginnala
A. micranthum
A. tschonoskii
A. australe
A. capillipes
A. rufinerve
A. morifolium
A. insulare
A. crataegifolium
A. pictum
A. miyabei
A. argutum
A. cissifolium
A. amamiense
A. diabolicum
A. maximowiczianum
A. oblongum
A. carpinifolium
A. pycnanthum
A. nipponicum
A. distylum
Dipteronia sinensis
Out group

Figure 2

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Supplementary information

Chapter 2

Table S1 Species with chloroplast regions sequenced and GenBank accession numbers

	matK gene	rbcL gene	rpl16 intron	trnL intron	trnL-F spacer
Acer amamiense	AB872503	AB872536	AB872569	AB872602	AB872635
A. amoenum	AB872504	AB872537	AB872570	AB872603	AB872636
A. argutum	AB872505	AB872538	AB872571	AB872604	AB872637
A. australe	AB872506	AB872539	AB872572	AB872605	AB872638
A. capillipes	AB872507	AB872540	AB872573	AB872606	AB872639
A. carpinifolium	AB872508	AB872541	AB872574	AB872607	AB872640
A. cissifolium	AB872509	AB872542	AB872575	AB872608	AB872641
A. crataegifolium	AB872510	AB872543	AB872576	AB872609	AB872642
A. diabolicum	AB872511	AB872544	AB872577	AB872610	AB872643
A. distylum	AB872512	AB872545	AB872578	AB872611	AB872644
A. ginnala	AB872513	AB872546	AB872579	AB872612	AB872645
A. insulare	AB872514	AB872547	AB872580	AB872613	AB872646
A. japonicum	AB872515	AB872548	AB872581	AB872614	AB872647
A. maximowiczianum	AB872516	AB872549	AB872582	AB872615	AB872648
A. micranthum	AB872517	AB872550	AB872583	AB872616	AB872649
A. miyabei	AB872518	AB872551	AB872584	AB872617	AB872650
A. morifolium	AB872519	AB872552	AB872585	AB872618	AB872651
A. nipponicum subsp. *nipponicum*	AB872520	AB872553	AB872586	AB872619	AB872652
A. oblongum subsp. *itoanum*	AB872521	AB872554	AB872587	AB872620	AB872653
A. palmatum	AB872522	AB872555	AB872588	AB872621	AB872654

	matK gene	rbcL gene	rpl16 intron	trnL intron	trnL-F spacer
A. pictum subsp. *dissectum*	AB872523	AB872556	AB872589	AB872622	AB872655
A. pictum subsp. *pictum*	AB872524	AB872557	AB872590	AB872623	AB872656
A. pictum subsp. *savatieri*	AB872525	AB872558	AB872591	AB872624	AB872657
A. pycnanthum	AB872526	AB872559	AB872592	AB872625	AB872658
A. rufinerve	AB872527	AB872560	AB872593	AB872626	AB872659
A. shirasawanum	AB872528	AB872561	AB872594	AB872627	AB872660
A. sieboldianum	AB872529	AB872562	AB872595	AB872628	AB872661
A. tenuifolium	AB872530	AB872563	AB872596	AB872629	AB872662
A. tschonoskii	AB872531	AB872564	AB872597	AB872630	AB872663
A. ukurunduense	AB872532	AB872565	AB872598	AB872631	AB872664
Dipteronia sinensis	AB872533	AB872566	AB872599	AB872632	AB872665
Aesculus hippocastanum	AB872534	AB872567	AB872600	AB872633	AB872666
Koelreuteria paniculata	AB872535	AB872568	AB872601	AB872634	AB872667

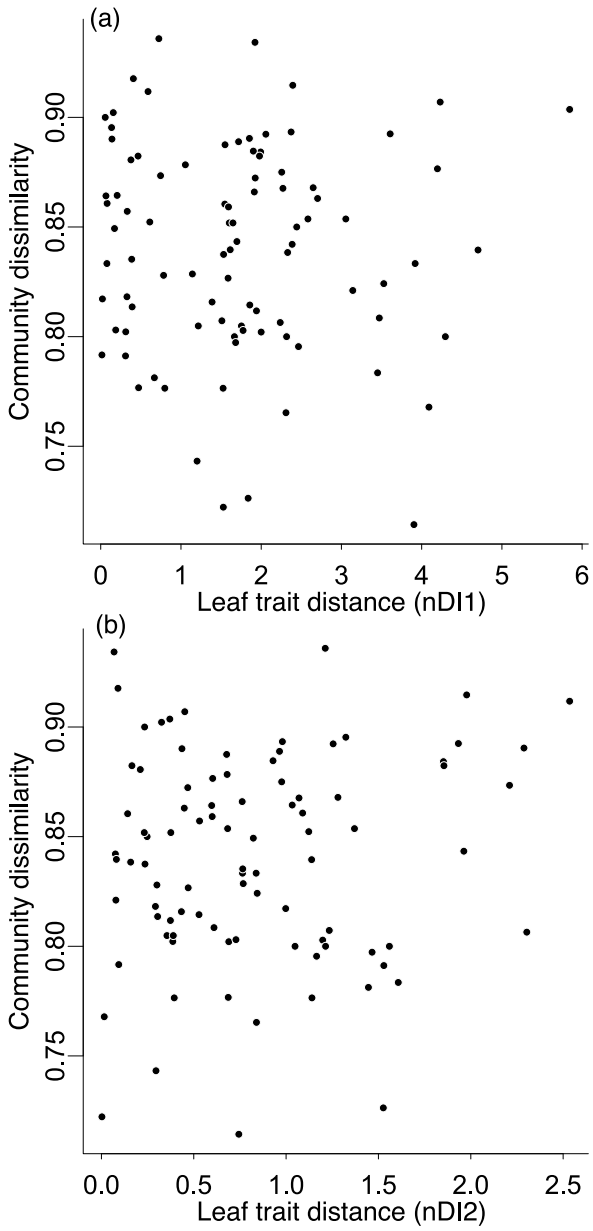


Figure S1 Relationships between the distance of the index of leaf defenses of nDI1 (a) and nDI2 (b) and community dissimilarity. Neither relationship was significant ((a): Pearson r = –0.052, P = 0.370), (b): Pearson r = 0.102, P = 0.235), respectively; Mantel test)

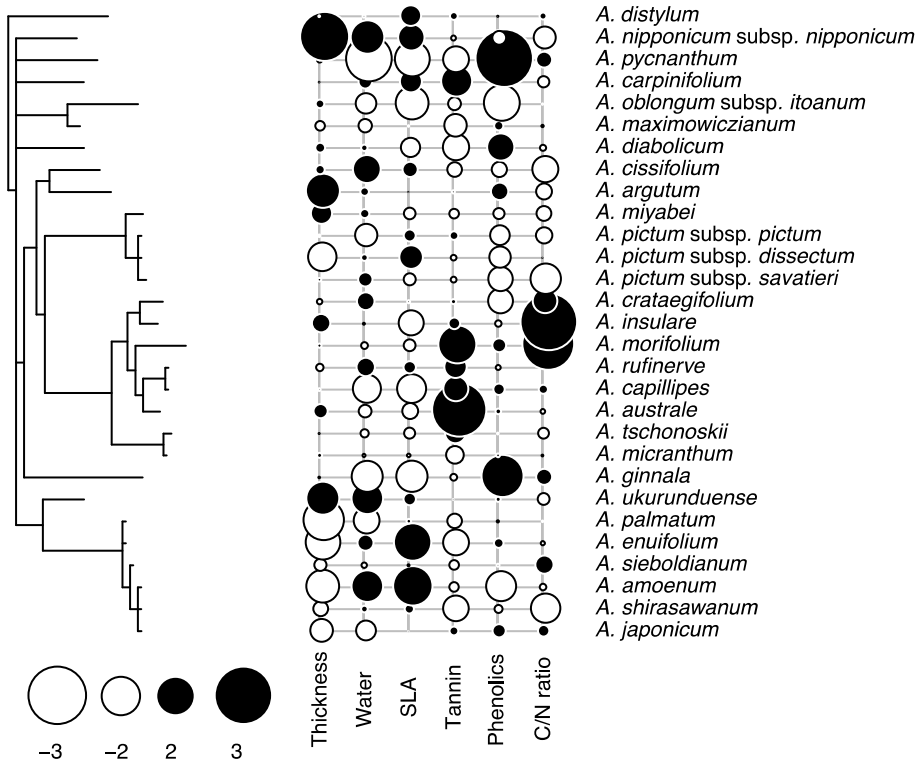


Figure S2-1 The distribution of leaf traits across the phylogeny

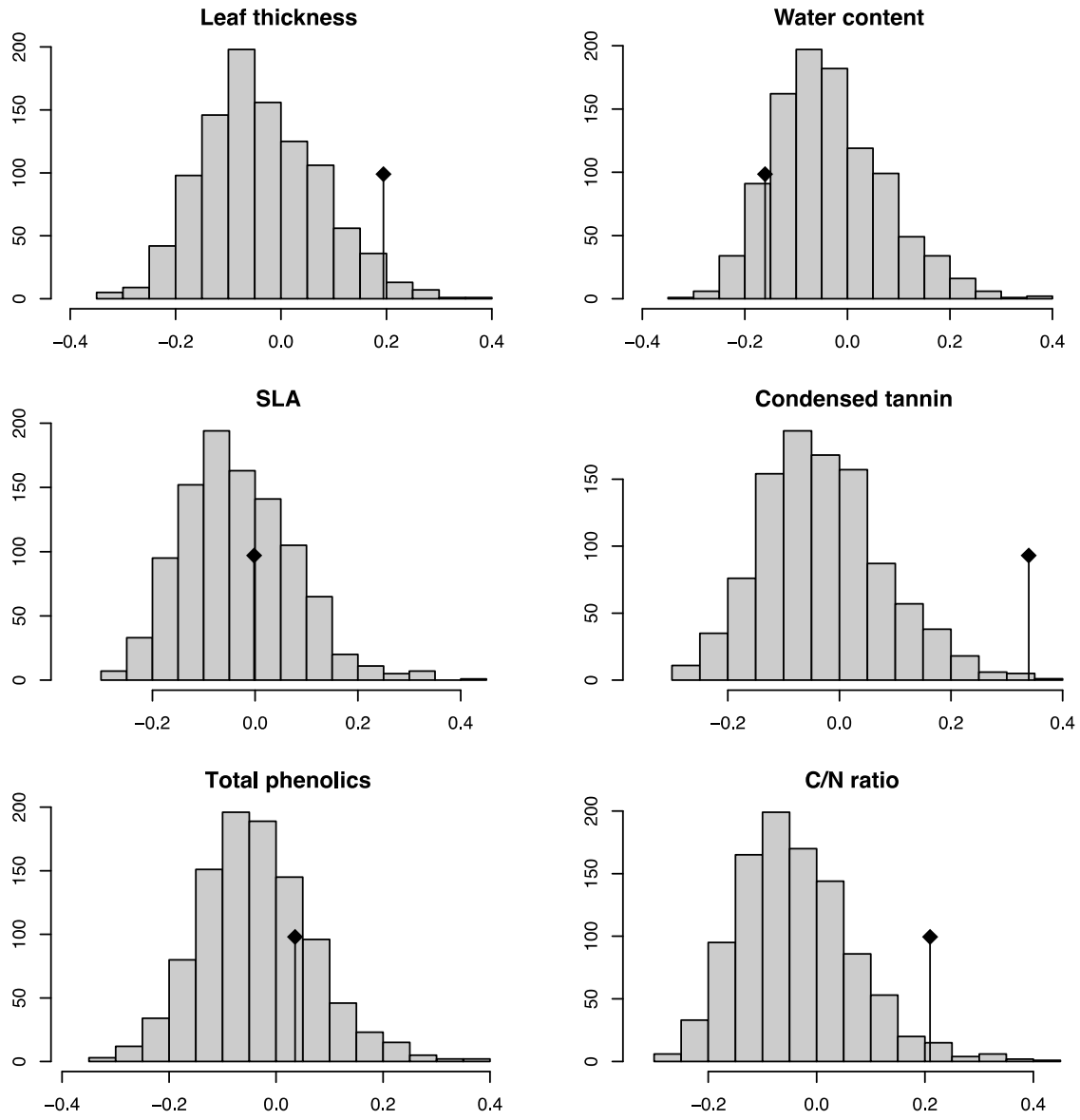


Figure S2-2 Moran's I test results for plant leaf traits

Chapter 3

Table S1 Specimen information.

