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

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Abstract

The diversity of herbivorous insects is often considered a function of host plant diversity. However, recent research has uncovered many examples of closely related herbivores using the same host plant(s), suggesting that partitioning of host plants is not the only mechanism generating diversity. Herbivores sharing hosts may utilize different parts of the same plant, but such resource partitioning is often not apparent; hence, the factors that allow closely related herbivores to coexist are still largely undetermined. We examined whether partitioning of phenology or natural enemies may explain the coexistence of leaf cone moths (*Caloptilia*; Gracillariidae) associated with maples (*Acer*; Sapindaceae). Larval activity of 10 sympatric *Caloptilia* species found on nine maple species was monitored every 2–3 weeks for a total of 13 sampling events, and an exhaustive search for internal parasitoid wasps was conducted using high-throughput sequencing. Blocking primers were used to facilitate the detection of wasp larvae inside moth tissue. We found considerable phenological overlap among *Caloptilia* species, with two clear peaks in July and September–October. Coexisting *Caloptilia* species also had largely overlapping parasitoid communities; a total of 13 wasp species belonging to four families attacked *Caloptilia* in a non-specific fashion at an overall parasitism rate of 46.4%. Although coexistence may be facilitated by factors not accounted for in this study, it appears that niche partitioning is not necessary for closely related herbivores to stably coexist on shared hosts. Co-occurrence without resource partitioning may provide an additional axis along which herbivorous insects attain increased species richness.

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

66 species coexistence (MacArthur & Levins 1967; Benson 1978). However, there are
67 many examples where closely related herbivores overlap in their use of host plants.
68 Herbivores that share hosts sometimes partition resources by using different parts of the
69 same plant or leaves of different ages (Benson 1978; Bailey *et al*. 2009; Condon *et al*.
70 2014). For example, Benson (1978) confirmed niche partitioning among *Heliconiini*
71 butterflies along three different niche axes (plant species, plant habitat, and plant part).
72 However, in many instances, closely related herbivores co-occur on the same host
73 without any apparent means of resource partitioning (Strong *et al.* 1982), indicating that
74 there are other factors besides resource partitioning that facilitate coexistence of species
75 sharing the same host plant.
76 One mechanism that allows coexistence of species with similar resource use is
77 phenological partitioning. For example, the geometrid winter moth *Inurois punctigera*
78 has two allochronic races that coexist stably without partitioning resources; allochrony
79 is even postulated as the direct cause of divergence in this case (Yamamoto & Sota
80 2009). Alternatively, species that share the same food resources can have different
81 natural enemies and thereby occupy non-overlapping niches. Condon *et al*. (2014)
82 demonstrated that species-specific parasitoids increase the niche diversity of
83 *Blepharoneura* flies co-existing on the same-sex flowers of curcurbit host plants. Also,
84 the more than 20 *Andricus* gall wasp species that coexist on shared oak hosts display
85 remarkable diversity of gall forms; because gall morphology is a major determinant of
86 parasitoid community structure, differences in natural enemies also provide a
87 comprehensive explanation for the coexistence of multiple gall wasp species on oaks
88 (Bailey *et al*. 2009). However, analysis of parasitoid communities among closely related

89 herbivores is still limited, and our understanding of the role of natural enemies will
90 increase with additional data.
91 In this study, we examined whether differences in phenology or natural
92 enemies explain the coexistence of closely related herbivorous insects on shared host
93 plants. We focused on interactions between a group of leaf cone moths (*Caloptilia*,
94 Gracillariidae) and their maple hosts (*Acer*, Sapindaceae) because previous studies have
95 identified multiple pairs of species that occur sympartrically with a great deal of overlap
96 in host use (Kumata 1982; Nakadai & Murakami 2015). With 124 species, the genus
97 *Acer* is one of the most species-rich groups of trees in the northern hemisphere,
98 particularly in the temperate regions of East Asia, eastern North America, and Europe
99 (van Gelderen *et al.* 1994). In temperate Japan, as many as 20 *Acer* species can occur in
100 a single location (Nakadai et al. 2014), which may host to up to 10 sympatric *Caloptilia*
101 species, as predicted from the geographic distribution of leaf cone moths (Nakadai &
102 Kawakita 2016). Twenty-eight *Acer* species occur in Japan (Nakadai et al. 2014), and a
103 previous study confirmed 14 *Acer*-feeding *Caloptilia* species; 13 of the 14 *Caloptilia*
104 species formed a monophyletic group, together with a *Toxicodendron*-feeding *Caloptilia*,
105 in the global *Caloptilia* phylogeny and thus are very closely related (Nakadai &
106 Kawakita 2016). We investigated the phenology (i.e., temporal niche) and parasitoid
107 community (i.e., enemy niche) of locally co-occurring, maple-feeding *Caloptilia* species
108 by sampling *Acer* leaves containing *Caloptilia* larvae every 2–3 weeks for a total of 13
109 sampling events, yielding 274 moth larvae. Species identification of moth larvae and
110 detection of internal parasitoids were based on a simultaneous barcoding
111 (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