Family	Genus	Species	Sampling country	Sampling site	Host plant order	Host plant family
Gracillariidae	*Caloptilia*	*acericola*	Japan	Hokkaido	Sapindales	Sapindaceae
	Caloptilia	*aceris*	Japan	Hokkaido	Sapindales	Sapindaceae
	Caloptilia	*alni*	Japan	Nara	Fagales	Betulaceae
	Caloptilia	*aurifasciata*	Japan	Wakayama	Sapindales	Anacardiaceae
	Caloptilia	*azaleella*	Japan	Shiga	Ericales	Ericaceae
	Caloptilia	*bipunctata*	Japan	Kyoto	Laurales	Lauraceae
	Caloptilia	*camphorae*	Japan	Wakayama	Laurales	Lauraceae
	Caloptilia	*cecidophora*	Japan	Wakayama	Malpighiales	Phyllanthaceae
	Caloptilia	*celtidis*	Japan	Wakayama	Rosales	Ulmaceae
	Caloptilia	*chrysolampra*	Japan	Miyagi	Malpighiales	Salicaceae
	Caloptilia	*crinotibialis*	Japan	Wakayama	Laurales	Lauraceae
	Caloptilia	sp. cf. *geminata*	Japan	Shiga	Ericales	Ericaceae
	Caloptilia	*gloriosa*	Japan	Fukui	Sapindales	Sapindaceae
	Caloptilia	sp. cf. *heringi*	Japan	Hokkaido	Sapindales	Sapindaceae
	Caloptilia	*hidakensis*	Japan	Akita	Sapindales	Sapindaceae
	Caloptilia	*illicii*	Japan	Wakayama	Austrobaileyales	Schisandraceae
	Caloptilia	*isochrysa*	Japan	Wakayama	Ericales	Pentaphylacaceae
	Caloptilia	*kadsurae*	Japan	Wakayama	Austrobaileyales	Schisandraceae
	Caloptilia	*kisoensis*	Japan	Nagano	Sapindales	Sapindaceae
	Caloptilia	*kurokoi*	Japan	Kyoto	Sapindales	Sapindaceae
	Caloptilia	*magnifica*	Japan	Fukui	Ranunculales	Berberidaceae

Family	Genus	Species	Sampling country	Sampling site	Host plant order	Host plant family
	Caloptilia	*matsumurai*	Japan	Shiga	Sapindales	Anacardiaceae
	Caloptilia	*monticola*	Japan	Nagano	Sapindales	Sapindaceae
	Caloptilia	*obliquatella*	From database	—	Fagales	Fagaceae
	Caloptilia	sp. cf. *protiella*	Japan	Amami-oshima Island	Sapindales	Anacardiaceae
	Caloptilia	*querci*	Japan	Shiga	Fagales	Fagaceae
	Caloptilia	*recitata*	Japan	Fukui	Sapindales	Anacardiaceae
	Caloptilia	*rhois*	Japan	Nagano	Sapindales	Anacardiaceae
	Caloptilia	*ryukyuensis*	Japan	Amami-oshima Island	Malpighiales	Phyllanthaceae
	Caloptilia	*sapiivora*	Japan	Kyoto	Malpighiales	Euphorbiaceae
	Caloptilia	*sapporella*	Japan	Shiga	Fagales	Fagaceae
	Caloptilia	*semifasciella*	Japan	Kyoto	Sapindales	Sapindaceae
	Caloptilia	*soyella*	Japan	Oita	Fabales	Fabaceae
	Caloptilia	*stigmatella*	Japan	Hokkaido	Malpighiales	Salicaceae
	Caloptilia	*syrphetias*	Japan	Mie	Laurales	Lauraceae
	Caloptilia	*theivora*	Cultivated	—	Theales	Theaceae
	Caloptilia	*wakayamensis*	Japan	Nara	Sapindales	Sapindaceae
	Caloptilia	sp. cf. *yasudai*	Japan	Hokkaido	Sapindales	Sapindaceae
	Caloptilia	sp. cf. *zachrysa*	Japan	Shiga	Rosales	Rosaceae
	Caloptilia	sp. 1	Japan	Shiga	Sapindales	Sapindaceae
	Caloptilia	sp. 2	Japan	Hokkaido	Sapindales	Sapindaceae
	Caloptilia	sp. 3	Japan	Kyoto	Sapindales	Sapindaceae
	Caloptilia	sp. 4	Japan	Ishikawa	Fagales	Fagaceae
	Caloptilia	sp. 5	Japan	Wakayama	Laurales	Lauraceae

Family	Genus	Species	Sampling country	Sampling site	Host plant order	Host plant family
	Caloptilia	sp. 6	Peru	Huancabamba	Fagales	Myricaceae
	Caloptilia	sp. 7	Taiwan	Taichung	Malpighiales	Phyllanthaceae
	Caloptilia	sp. 8	Australia	New South Wales	Malpighiales	Phyllanthaceae
	Caloptilia	sp. 9	Australia	Tasmania	—	—
	Caloptilia	sp. 10	Japan	Shiga	Fagales	Fagaceae
	Caloptilia	sp. 11	Australia	Queensland	Myrtales	Myrtaceae
	Caloptilia	sp. 12	Japan	Yonaguni-jima Island	Malpighiales	Euphorbiaceae
	Caloptilia	sp. 13	Japan	Okinawa Island	Lamiales	Lamiaceae
	Caloptilia	sp. 14	Laos	Phonsavan	Laurales	Lauraceae
	Caloptilia	sp. 15	Laos	Phonsavan	Lamiales	Lamiaceae
	Caloptilia	sp. 16	Laos	Sam Neua	Proteales	Proteaceae
	Caloptilia	sp. 17	Australia	New South Wales	Myrtales	Myrtaceae
	Caloptilia	sp. 18	China	Hainan Island	Malpighiales	Phyllanthaceae
	Caloptilia	sp. 19	Japan	Shiga	Fagales	Betulaceae
	Caloptilia	sp. 20	USA	New Hampshire	Malpighiales	Salicaceae
	Calybites	*phasianipennella*	Japan	Hokkaido	Caryophyllales	Polygonaceae
	Calybites	*trimaculata*	Japan	Amami-oshima Island	Caryophyllales	Polygonaceae
	Eucalybites	*aureola*	From database	—	Theales	Clusiaceae
	Gracillaria	sp. cf. *japonica*	Japan	Shiga	Lamiales	Oleaceae
	Gracillaria	*ussuriella*	Japan	Hokkaido	Lamiales	Oleaceae
	Gracillaria	sp. 1	Japan	Shiga	Lamiales	Oleaceae
	Gracillaria	sp. 2	Japan	Nagano	Lamiales	Oleaceae

Family	Genus	Species	Sampling country	Sampling site	Host plant order	Host plant family
	Callisto	*denticulella*	From database	—	Rosales	Rosaceae
	Marmara	*serotinella*	From database	—	Rosales	Rosaceae
	Acrocercops	*brongniardella*	From database	—	Fagales	Fagaceae
	Deoptilia	*heptadeta*	From database	—	Malpighiales	Euphorbiaceae
	Epicephala	*relictella*	From database	—	Malpighiales	Phyllanthaceae

Table S1 Continued

Sampled host species	Known host genera
Acer amoenum	*Acer*
Acer pictum	*Acer*
Alnus hirsuta var. *sibirica*	*Alnus*
Toxicodendron vernicifluum	*Rhus, Toxicodendron*
Rhododendron kaempferi	*Rhododendron*
Neolitsea sericea	*Neolitsea*
Cinnamomum camphora	*Cinnamomum, Lindera, Litsea*
Glochidion obovatum	*Glochidion*
Celtis sinensis	*Celtis*
Salix babylonica	*Populus, Salix*
Cinnamomum tenuifolium	*Persea*
Vaccinium oldhamii	*Vaccinium*
Acer pictum	*Acer*
Acer pictum	*Acer*
Acer pictum	*Acer*

Sampled host species	Known host genera
Illicium anisatum	*Illicium*
Eurya japonica	*Cleyera*
Kadsura japonica	*Kadsura*
Acer ginnala	*Acer*
Acer rufinerve	*Acer*
Epimedium grandiflorum var. *thunbergianum*	*Epimedium*
Rhus javanica	*Toxicodendron*
Acer rufinerve	*Acer*
—	*Quercus*
Toxicodendron succedaneum	*Anacardium, Toxicodendron, Protium*
Quercus glauca	*Castanea, Quercus*
Toxicodendron orientale	*Cotinus, Rhus, Toxicodendron*
Toxicodendron trichocarpum	*Rhus, Toxicodendron*
Glochidion zeylanicum	*Glochidion*
Neoshirakia japonica	*Neoshirakia*
Quercus serrata	*Castanea, Quercus*
Acer rufinerve	*Acer*
Rhynchosia volubilis	*Cajanus, Dolichos, Glycine, Lespedeza, Phaseolus, Pueraria, Soja, Vigna*
Salix sp. 1	*Chosenia, Myrica, Populus, Robinia, Salix*
Cinnamomum camphora	*Machilus*
—	*Camellia, Thea*
Acer palmatum	*Acer*
Acer miyabei	*Acer*

Sampled host species	Known host genera
Photinia glabra	*Malus, Photinia, Rubus*
Acer amoenum	—
Acer cissifolium	—
Acer maximowiczianum	—
Fagus crenata	—
Litsea acuminata	—
Myrica pubescens	—
Bridelia tomentosa	—
Glochidion ferdinaadii	—
Collected adult specimen	—
Fagus japonica	—
Eucalyptus sp. 1	—
Securinega suffruticosa var. *amamiensis*	—
Clerodendrum trichotomum	—
Lauraceae sp.	—
Callicarpa japonica	—
Helicia cochinchinensis	—
Eucalyptus sp. 2	—
Glochidion sp.	—
Alnus trabeculosa	—
Salix sp. 2	—
Persicaria filiformis	*Symphytum, Chenopodium, Hypericum, Lythrum, Calamagrostis, Oxyria, Persicaria, Rumex, Lysimachia*
Persicaria chinensis	*Persicaria*

Sampled host species	Known host genera
—	*Hypericum*
Ligustrum obtusifolium	*Ligustrum*
Fraxinus mandshurica	*Fraxinus*
Fraxinus sieboldiana	—
Fraxinus apertisquamifera	—
—	*Amelanchier, Malus, Pyrus*
—	*Prunus*
—	*Castanea, Quercus*
—	*Mallotus*
—	*Flueggea*

Table S1 Continued

Feeding habit
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Blotch-miner
Leaf-miner/Blotch-miner
Leaf-miner/Leaf-galler
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller

Feeding habit
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller on cut leaf margin
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller

Feeding habit
Leaf-miner/Leaf-roller on dropped leaf
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Feeding habit
Leaf-miner/Leaf-roller
Leaf-miner/Blotch-miner
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller

Feeding habit
Leaf-miner/Leaf-galler
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller on cut leaf margin
Leaf-miner/Leaf-roller on cut leaf margin
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Leaf-miner/Leaf-roller
Feeding habit
Leaf-miner/Leaf-roller
Branch-miner
Leaf-miner/Blotch-miner
Leaf-miner/Leaf-roller
Fruit-feeder

Table S2 DDBJ accession numbers.

Family	Genus	Species	COI	ArgK	CAD	EF-1α
Gracillariidae	*Caloptilia*	*acericola*	LC127790	LC127852	LC127904	LC127966
	Caloptilia	*aceris*	LC127805	—	LC127919	LC127981
	Caloptilia	*alni*	LC127783	LC127847	LC127897	LC127960
	Caloptilia	*aurifasciata*	LC127784	LC127848	LC127898	LC127961
	Caloptilia	*azaleella*	LC127813	LC127871	LC127927	LC127989
	Caloptilia	*bipunctata*	LC127833	LC127889	LC127947	LC128009
	Caloptilia	*camphorae*	LC127795	LC127857	LC127909	LC127971
	Caloptilia	*cecidophora*	LC127827	LC127884	LC127941	LC128003
	Caloptilia	*celtidis*	LC127779	LC127843	LC127893	LC127956
	Caloptilia	*chrysolampra*	LC127829	LC127886	LC127943	LC128005
	Caloptilia	*crinotibialis*	LC127811	LC127869	LC127925	LC127987
	Caloptilia	sp. cf. *geminata*	LC127785	LC127849	LC127899	LC127962
	Caloptilia	*gloriosa*	LC127789	—	LC127903	LC127965
	Caloptilia	sp. cf. *heringi*	LC127793	LC127855	LC127907	LC127969
	Caloptilia	*hidakensis*	LC127816	LC127874	LC127930	LC127992
	Caloptilia	*illicii*	LC127786	—	LC127900	—
	Caloptilia	*isochrysa*	LC127830	LC127887	LC127944	LC128006
	Caloptilia	*kadsurae*	LC127782	LC127846	LC127896	LC127959
	Caloptilia	*kisoensis*	LC127787	LC127850	LC127901	LC127963
	Caloptilia	*kurokoi*	LC127788	LC127851	LC127902	LC127964
	Caloptilia	*magnifica*	LC127834	LC127890	LC127948	LC128010
	Caloptilia	*matsumurai*	LC127814	LC127872	LC127928	LC127990

Family	Genus	Species	COI	ArgK	CAD	EF-1α
	Caloptilia	*monticola*	LC127791	LC127853	LC127905	LC127967
	Caloptilia	*obliquatella*	—	—	JN125084	—
	Caloptilia	sp. cf. *protiella*	LC127815	LC127873	LC127929	LC127991
	Caloptilia	*querci*	LC127799	LC127861	LC127913	LC127975
	Caloptilia	*recitata*	LC127797	LC127859	LC127911	LC127973
	Caloptilia	*rhois*	LC127781	LC127845	LC127895	LC127958
	Caloptilia	*ryukyuensis*	LC127812	LC127870	LC127926	LC127988
	Caloptilia	*sapiivora*	LC127780	LC127844	LC127894	LC127957
	Caloptilia	*sapporella*	LC127806	LC127867	LC127920	LC127982
	Caloptilia	*semifasciella*	LC127792	LC127854	LC127906	LC127968
	Caloptilia	*soyella*	LC127842	—	LC127955	—
	Caloptilia	*stigmatella*	LC127809	—	LC127923	LC127985
	Caloptilia	*syrphetias*	LC127800	LC127862	LC127914	LC127976
	Caloptilia	*theivora*	LC127837	—	LC127950	LC128013
	Caloptilia	*wakayamensis*	LC127802	LC127864	LC127916	LC127978
	Caloptilia	sp. cf. *yasudai*	LC127839	LC127892	LC127952	—
	Caloptilia	sp. cf. *zachrysa*	LC127807	LC127868	LC127921	LC127983
	Caloptilia	sp. 1	LC127794	LC127856	LC127908	LC127970
	Caloptilia	sp. 2	LC127838	—	LC127951	—
	Caloptilia	sp. 3	LC127808	—	LC127922	LC127984
	Caloptilia	sp. 4	LC127801	LC127863	LC127915	LC127977
	Caloptilia	sp. 5	LC127803	LC127865	LC127917	LC127979
	Caloptilia	sp. 6	LC127817	LC127875	LC127931	LC127993

Family	Genus	Species	COI	ArgK	CAD	EF-1α
	Caloptilia	sp. 7	LC127818	LC127876	LC127932	LC127994
	Caloptilia	sp. 8	LC127820	—	LC127934	LC127996
	Caloptilia	sp. 9	LC127821	LC127878	LC127935	LC127997
	Caloptilia	sp. 10	LC127823	LC127880	LC127937	LC127999
	Caloptilia	sp. 11	LC127824	LC127881	LC127938	LC128000
	Caloptilia	sp. 12	LC127825	LC127882	LC127939	LC128001
	Caloptilia	sp. 13	LC127826	LC127883	LC127940	LC128002
	Caloptilia	sp. 14	LC127828	LC127885	LC127942	LC128004
	Caloptilia	sp. 15	LC127831	LC127888	LC127945	LC128007
	Caloptilia	sp. 16	LC127832	—	LC127946	LC128008
	Caloptilia	sp. 17	LC127835	—	—	LC128011
	Caloptilia	sp. 18	LC127836	LC127891	LC127949	LC128012
	Caloptilia	sp. 19	LC127840	—	LC127953	—
	Caloptilia	sp. 20	LC127841	—	LC127954	—
	Calybites	*phasianipennella*	LC127804	LC127866	LC127918	LC127980
	Calybites	*trimaculata*	LC127819	LC127877	LC127933	LC127995
	Eucalybites	*aureola*	GU073228	—	JN125067	—
	Gracillaria	sp. cf. *japonica*	LC127810	—	LC127924	LC127986
	Gracillaria	ussuriella	LC127798	LC127860	LC127912	LC127974
	Gracillaria	sp. 1	LC127796	LC127858	LC127910	LC127972
	Gracillaria	sp. 2	LC127822	LC127879	LC127936	LC127998
	Callisto	*denticulella*	—	—	JN125056	JN125113
	Marmara	*serotinella*	KR446487	—	JN125075	—

Family	Genus	Species	COI	ArgK	CAD	EF-1α
	Acrocercops	*brongniardella*	—	—	JN125047	JN125106
	Deoptilia	*heptadeta*	GU816425	GU816518	JN125063	JN125122
	Epicephala	*relictella*	JF797232	—	JN125066	—

Table S3 Primers used in this study.

Locus	Primer name	Sequence	Reference
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al. 1994
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer et al. 1994
ArgK	F7	5'-AACTGGGGWGAYGTYGAGACYCTYGG-3'	this study
	F9	5'-ATGGARGGHTAYCCBTTCAACC-3'	this study
	R8	5'-GCACCTGCAGGTGGTACTTG-3'	this study
	R9	5'-CAGCTTVGGYAGCTTRATGTG-3'	this study
CAD	f222	5'-GGDTCWCAAGCWGTDAARGCDATGC-3'	this study
	r221	5'-GCHACNACDATYGATTCNCC-3'	this study
	r222	5'-CCDAGDGGRTCAACRTTYTCCAT-3'	this study
EF-1α	f2	5'-CCCATTTCKGGCTGGCAYGGAGA-3'	Kawakita et al. 2004
	r2	5'-GATTTACCRGWACGACGRTC-3'	Kawakita et al. 2004
	r3	5'-GACTTKCCRGTACGRCGGTC-3'	this study

Reference

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3:294–299.

Kawakita, A., A. Takimura, T. Terachi, T. Sota, and M. Kato. 2004. Cospeciation analysis of an obligate pollination mutualism: have *Glochidion* trees (Euphorbiaceae) and pollinating *Epicephala* moths (Gracillariidae) diversified in parallel? Evolution 58:2201–2214.

Table S4 The results of randomization tests including *C. aurifasciata* that feeds on *Toxicodendron*.

Significance level: n.s., $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$.

		Turnover + Nestedness			Turnover			Nestedness		
Dataset		Sign	SES		Sign	SES		Sign	SES	
All-genes dataset	Jaccard index	+	1.93	*	+	2.25	*	−	-1.54	n.s.
	Unifrac index	+	2.64	**	+	2.66	**	−	-1.19	n.s.
Nucear-only dataset	Jaccard index	+	2.11	*	+	2.17	*	−	-1.25	n.s.
	Unifrac index	+	3.09	**	+	2.74	**	−	-0.76	n.s.

Figure S1 Phylogeny of *Caloptilia* moths feeding on *Acer* based on mitochondrial COI with information on sampling site.

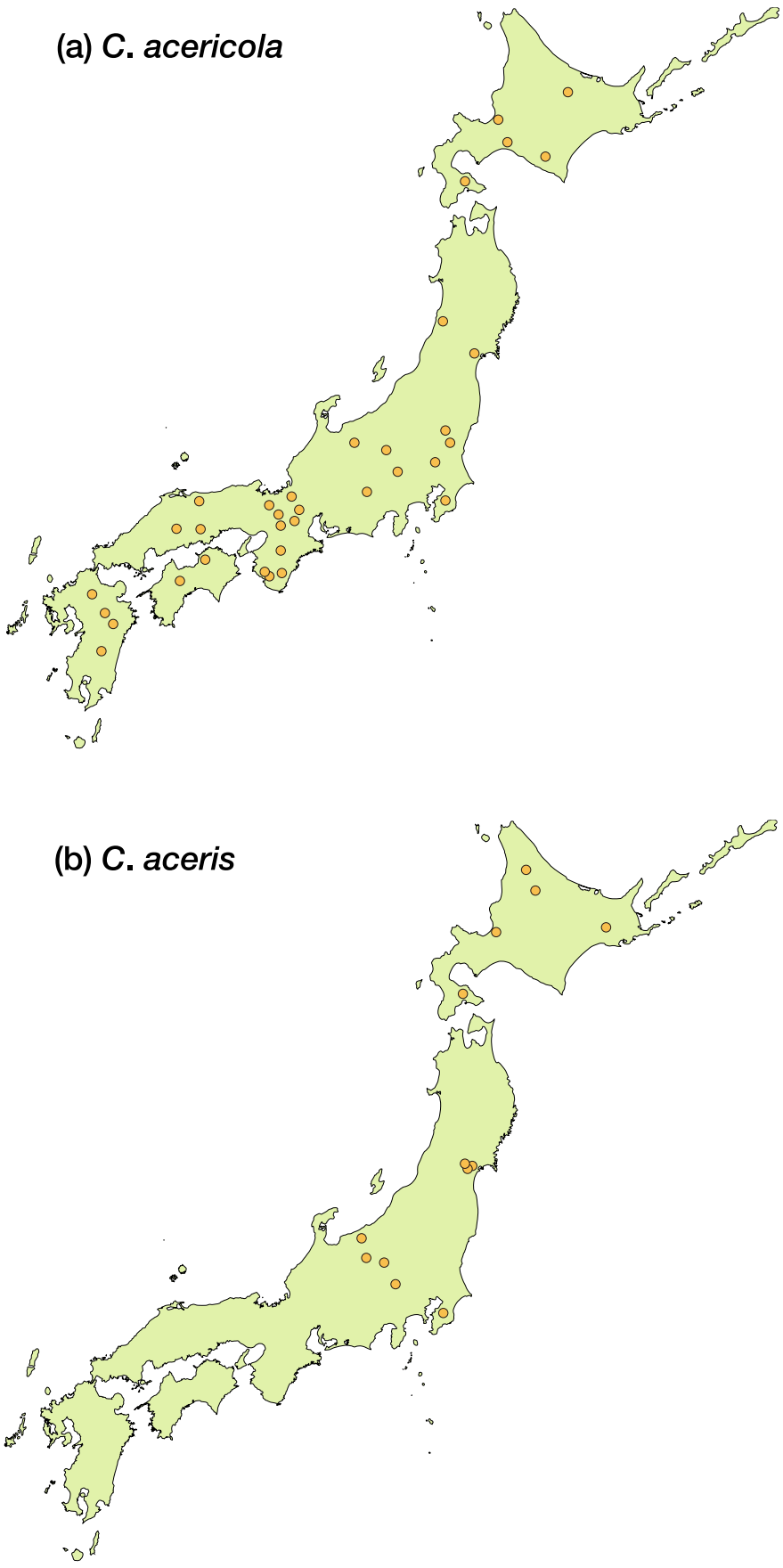
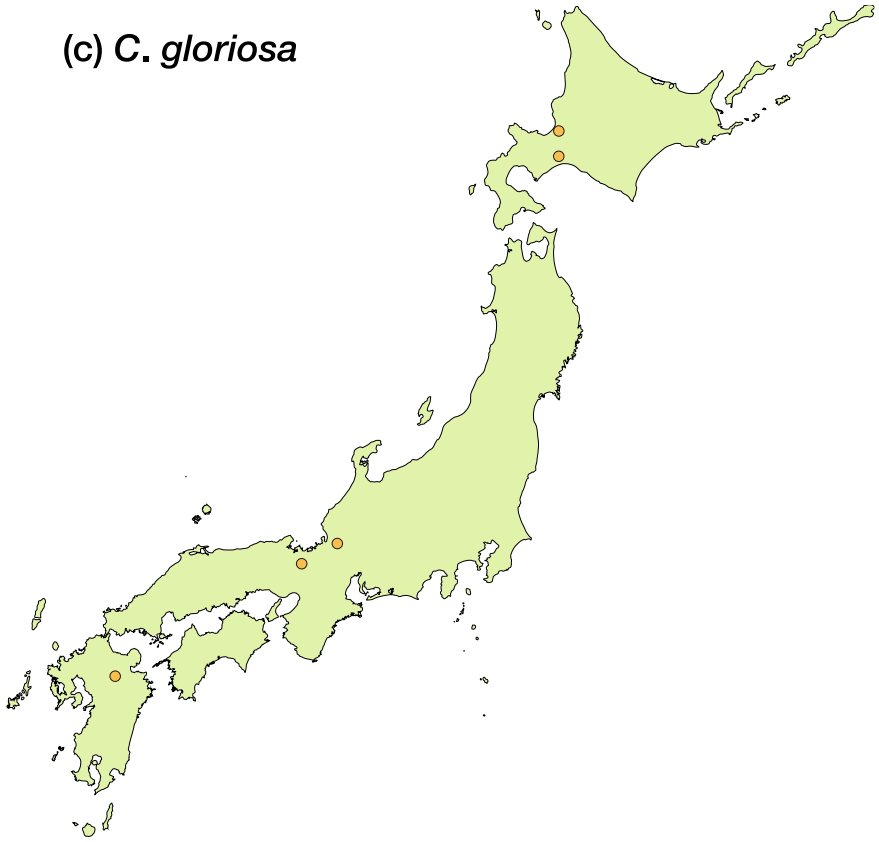


Figure S2 Distributions of 14 *Caloptilia* moth species feeding on *Acer*



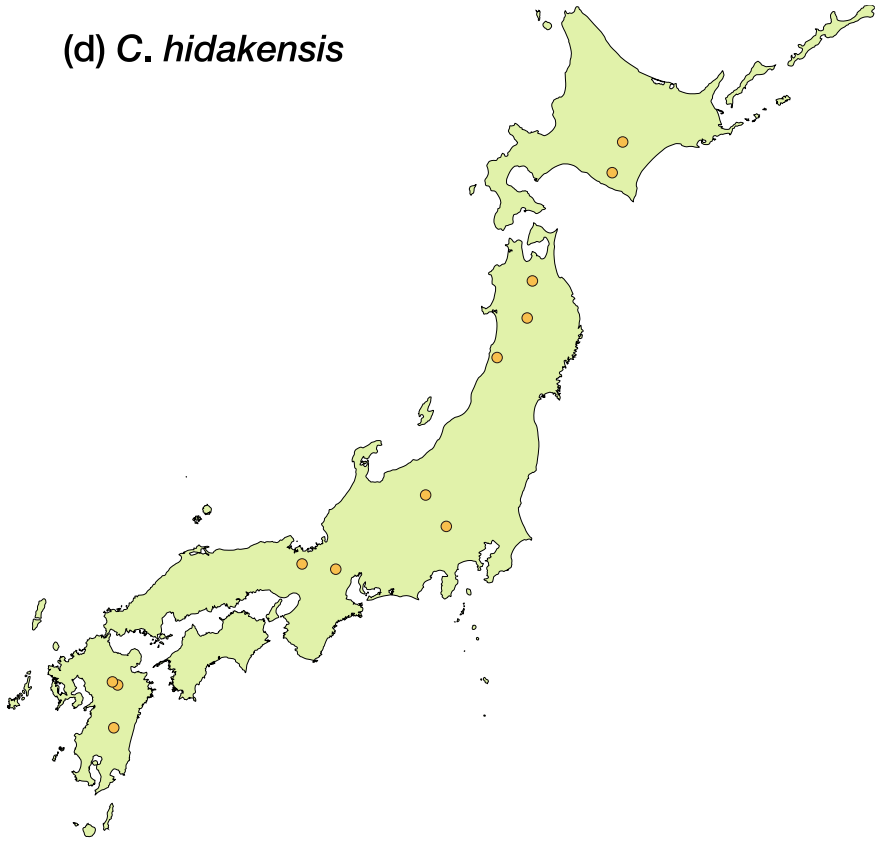


Figure S2 Continued

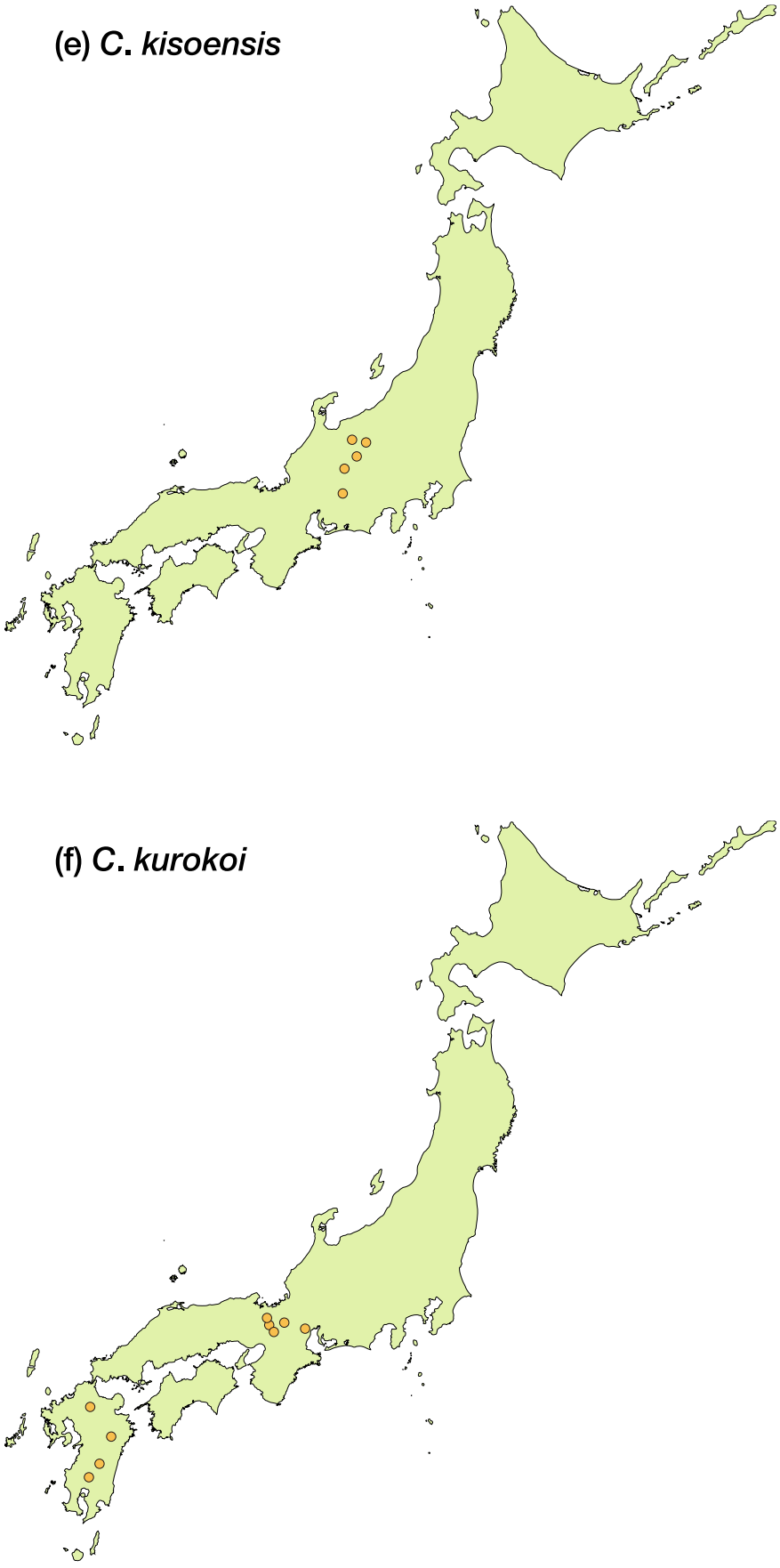


Figure S2 Continued

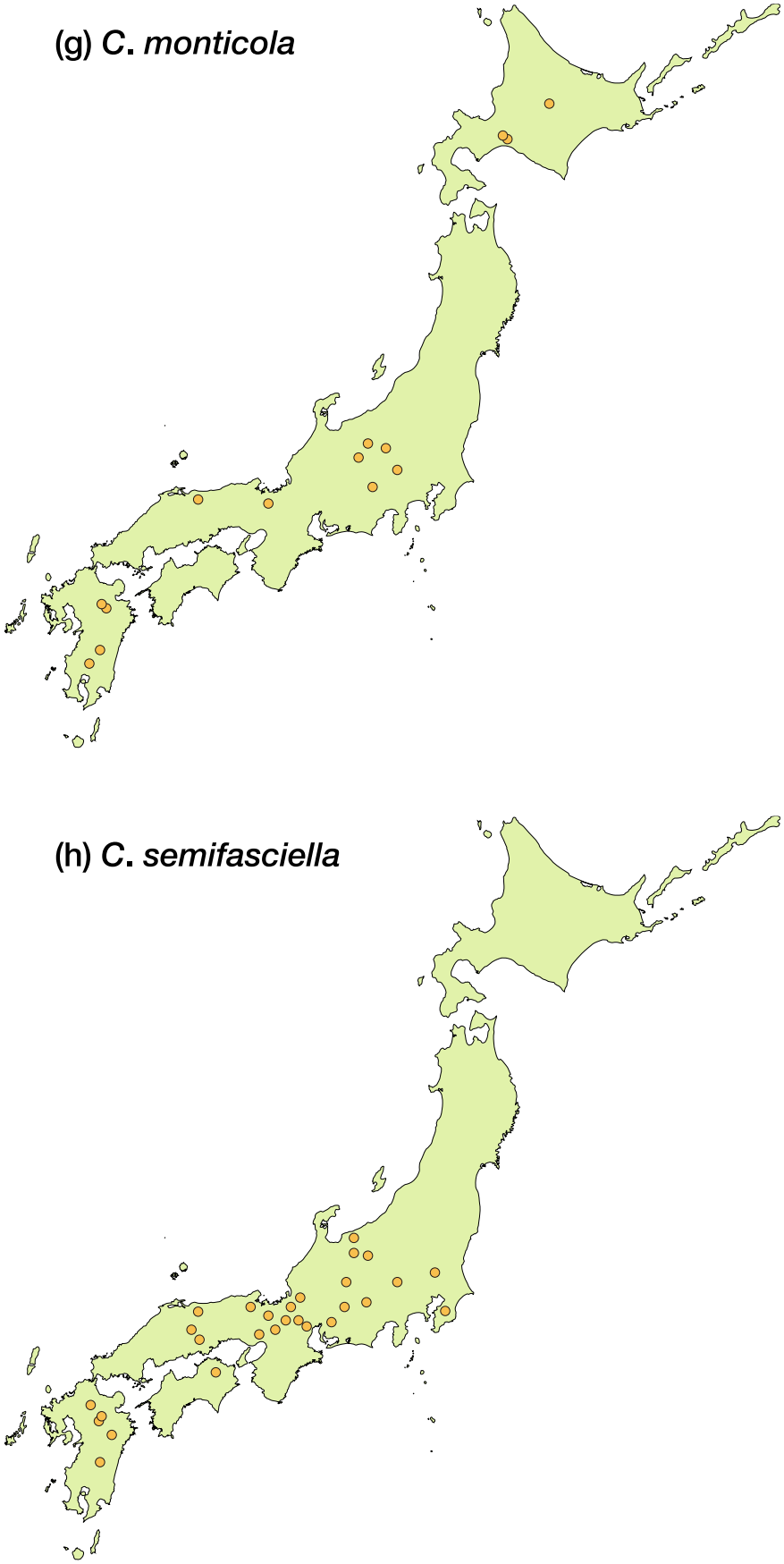
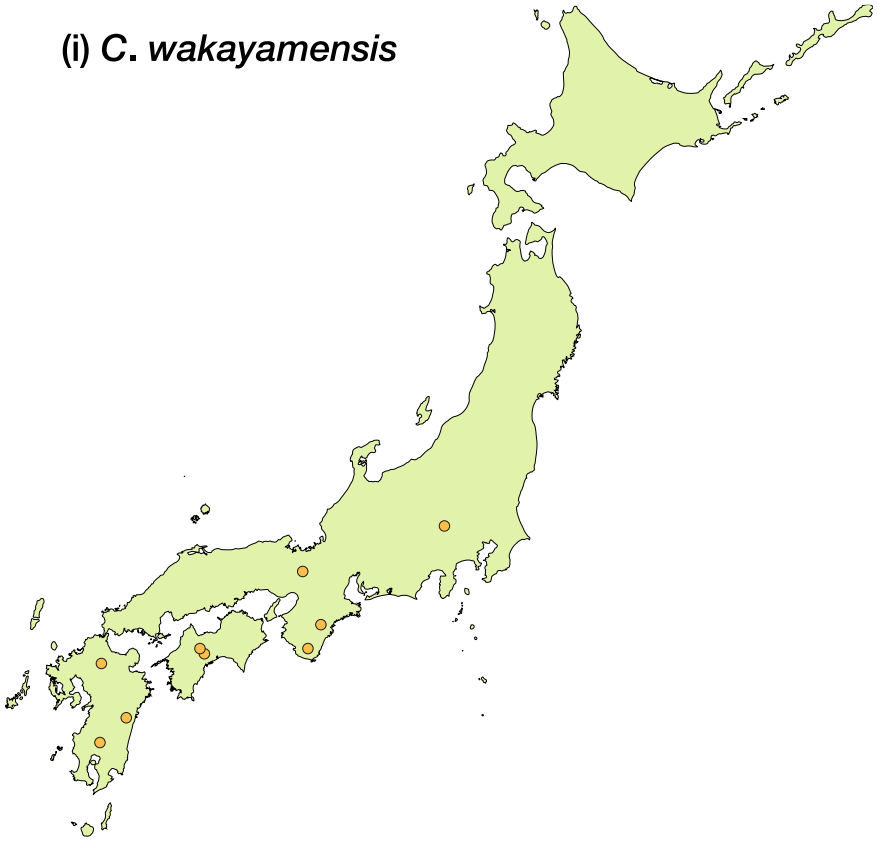


Figure S2 Continued



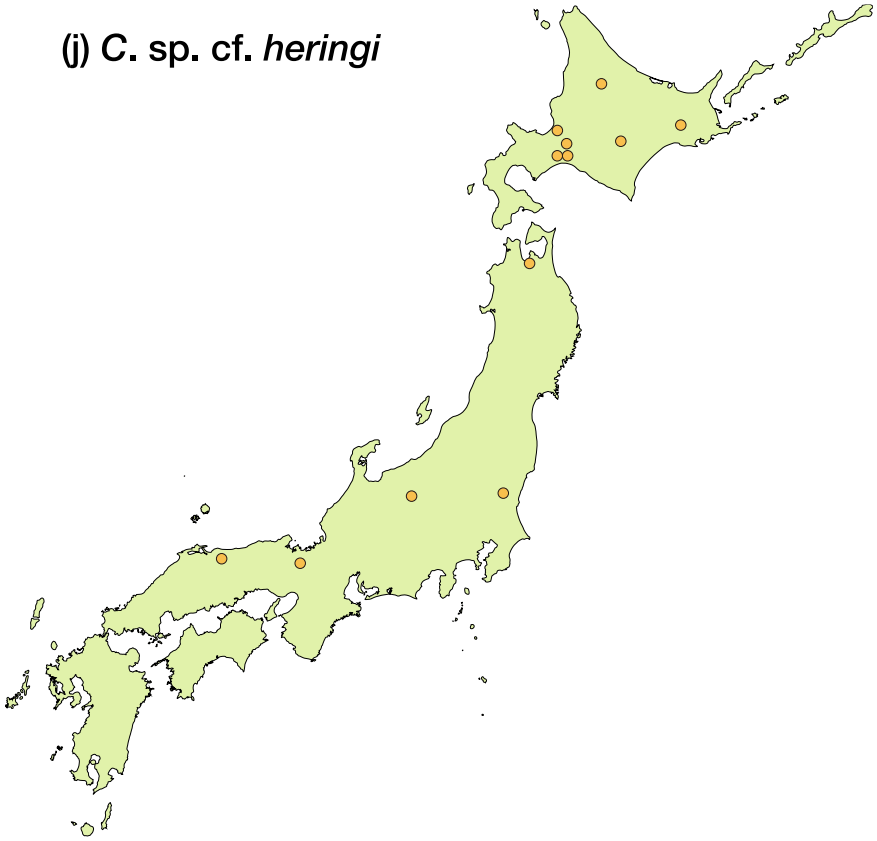


Figure S2 Continued

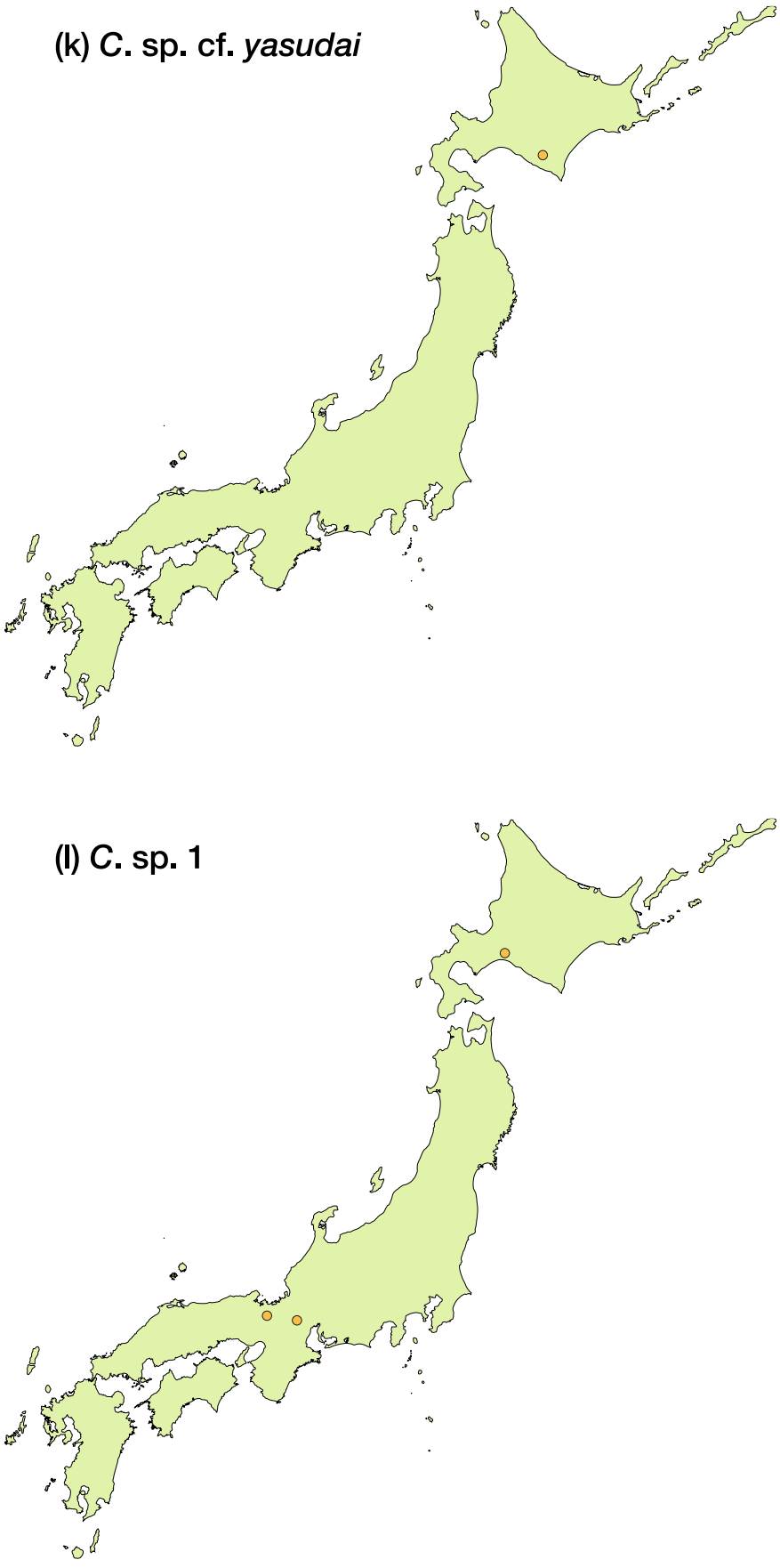
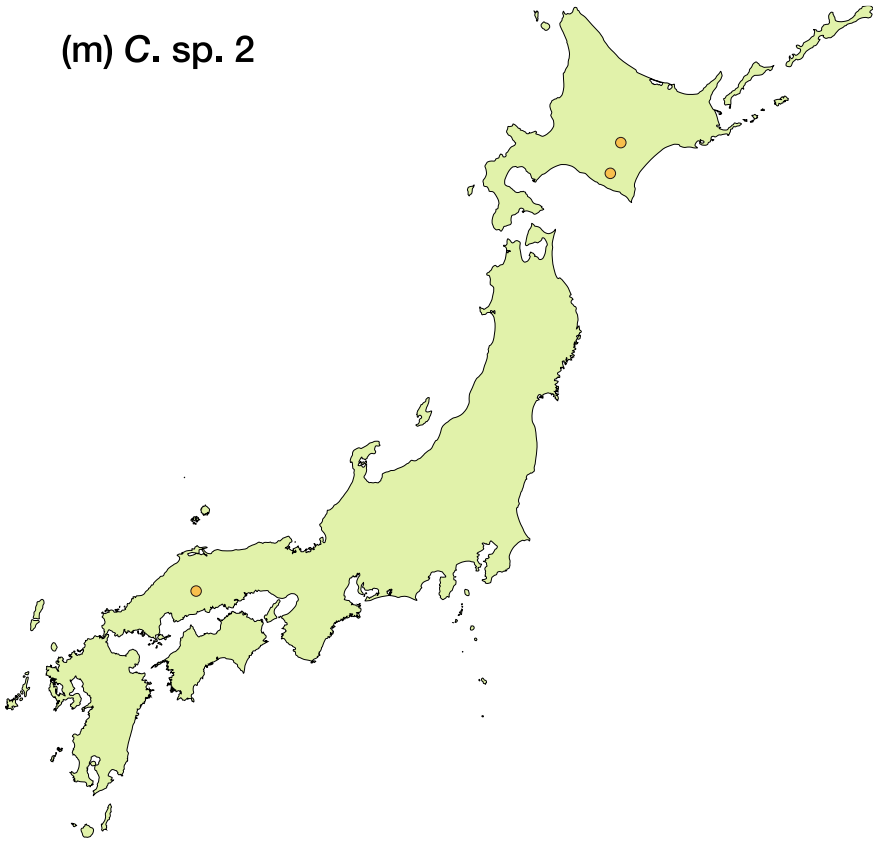


Figure S2 Continued



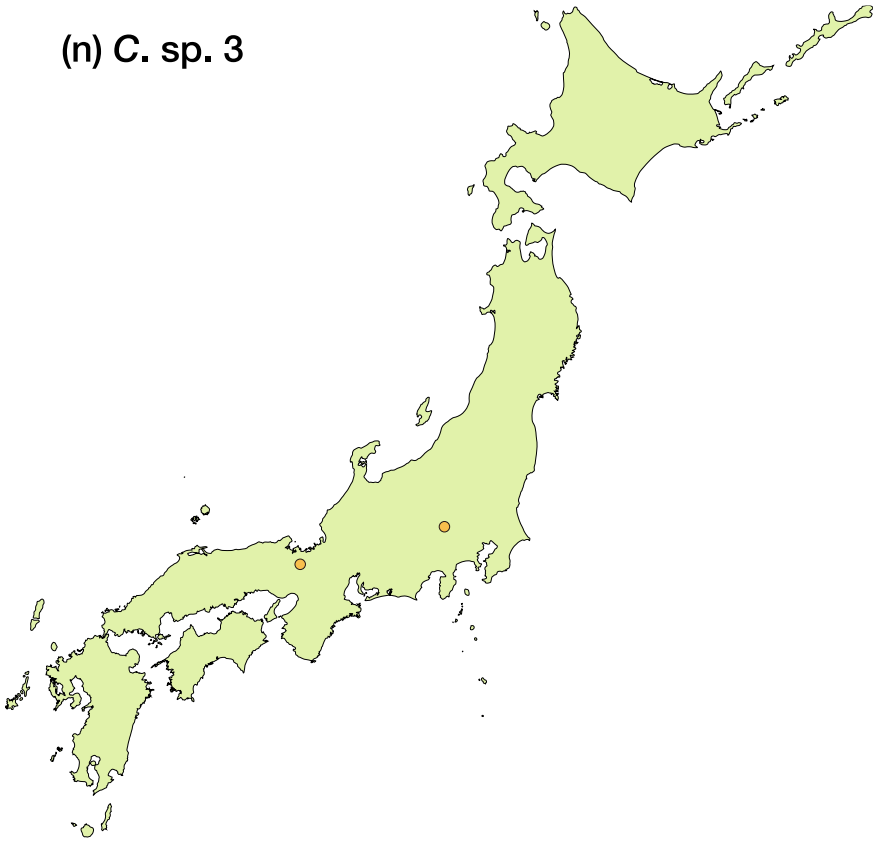


Figure S2 Continued

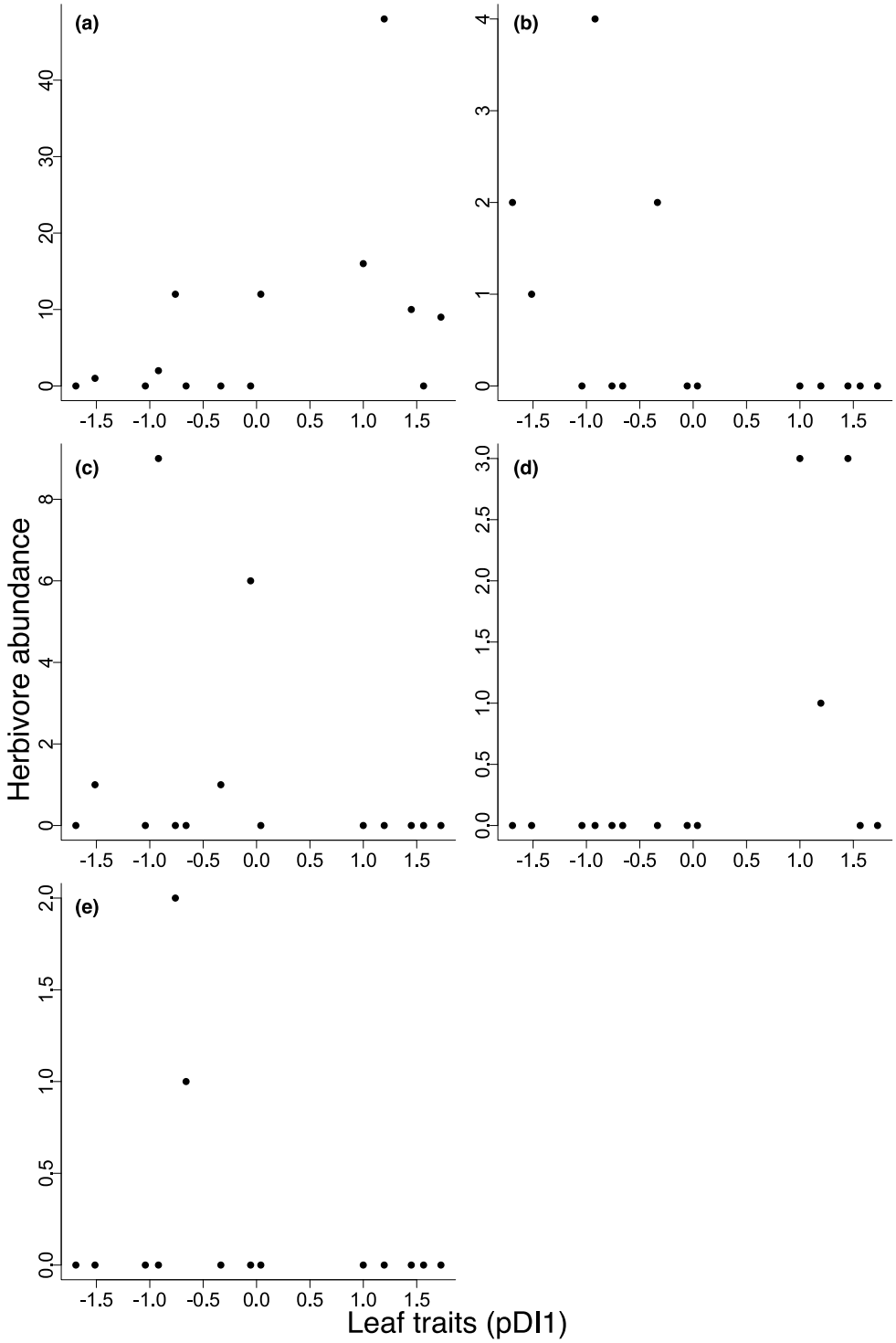
File S1 Text file containing the command for running the analysis in R.

```
library(ade4)
library(betapart)
library(picante)
library(vegan)

#import host use information
host.use.data <- read.csv("host_use_information.csv", header = TRUE, na.strings = "")
#import host phylogeny (edited from Nakadai et al. 2014)
host.new <- read.tree("host_phylogeny.new")
#import herbivorous insect phylogeny (all-genes dataset)
herb.new <- read.tree("herbivore_phylogeny.new")

#edit host use information
host.use <- tapply(host.use.data$num, host.use.data [, c("herb_sp", "host_sp")], sum)
host.use <- replace(host.use, is.na(host.use), 0)
host.use <- decostand(host.use,"pa")

#calculate phylogenetic distance between all pairs of herbivorous insects
herb.dis <- cophenetic(herb.new)
herb.dis <- as.matrix(herb.dis)
tmp <- herb.dis[order(colnames(herb.dis)),]
herb.dis <- tmp[,order(colnames(herb.dis))]
herb.dis <- as.dist(herb.dis)

#calculate Jaccard index
jaccard.index <- beta.pair(host.use,index.family="jaccard")

#calculate Unifrac index
unifrac.index <- phylo.beta.pair(host.use, host.new,index.family="jaccard")

#test
test <- function(x, y, z) {      #x phylogenetic distance between herbivore
                                                                    #y host use dissimilarity
                                                                    #z randomization
std <- sum(x*y)
a <- 0
```

```
b <- NULL
result <- NULL
for (k in 1: z){
herb.dis <- as.dist(x)
vec.host.dis <- as.vector(as.dist(y))
null.model <- sample(vec.host.dis, length(vec.host.dis))
null.model.value <- sum(herb.dis*null.model)
b <- c(b, null.model.value)

if (as.numeric(null.model.value) < as.numeric(std)){a <- a+1
} else {a <- a}
}

if (a < z/2){pval <- 2*(a/z)
} else {pval <- 2*(1-(a/z))}
ses <- (std-mean(b))/sd(b) #standardized effect size
result <- c(pval,ses)
names(result) <- c("p-value","ses-value")
return(result)

}

#calculate the trend of host use changes by turnover component of unifrac index
test(herb.dis,unifrac.index$phylo.beta.jtu,10000)
#calculate the trend of host use changes by turnover component of jaccard index
test(herb.dis,jaccard.index$beta.jtu,10000)
```

Section 4-1

Figure S1. Relationships between leaf traits with phylogenetic signals (pDI1) and herbivore abundance of each *Caloptilia* species: (a) *C. monticola*, (b) *C. acericola*, (c) *C. sp*, (d) *C. semifasciella*, (e) *C. aceris*.



Section 4-2

Table S1 OTUs used in the analysis.

Caloptilia species	OTU_name	Top hit in blast search
C. acericola	OTU_1	Caloptilia acericola mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN12
C. aceris	OTU_22	Caloptilia aceris mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN30
C. gloriosa	OTU_18	Caloptilia gloriosa mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN11
C. heringi	OTU_12	Caloptilia cf. heringi RN-2016 mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN266
C. hidakensis	OTU_8	Caloptilia hidakensis mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: HIOJ016-14
C. kurokoi	OTU_14	Caloptilia kurokoi mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN345
C. monticola	OTU_3	Caloptilia monticola mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN317
C. semifasciella	OTU_2	Caloptilia semifasciella mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: HIOJ004-14
C. sp. 1	OTU_9	Caloptilia sp. RN-2016 mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN16
C. sp. 3	OTU_4	Caloptilia sp. RN-2016 mitochondrial gene for cytochrome oxidase subunit 1, partial cds, isolate: RN34

Parasitoid species	OTU_name	Top hit in blast search
P1	OTU_59	Baryscapus pallidae isolate Bapa2 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial
P2	OTU_20	Baryscapus pallidae isolate Bpal28 cytochrome c oxidase subunit I gene, partial cds; mitochondrial
P3	OTU_10	Baryscapus servadeii haplotype 78 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial
P4	OTU_21	Baryscapus pallidae isolate Bpal146 cytochrome c oxidase subunit I gene, partial cds; mitochondrial
P5	OTU_16	Trichogramma brassicae cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
P6	OTU_5	Achrysocharoides cilla isolate Acil1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial
P7	OTU_7	Ichneumonidae sp. BOLD:ACI6750 voucher BIOUG06519-E12 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
P8	OTU_24	Mesochorinae sp. BOLD:ACN4221 voucher BIOUG12651-H02 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

Parasitoid species	OTU_name	Top hit in blast search
P9	OTU_29	Rhysipolis temporalis cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
P10	OTU_13	Braconidae sp. ex Phyllonorycter ringoniella cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
P11	OTU_35	Glyptapanteles sp. G-14912A cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
P12	OTU_11	Microgastrinae sp. BOLD:AAV2119 voucher JMIC 0482 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
P13	OTU_17	Microgastrinae voucher USNM00681734 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

Table S1 Continued

Accession number	Identities
LC127790.1	100
LC127805.1	98.4
LC127789.1	100
LC127671.1	99.68
LC127750.1	100
LC127725.1	100
LC127704.1	100
LC127770.1	100
LC127794.1	99.68
LC127808.1	100

Accession number	Identities	Previous confirmation of emerging adults from *Caloptilia* moths
HM573727.1	92.16	
JQ416788.1	92.16	
KP420109.1	93.14	*
JQ416796.1	92.81	
KM242284.1	88.6	
HM573686.1	97.39	*
KM564477.1	93.89	*
KR411552.1	97.54	*
AY935376.1	95.92	

Accession number	Identities	Previous confirmation of emerging adults from *Caloptilia* moths
HQ538464.1	99.68	*
KT284341.1	96.49	
JN659992.1	91.99	*
HM421299.1	97.76	

Table S2 DDBJ accession numbers.

(a) For main analysis

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A1	Late May.	*Acer rufinerve*	DRX072707
A2	Late May.	*Acer pictum*	DRX072708
A3	Late May.	*Acer pictum*	DRX072709
A4	Late May.	*Acer amoenum*	DRX072710
A5	Late May.	*Acer amoenum*	DRX072711
A6	Late May.	*Acer amoenum*	DRX072712
A7	Mid Jun.	*Acer rufinerve*	DRX072713
A8	Mid Jun.	*Acer rufinerve*	DRX072714
A9	Mid Jun.	*Acer micranthum*	DRX072715
A10	Mid Jun.	*Acer micranthum*	DRX072716
A11	Mid Jun.	*Acer micranthum*	DRX072717
A12	Mid Jun.	*Acer amoenum*	DRX072718
A13	Mid Jun.	*Acer nipponicum*	DRX072719
A14	Mid Jun.	*Acer nipponicum*	DRX072720
A15	Late Jun.	*Acer micranthum*	DRX072721
A16	Late Jun.	*Acer maximowiczianum*	DRX072722
A17	Late Jun.	*Acer maximowiczianum*	DRX072723
A18	Late Jun.	*Acer amoenum*	DRX072724
A19	Late Jun.	*Acer amoenum*	DRX072725
A20	Late Jun.	*Acer amoenum*	DRX072726
A21	Late Jun.	*Acer amoenum*	DRX072727

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A22	Mid Jul.	*Acer rufinerve*	DRX072728
A23	Mid Jul.	*Acer micranthum*	DRX072729
A24	Mid Jul.	*Acer pictum*	DRX072730
A25	Mid Jul.	*Acer pictum*	DRX072731
A26	Mid Jul.	*Acer pictum*	DRX072732
A27	Mid Jul.	*Acer pictum*	DRX072733
A28	Mid Jul.	*Acer pictum*	DRX072734
A29	Mid Jul.	*Acer pictum*	DRX072735
A30	Mid Jul.	*Acer pictum*	DRX072736
A31	Mid Jul.	*Acer pictum*	DRX072737
A32	Mid Jul.	*Acer amoenum*	DRX072738
A33	Mid Jul.	*Acer amoenum*	DRX072739
A34	Mid Jul.	*Acer amoenum*	DRX072740
A35	Mid Jul.	*Acer amoenum*	DRX072741
A36	Mid Jul.	*Acer amoenum*	DRX072742
A37	Mid Jul.	*Acer amoenum*	DRX072743
A38	Mid Jul.	*Acer amoenum*	DRX072744
A39	Mid Jul.	*Acer maximowiczianum*	DRX072745
A40	Mid Jul.	*Acer maximowiczianum*	DRX072746
A41	Mid Jul.	*Acer maximowiczianum*	DRX072747
A42	Mid Jul.	*Acer maximowiczianum*	DRX072748
A43	Mid Jul.	*Acer maximowiczianum*	DRX072749
A44	Mid Jul.	*Acer maximowiczianum*	DRX072750

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A45	Mid Jul.	*Acer maximowiczianum*	DRX072751
A46	Mid Jul.	*Acer maximowiczianum*	DRX072752
A47	Mid Jul.	*Acer maximowiczianum*	DRX072753
A48	Mid Jul.	*Acer maximowiczianum*	DRX072754
A49	Mid Jul.	*Acer maximowiczianum*	DRX072755
A50	Mid Jul.	*Acer maximowiczianum*	DRX072756
A51	Mid Jul.	*Acer maximowiczianum*	DRX072757
A52	Mid Jul.	*Acer maximowiczianum*	DRX072758
A53	Mid Jul.	*Acer maximowiczianum*	DRX072759
A54	Mid Jul.	*Acer maximowiczianum*	DRX072760
A55	Mid Jul.	*Acer maximowiczianum*	DRX072761
A56	Mid Jul.	*Acer maximowiczianum*	DRX072762
A57	Mid Jul.	*Acer maximowiczianum*	DRX072763
A58	Mid Jul.	*Acer maximowiczianum*	DRX072764
A59	Mid Jul.	*Acer maximowiczianum*	DRX072765
A60	Mid Jul.	*Acer maximowiczianum*	DRX072766
A61	Mid Jul.	*Acer maximowiczianum*	DRX072767
A62	Mid Jul.	*Acer maximowiczianum*	DRX072768
A63	Mid Jul.	*Acer maximowiczianum*	DRX072769
A64	Late Jul.	*Acer crataegifolium*	DRX072770
A65	Late Jul.	*Acer crataegifolium*	DRX072771
A66	Late Jul.	*Acer rufinerve*	DRX072772
A67	Late Jul.	*Acer rufinerve*	DRX072773

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A68	Late Jul.	*Acer rufinerve*	DRX072774
A69	Late Jul.	*Acer rufinerve*	DRX072775
A70	Late Jul.	*Acer micranthum*	DRX072776
A71	Late Jul.	*Acer micranthum*	DRX072777
A72	Late Jul.	*Acer micranthum*	DRX072778
A73	Late Jul.	*Acer micranthum*	DRX072779
A74	Late Jul.	*Acer micranthum*	DRX072780
A75	Late Jul.	*Acer micranthum*	DRX072781
A76	Late Jul.	*Acer micranthum*	DRX072782
A77	Late Jul.	*Acer micranthum*	DRX072783
A78	Late Jul.	*Acer micranthum*	DRX072784
A79	Late Jul.	*Acer maximowiczianum*	DRX072785
A80	Late Jul.	*Acer maximowiczianum*	DRX072786
A81	Late Jul.	*Acer maximowiczianum*	DRX072787
A82	Late Jul.	*Acer maximowiczianum*	DRX072788
A83	Late Jul.	*Acer maximowiczianum*	DRX072789
A84	Late Jul.	*Acer maximowiczianum*	DRX072790
A85	Late Jul.	*Acer maximowiczianum*	DRX072791
A86	Late Jul.	*Acer maximowiczianum*	DRX072792
A87	Late Jul.	*Acer maximowiczianum*	DRX072793
A88	Late Jul.	*Acer maximowiczianum*	DRX072794
A89	Late Jul.	*Acer maximowiczianum*	DRX072795
A90	Late Jul.	*Acer maximowiczianum*	DRX072796

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A91	Late Jul.	*Acer maximowiczianum*	DRX072797
A92	Late Jul.	*Acer maximowiczianum*	DRX072798
A93	Late Jul.	*Acer maximowiczianum*	DRX072799
A94	Late Jul.	*Acer maximowiczianum*	DRX072800
A95	Late Jul.	*Acer maximowiczianum*	DRX072801
A96	Late Jul.	*Acer maximowiczianum*	DRX072802
A97	Late Jul.	*Acer pictum*	DRX072803
A98	Late Jul.	*Acer pictum*	DRX072804
A99	Late Jul.	*Acer pictum*	DRX072805
A100	Late Jul.	*Acer pictum*	DRX072806
A101	Late Jul.	*Acer pictum*	DRX072807
A102	Late Jul.	*Acer pictum*	DRX072808
A103	Late Jul.	*Acer pictum*	DRX072809
A104	Late Jul.	*Acer pictum*	DRX072810
A105	Late Jul.	*Acer pictum*	DRX072811
A106	Late Jul.	*Acer sieboldianum*	DRX072812
A107	Late Jul.	*Acer sieboldianum*	DRX072813
A108	Late Jul.	*Acer sieboldianum*	DRX072814
A109	Late Jul.	*Acer sieboldianum*	DRX072815
A110	Late Jul.	*Acer amoenum*	DRX072816
A111	Late Jul.	*Acer amoenum*	DRX072817
A112	Late Jul.	*Acer amoenum*	DRX072818
A113	Late Jul.	*Acer amoenum*	DRX072819

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A114	Late Jul.	*Acer japonicum*	DRX072820
A115	Late Jul.	*Acer japonicum*	DRX072821
A116	Late Jul.	*Acer japonicum*	DRX072822
A117	Late Jul.	*Acer japonicum*	DRX072823
A118	Late Jul.	*Acer japonicum*	DRX072824
A119	Late Jul.	*Acer nipponicum*	DRX072825
A120	Late Jul.	*Acer nipponicum*	DRX072826
A121	Late Jul.	*Acer nipponicum*	DRX072827
A122	Late Jul.	*Acer nipponicum*	DRX072828
A123	Mid Aug.	*Acer crataegifolium*	DRX072829
A124	Mid Aug.	*Acer micranthum*	DRX072830
A125	Mid Aug.	*Acer maximowiczianum*	DRX072831
A126	Mid Aug.	*Acer pictum*	DRX072832
A127	Mid Aug.	*Acer pictum*	DRX072833
A128	Mid Aug.	*Acer sieboldianum*	DRX072834
A129	Mid Aug.	*Acer nipponicum*	DRX072835
A130	Late Aug.	*Acer micranthum*	DRX072836
A131	Late Aug.	*Acer maximowiczianum*	DRX072837
A132	Late Aug.	*Acer sieboldianum*	DRX072838
A133	Late Aug.	*Acer amoenum*	DRX072839
A134	Mid Sep.	*Acer micranthum*	DRX072840
A135	Mid Sep.	*Acer micranthum*	DRX072841
A136	Mid Sep.	*Acer micranthum*	DRX072842

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A137	Mid Sep.	*Acer micranthum*	DRX072843
A138	Mid Sep.	*Acer micranthum*	DRX072844
A139	Mid Sep.	*Acer amoenum*	DRX072845
A140	Late Sep.	*Acer rufinerve*	DRX072846
A141	Late Sep.	*Acer rufinerve*	DRX072847
A142	Late Sep.	*Acer rufinerve*	DRX072848
A143	Late Sep.	*Acer rufinerve*	DRX072849
A144	Late Sep.	*Acer rufinerve*	DRX072850
A145	Late Sep.	*Acer rufinerve*	DRX072851
A146	Late Sep.	*Acer rufinerve*	DRX072852
A147	Late Sep.	*Acer micranthum*	DRX072853
A148	Late Sep.	*Acer micranthum*	DRX072854
A149	Late Sep.	*Acer micranthum*	DRX072855
A150	Late Sep.	*Acer micranthum*	DRX072856
A151	Late Sep.	*Acer micranthum*	DRX072857
A152	Late Sep.	*Acer micranthum*	DRX072858
A153	Late Sep.	*Acer micranthum*	DRX072859
A154	Late Sep.	*Acer micranthum*	DRX072860
A155	Late Sep.	*Acer micranthum*	DRX072861
A156	Late Sep.	*Acer micranthum*	DRX072862
A157	Late Sep.	*Acer micranthum*	DRX072863
A158	Late Sep.	*Acer micranthum*	DRX072864
A159	Late Sep.	*Acer micranthum*	DRX072865

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A160	Late Sep.	*Acer micranthum*	DRX072866
A161	Late Sep.	*Acer micranthum*	DRX072867
A162	Late Sep.	*Acer micranthum*	DRX072868
A163	Late Sep.	*Acer micranthum*	DRX072869
A164	Late Sep.	*Acer micranthum*	DRX072870
A165	Late Sep.	*Acer micranthum*	DRX072871
A166	Late Sep.	*Acer micranthum*	DRX072872
A167	Late Sep.	*Acer micranthum*	DRX072873
A168	Late Sep.	*Acer micranthum*	DRX072874
A169	Late Sep.	*Acer micranthum*	DRX072875
A170	Late Sep.	*Acer micranthum*	DRX072876
A171	Late Sep.	*Acer micranthum*	DRX072877
A172	Late Sep.	*Acer micranthum*	DRX072878
A173	Late Sep.	*Acer micranthum*	DRX072879
A174	Late Sep.	*Acer micranthum*	DRX072880
A175	Late Sep.	*Acer pictum*	DRX072881
A176	Late Sep.	*Acer pictum*	DRX072882
A177	Late Sep.	*Acer pictum*	DRX072883
A178	Late Sep.	*Acer maximowiczianum*	DRX072884
A179	Late Sep.	*Acer maximowiczianum*	DRX072885
A180	Late Sep.	*Acer maximowiczianum*	DRX072886
A181	Late Sep.	*Acer maximowiczianum*	DRX072887
A182	Late Sep.	*Acer maximowiczianum*	DRX072888

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A183	Late Sep.	*Acer maximowiczianum*	DRX072889
A184	Late Sep.	*Acer sieboldianum*	DRX072890
A185	Late Sep.	*Acer sieboldianum*	DRX072891
A186	Late Sep.	*Acer sieboldianum*	DRX072892
A187	Late Sep.	*Acer sieboldianum*	DRX072893
A188	Late Sep.	*Acer sieboldianum*	DRX072894
A189	Late Sep.	*Acer sieboldianum*	DRX072895
A190	Late Sep.	*Acer sieboldianum*	DRX072896
A191	Late Sep.	*Acer sieboldianum*	DRX072897
A192	Late Sep.	*Acer amoenum*	DRX072898
A193	Late Sep.	*Acer amoenum*	DRX072899
A194	Late Sep.	*Acer amoenum*	DRX072900
A195	Late Sep.	*Acer amoenum*	DRX072901
A196	Late Sep.	*Acer amoenum*	DRX072902
A197	Late Sep.	*Acer amoenum*	DRX072903
A198	Late Sep.	*Acer amoenum*	DRX072904
A199	Late Sep.	*Acer amoenum*	DRX072905
A200	Late Sep.	*Acer amoenum*	DRX072906
A201	Late Sep.	*Acer amoenum*	DRX072907
A202	Late Sep.	*Acer amoenum*	DRX072908
A203	Late Sep.	*Acer amoenum*	DRX072909
A204	Mid Oct.	*Acer crataegifolium*	DRX072910
A205	Mid Oct.	*Acer micranthum*	DRX072911

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A206	Mid Oct.	*Acer micranthum*	DRX072912
A207	Mid Oct.	*Acer micranthum*	DRX072913
A208	Mid Oct.	*Acer micranthum*	DRX072914
A209	Mid Oct.	*Acer micranthum*	DRX072915
A210	Mid Oct.	*Acer micranthum*	DRX072916
A211	Mid Oct.	*Acer micranthum*	DRX072917
A212	Mid Oct.	*Acer micranthum*	DRX072918
A213	Mid Oct.	*Acer micranthum*	DRX072919
A214	Mid Oct.	*Acer micranthum*	DRX072920
A215	Mid Oct.	*Acer micranthum*	DRX072921
A216	Mid Oct.	*Acer micranthum*	DRX072922
A217	Mid Oct.	*Acer micranthum*	DRX072923
A218	Mid Oct.	*Acer micranthum*	DRX072924
A219	Mid Oct.	*Acer micranthum*	DRX072925
A220	Mid Oct.	*Acer micranthum*	DRX072926
A221	Mid Oct.	*Acer maximowiczianum*	DRX072927
A222	Mid Oct.	*Acer maximowiczianum*	DRX072928
A223	Mid Oct.	*Acer maximowiczianum*	DRX072929
A224	Mid Oct.	*Acer maximowiczianum*	DRX072930
A225	Mid Oct.	*Acer maximowiczianum*	DRX072931
A226	Mid Oct.	*Acer maximowiczianum*	DRX072932
A227	Mid Oct.	*Acer maximowiczianum*	DRX072933
A228	Mid Oct.	*Acer pictum*	DRX072934

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A229	Mid Oct.	*Acer pictum*	DRX072935
A230	Mid Oct.	*Acer sieboldianum*	DRX072936
A231	Mid Oct.	*Acer amoenum*	DRX072937
A232	Mid Oct.	*Acer amoenum*	DRX072938
A233	Mid Oct.	*Acer amoenum*	DRX072939
A234	Mid Oct.	*Acer amoenum*	DRX072940
A235	Mid Oct.	*Acer amoenum*	DRX072941
A236	Mid Oct.	*Acer amoenum*	DRX072942
A237	Mid Oct.	*Acer amoenum*	DRX072943
A238	Mid Oct.	*Acer amoenum*	DRX072944
A239	Mid Oct.	*Acer amoenum*	DRX072945
A240	Mid Oct.	*Acer amoenum*	DRX072946
A241	Mid Oct.	*Acer amoenum*	DRX072947
A242	Mid Oct.	*Acer amoenum*	DRX072948
A243	Mid Oct.	*Acer amoenum*	DRX072949
A244	Mid Oct.	*Acer amoenum*	DRX072950
A245	Mid Oct.	*Acer amoenum*	DRX072951
A246	Mid Oct.	*Acer amoenum*	DRX072952
A247	Mid Oct.	*Acer amoenum*	DRX072953
A248	Mid Oct.	*Acer amoenum*	DRX072954
A249	Mid Oct.	*Acer amoenum*	DRX072955
A250	Mid Oct.	*Acer amoenum*	DRX072956
A251	Mid Oct.	*Acer amoenum*	DRX072957

Individual ID of leaf cone moths	Season	Host plant of prey moth	Accession number
A252	Mid Oct.	*Acer japonicum*	DRX072958
A253	Mid Oct.	*Acer japonicum*	DRX072959
A254	Mid Oct.	*Acer japonicum*	DRX072960
A255	Late Oct.	*Acer amoenum*	DRX072961
A256	Late Oct.	*Acer amoenum*	DRX072962
A257	Late Oct.	*Acer amoenum*	DRX072963
A258	Late Oct.	*Acer amoenum*	DRX072964
A259	Late Oct.	*Acer amoenum*	DRX072965
A260	Late Oct.	*Acer amoenum*	DRX072966
A261	Late Oct.	*Acer amoenum*	DRX072967
A262	Late Oct.	*Acer amoenum*	DRX072968
A263	Late Oct.	*Acer amoenum*	DRX072969
A264	Late Oct.	*Acer amoenum*	DRX072970
A265	Late Oct.	*Acer amoenum*	DRX072971
A266	Late Oct.	*Acer amoenum*	DRX072972
A267	Late Oct.	*Acer amoenum*	DRX072973
A268	Late Oct.	*Acer amoenum*	DRX072974
A269	Late Oct.	*Acer amoenum*	DRX072975
A270	Late Oct.	*Acer amoenum*	DRX072976
A271	Late Oct.	*Acer maximowiczianum*	DRX072977
A272	Late Oct.	*Acer maximowiczianum*	DRX072978
A273	Late Oct.	*Acer maximowiczianum*	DRX072979
A274	Late Oct.	*Acer maximowiczianum*	DRX072980

(b) For test of blocking primer (Supplementaly file 2)

Sample ID	Blocking primer	Individual ID	Species name	Replicate num.	Accession number
BA1-1	absence	A20	*acericola*	1	DRX072981
BA1-2	absence	A229	*aceris*	1	DRX072982
BA1-3	absence	A6	*gloriosa*	1	DRX072983
BA1-4	absence	A98	*heringi*	1	DRX072984
BA1-5	absence	A2	*hidakensis*	1	DRX072985
BA1-6	absence	A145	*kurokoi*	1	DRX072986
BA1-7	absence	A9	*monticola*	1	DRX072987
BA1-8	absence	A8	*semifasciella*	1	DRX072988
BA1-9	absence	A4	sp. 1	1	DRX072989
BA1-10	absence	A227	sp. 3	1	DRX072990
BP1-1	presence	A20	*acericola*	1	DRX072991
BP1-2	presence	A229	*aceris*	1	DRX072992
BP1-3	presence	A6	*gloriosa*	1	DRX072993
BP1-4	presence	A98	*heringi*	1	DRX072994
BP1-5	presence	A2	*hidakensis*	1	DRX072995
BP1-6	presence	A145	*kurokoi*	1	DRX072996
BP1-7	presence	A9	*monticola*	1	DRX072997
BP1-8	presence	A8	*semifasciella*	1	DRX072998
BP1-9	presence	A4	sp. 1	1	DRX072999
BP1-10	presence	A227	sp. 3	1	DRX073000
BA2-1	absence	A20	*acericola*	2	DRX073001

Sample ID	Blocking primer	Individual ID	Species name	Replicate num.	Accession number
BA2-2	absence	A229	*aceris*	2	DRX073002
BA2-3	absence	A6	*gloriosa*	2	DRX073003
BA2-4	absence	A98	*heringi*	2	DRX073004
BA2-5	absence	A2	*hidakensis*	2	DRX073005
BA2-6	absence	A145	*kurokoi*	2	DRX073006
BA2-7	absence	A9	*monticola*	2	DRX073007
BA2-8	absence	A8	*semifasciella*	2	DRX073008
BA2-9	absence	A4	sp. 1	2	DRX073009
BA2-10	absence	A227	sp. 3	2	DRX073010
BP2-1	presence	A20	*acericola*	2	DRX073011
BP2-2	presence	A229	*aceris*	2	DRX073012
BP2-3	presence	A6	*gloriosa*	2	DRX073013
BP2-4	presence	A98	*heringi*	2	DRX073014
BP2-5	presence	A2	*hidakensis*	2	DRX073015
BP2-6	presence	A145	*kurokoi*	2	DRX073016
BP2-7	presence	A9	*monticola*	2	DRX073017
BP2-8	presence	A8	*semifasciella*	2	DRX073018
BP2-9	presence	A4	sp. 1	2	DRX073019
BP2-10	presence	A227	sp. 3	2	DRX073020
BA3-1	absence	A20	*acericola*	3	DRX073021
BA3-2	absence	A229	*aceris*	3	DRX073022
BA3-3	absence	A6	*gloriosa*	3	DRX073023
BA3-4	absence	A98	*heringi*	3	DRX073024

Sample ID	Blocking primer	Individual ID	Species name	Replicate num.	Accession number
BA3-5	absence	A2	*hidakensis*	3	DRX073025
BA3-6	absence	A145	*kurokoi*	3	DRX073026
BA3-7	absence	A9	*monticola*	3	DRX073027
BA3-8	absence	A8	*semifasciella*	3	DRX073028
BA3-9	absence	A4	sp. 1	3	DRX073029
BA3-10	absence	A227	sp. 3	3	DRX073030
BP3-1	presence	A20	*acericola*	3	DRX073031
BP3-2	presence	A229	*aceris*	3	DRX073032
BP3-3	presence	A6	*gloriosa*	3	DRX073033
BP3-4	presence	A98	*heringi*	3	DRX073034
BP3-5	presence	A2	*hidakensis*	3	DRX073035
BP3-6	presence	A145	*kurokoi*	3	DRX073036
BP3-7	presence	A9	*monticola*	3	DRX073037
BP3-8	presence	A8	*semifasciella*	3	DRX073038
BP3-9	presence	A4	sp. 1	3	DRX073039
BP3-10	presence	A227	sp. 3	3	DRX073040
BA4-1	absence	A20	*acericola*	4	DRX073041
BA4-2	absence	A229	*aceris*	4	DRX073042
BA4-3	absence	A6	*gloriosa*	4	DRX073043
BA4-4	absence	A98	*heringi*	4	DRX073044
BA4-5	absence	A2	*hidakensis*	4	DRX073045
BA4-6	absence	A145	*kurokoi*	4	DRX073046
BA4-7	absence	A9	*monticola*	4	DRX073047

Sample ID	Blocking primer	Individual ID	Species name	Replicate num.	Accession number
BA4-8	absence	A8	*semifasciella*	4	DRX073048
BA4-9	absence	A4	sp. 1	4	DRX073049
BA4-10	absence	A227	sp. 3	4	DRX073050
BP4-1	presence	A20	*acericola*	4	DRX073051
BP4-2	presence	A229	*aceris*	4	DRX073052
BP4-3	presence	A6	*gloriosa*	4	DRX073053
BP4-4	presence	A98	*heringi*	4	DRX073054
BP4-5	presence	A2	*hidakensis*	4	DRX073055
BP4-6	presence	A145	*kurokoi*	4	DRX073056
BP4-7	presence	A9	*monticola*	4	DRX073057
BP4-8	presence	A8	*semifasciella*	4	DRX073058
BP4-9	presence	A4	sp. 1	4	DRX073059
BP4-10	presence	A227	sp. 3	4	DRX073060
BA5-1	absence	A20	*acericola*	5	DRX073061
BA5-2	absence	A229	*aceris*	5	DRX073062
BA5-3	absence	A6	*gloriosa*	5	DRX073063
BA5-4	absence	A98	*heringi*	5	DRX073064
BA5-5	absence	A2	*hidakensis*	5	DRX073065
BA5-6	absence	A145	*kurokoi*	5	DRX073066
BA5-7	absence	A9	*monticola*	5	DRX073067
BA5-8	absence	A8	*semifasciella*	5	DRX073068
BA5-9	absence	A4	sp. 1	5	DRX073069
BA5-10	absence	A227	sp. 3	5	DRX073070

Sample ID	Blocking primer	Individual ID	Species name	Replicate num.	Accession number
BP5-1	presence	A20	*acericola*	5	DRX073071
BP5-2	presence	A229	*aceris*	5	DRX073072
BP5-3	presence	A6	*gloriosa*	5	DRX073073
BP5-4	presence	A98	*heringi*	5	DRX073074
BP5-5	presence	A2	*hidakensis*	5	DRX073075
BP5-6	presence	A145	*kurokoi*	5	DRX073076
BP5-7	presence	A9	*monticola*	5	DRX073077
BP5-8	presence	A8	*semifasciella*	5	DRX073078
BP5-9	presence	A4	sp. 1	5	DRX073079
BP5-10	presence	A227	sp. 3	5	DRX073080

Table S3 Abundance and parasitoid rate for each species.

	C. acericola	*C. aceris*	*C. gloriosa*	*C. heringi*	*C. hidakensis*	*C. kurokoi*	*C. monticola*	*C. semifasciella*	*C.* sp. 1	*C.* sp. 3
abundance	84	21	2	3	2	1	53	36	8	64
parasite rate	0.37	0.86	0.50	0.00	1.00	0.00	0.15	0.39	0.75	0.73

Table S4 Information on parasitoid wasps that were used for constructing the blocking primer for *Caloptilia* moths.

Individual ID	Host plant of prey moth	Sampling country	Sampling site	Accession number
AP1	*Acer palmatum*	Japan	Wakayama	LC201483
AP2	*Acer palmatum*	Japan	Wakayama	LC201495
AP4	*Acer distylum*	Japan	Ishikawa	LC201501
AP10	*Acer sieboldianum*	Japan	Fukuoka	LC201498
AP11	*Acer pictum*	Japan	Hokkaido	LC201496
AP13	*Acer palmatum*	Japan	Kochi	LC201487
AP16	*Acer sieboldianum*	Japan	Fukuoka	LC201491
AP19	*Acer palmatum*	Japan	Shiga	LC201484
AP20	*Acer pictum*	Japan	Fukui	LC201488
AP23	*Acer rufinerve*	Japan	Mie	LC201489
AP28	*Acer crataegifolium*	Japan	Nagano	LC201494
AP29	*Acer rubrum*	USA	Vermont	LC201497
AP32	*Acer rufinerve*	Japan	Kagoshima	LC201490
AP42	*Acer maximowiczianum*	Japan	Kyoto	LC201493
AP62	*Acer rufinerve*	Japan	Kyoto	LC201492
AP66	*Acer crataegifolium*	Japan	Nagano	LC201500
AP75	*Acer pictum*	Japan	Hokkaido	LC201486
AP76	*Acer crataegifolium*	Japan	Hyogo	LC201485
AP83	*Acer pictum*	Japan	Hokkaido	LC201499

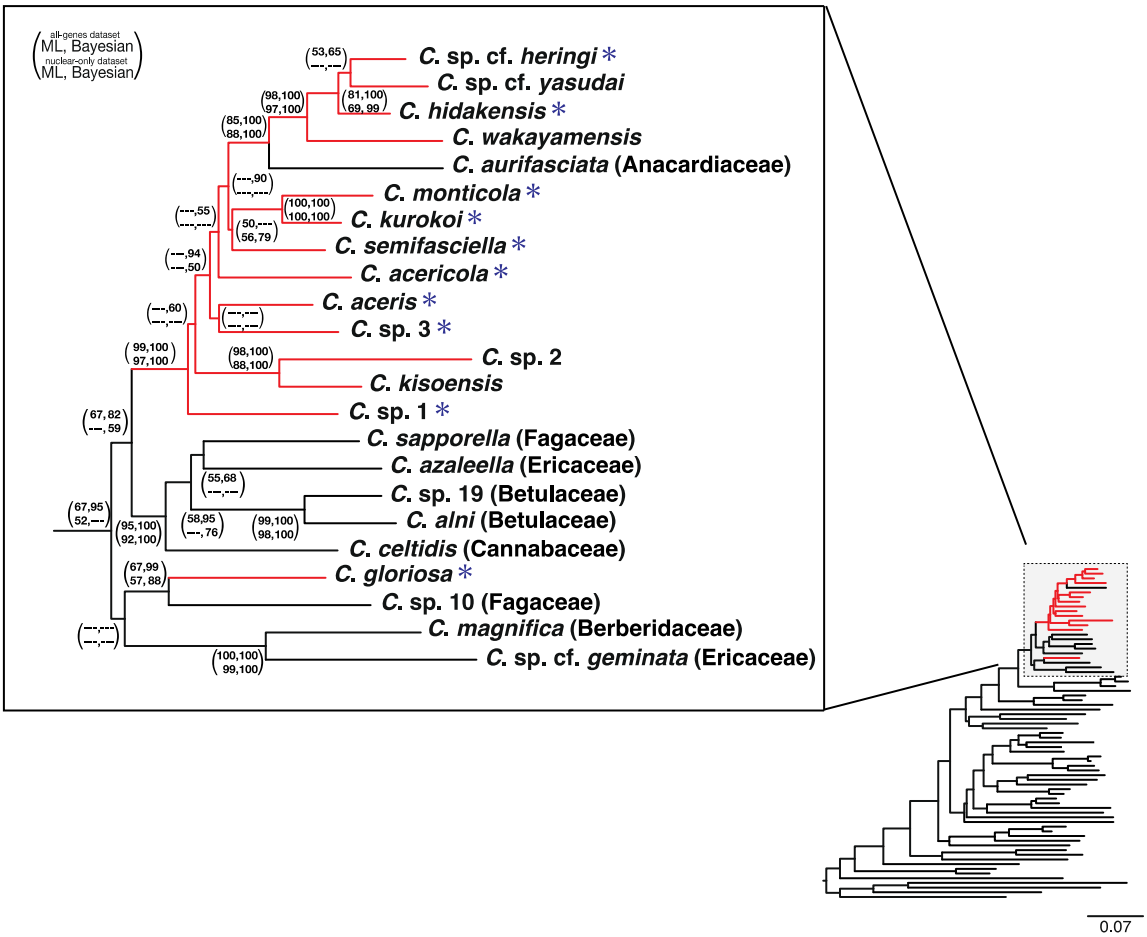


Figure S1 Phylogeny of *Caloptilia* moths and their related groups. The phylogeny was constructed by the maximum-likelihood method using four genomic regions (COI, ArgK, CAD, and EF-1a) of 71 species (Nakadai & Kawakita 2016). Red indicates the lineage of *Caloptilia* moths associated with maples.

Supplementary file 1 Fasta-formatted representative sequences of each OTU, created by the UPARSE pipeline.

>OTU_1
ACTTTCATCTAATATCGCTCATGGAGGAAGATCAGTAGATTTAGCAATTTTTTCTTTACATTTAGCTGGAATTTCTTCAATTTTAG
GAGCTATTAATTTTATTACTACAATTATTAATATACGAGTGAATGGAATAATATTTGATAGTATATCCTTATTTACATGAGCAGTAAG
TATTACTGCATTATTATTATTATTATCTTTACCTGTTTTAGCAGGTGCAATTACTATATTATTAACTGATCGAAATTTAAATACATCAT
TTTTTGATCCTGCTGGAGGAGGAGATCCTATTTTATATCAACATTTATTT
>OTU_2
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ATTACCGCTTTATTATTATTACTATCTTTACCTGTTTTAGCGGGTGCAATTACTATATTATTAACTGATCGAAATTTAAATACATCATT
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>OTU_3
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TATTTTATTATTATTATCATTACCTGTATTAGCTGGTGCAATTACTATATTATTATTTGATCGTAATTTAAATACATCATTTTTTGATCC
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Supplementary file 2 Performance assessment of the blocking primer created in this study for *Caloptilia* moths associated with maples.

I conducted a verification experiment to test the utility of the *Caloptila*-blocking primer that was designed in this study. I used the samples that were collected in this study (see Table 1 for details) for all *Caloptilia* species, and added the DNA that was extracted from adult parasitoid wasps collected in 2014 (AP 10). I normalized the concentration of DNA before PCR with a Nano Drop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) (50 ng/ml) and mixed the samples (*Caloptilia* DNA : parasitoid DNA = 50 : 1). I used the mixed DNA as a template for the first PCR protocol with or without blocking primer (blocking and control conditions, respectively). The methods are described in the main text. I conducted five replicates for each species under both control and blocking conditions. Figure 1 clearly shows that including blocking primers in the first PCR regulates the reaction. As a result, I were able to detect parasitoid DNA from all samples and replicates using the blocking primer, but I failed to detect the internal parasitoid when the blocking primer was not used (28 of 50 trials failed to detect parasitoid sequences in control situations). The relative read number of parasitoid wasps clearly increased with the use of the blocking primer (Table 1).

Figure 1 Electrophoresis images of the products of the first PCR with or without blocking primer

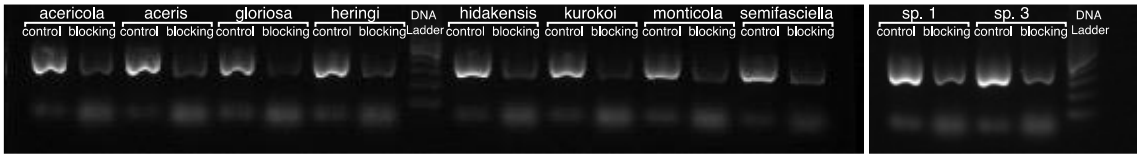

Table 1 The results of blocking primer verification experiments

species name	sample ID	Increasing rate of parasitoid reads using blocking primer (times)	
		average	standard error
acericola	A20	101.5729403	53.2370331
aceris	A229	119.0136099	89.13762705
gloriosa	A6	163.5356965	88.89274992
heringi	A98	239.2013174	371.8013004
hidakensis	A2	58.99979588	45.02161423
kurokoi	A145	78.96175101	26.39092581

species name	sample ID	Increasing rate of parasitoid reads using blocking primer (times)	
		average	standard error
monticola	A9	125.8553226	122.6384529
semifasciella	A8	161.7847638	102.8264766
sp. 1	A4	119.8546578	73.99450217
sp. 3	A227	66.89077743	41.09609608