We 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*

136 *crenata* and *Quercus crispula* above 600 m elevation and *Q. serrata*, *Q. salicina*, and
137 *Ilex pedunculosa* below 600 m (Ashiu Forest Research Station 2015). The average
138 annual temperature for 1981–2010 was 12.1°C, and the average annual rainfall for
139 1981–2010 was 2,257 mm (Ashiu Forest Research Station 2015).
140
141 *Acer species*
142 Fourteen *Acer* species have been confirmed in the Ashiu forest (Yasuda & Nagamasu
143 1995), but because four of these species are rare (*A. cissifolium*, *A. diabolicum*, *A.*
144 *palmatum*, and *A. tenuifolium*), we targeted the following ten species: *Acer amoenum*, *A.*
145 *carpinifolium*, *A. crataegifolium*, *A. japonicum*, *A. maximowiczianum*, *A. micranthum*, *A.*
146 *nipponicum* subsp. *nipponicum*, *A. pictum*, *A. rufinerve*, and *A. sieboldianum*.
147
148 *Sampling and species identification of leaf cone moths and the search for internal*
149 *parasitoid wasps*
150 *Caloptilia* moths feeding on *Acer* trees were sampled every 2–3 weeks by searching for
151 active larvae in leaf rolls (i.e., fourth or fifth instar) on the foliage of 10 *Acer* species
152 from mid-May to mid-November of 2015 (Fig. 3). We sampled only larvae in leaf rolls
153 because some leaf-mining larvae die early due to inconsistency between maternal
154 oviposition and larval performance, and host use cannot be assessed precisely in such
155 cases. This also enabled us to avoid sampling artifacts caused by the difficulty of
156 conducting an exhaustive search for leaf miners. To standardize sampling effort, we
157 sampled *Caloptilia* moths from branches with a diameter of 2.1 ± 4 mm from five
158 individuals of each tree species. After sampling, moths were preserved in 99.5% ethanol
159 and stored at –20°C.

Delimitation of species was based on sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene for all samples. We 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, we 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). We 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. We 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

184 µl including 1 µl of the template DNA amplified in the first PCR, 5 µl of Kapa HiFi
185 Hotstart ReadyMix, and 0.3 µM each of forward and reverse primers for the second
186 PCR. The protocol for the second PCR was 2 min at 95°C, followed by 12 cycles of 20
187 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.
188 Purification of products of the second PCR was also done with Agencourt AMPure XP.
189 PCR products were normalized and pooled. We normalized PCR products after
190 quantifying them with a Nano Drop ND-1000 (Thermo Fisher Scientific, Waltham, MA,
191 USA).
192
193 *Design of Caloptilia moth-specific annealing blocking primer*
194 The bodies of *Caloptilia* moths include both their own tissues and, occasionally, those
195 of internal parasitoids; the ratio of parasitoid tissue to moth tissue is very small. Thus,
196 the amplification of *Caloptilia* moth COI sequences must be suppressed to allow
197 detection of the sequences of internal parasitoids. We used the blocking primer
198 approach for this purpose (Vestheim & Jarman 2008). A blocking primer is a modified
199 primer that overlaps with one of the binding sites of the versatile primer. Blocking
200 primers are usually designed for only one species (Leray *et al.* 2015), so it is difficult to
201 apply them to multiple closely related species, as in this study, where we were unable to
202 identify the larvae morphologically. We designed the blocking primer
203 5'-CCCCCCHCTTTCATCWAAYATYGCHCATRGWGGWAGATC-3' to block
204 sequences of *Caloptilia* moths feeding on *Acer* based on known information about the
205 COI sequences of the moths and already-confirmed parasitoids (Table S4). The blocking
206 primer overlaps six bases with mlCOIlintF. The blocking primer was modified at the
207 3'-end with a Spacer C3 CPG (three hydrocarbons) to prevent elongation without

208 affecting its annealing properties (Vestheim & Jarman 2008). The performance of the
209 blocking primer for *Caloptilia* moths feeding on *Acer* was also tested (Supplemental
210 files 2).
211
212 *Analysis of sequencing data*
213 We extracted the reads, which fully contain both primer sequences, from the
214 output FastaQ files of Miseq using FastX Toolkit (ver. 0.0.13.2; Gordon & Hannon
215 2010). The remaining adapter, primer region, and the last nucleotide were trimmed from
216 the reads using FastX Toolkit. Additionally, the reads were quality filtered using Sickle
217 (ver. 1.33; Joshi & Fass 2011) with a minimum Sanger quality of 20 and a minimum
218 length of 100. Paired reads were assembled using FLASH (ver. 1.2.10; Magoč &
219 Salzberg 2011) with a minimum overlap of 20, and then transformed into Fasta format
220 using FastX Toolkit. A de novo chimera removal was performed using the UCHIME
221 algorithm (Edgar *et al.* 2011) of USEARCH (ver. 8.0.1623_i86linux64; Edgar 2010).
222 Duplicate and singleton reads were removed using USEARCH. We used the
223 UPARSE-OTU algorithm (Edgar 2013) of USEARCH for clustering OTUs with an
224 identity threshold of 97%. Thereafter, taxonomic assignment of individual OTUs was
225 performed by BLAST+ (ver. 2.2.29; Camacho *et al.* 2009).
226 Subsequently, non-target OTUs (organisms other than *Caloptilia* moths and
227 Hymenoptera) were removed, and OTUs were filtered with a sequence length of 313 ±
228 15 base pairs. We found some artifacts in the region overlapping with the blocking
229 primer, so after excluding that region, we clustered OTUs manually with an identity
230 threshold of 97% using MEGA (ver. 6.06; Tamura *et al.* 2013) once again. Additionally,
231 rare OTUs, whose total read number in the whole sample was under 100, were also

232 removed. The most abundant OTU of *Caloptilia* moth in each sample was used for the
233 identification of *Caloptilia* moths, and the OTUs of internal parasitoid wasp over 10
234 reads in each sample were defined as states of presence.
235
236 *Statistical analysis*
237 To assess niche use trends (i.e., niche partitioning or overlap), we calculated the degree
238 of niche overlap using the Pianka (Pianka 1973) and Czekanowski (Feinsinger *et al.*
239 1981) indices, which are common measures of niche overlap, and compared the
240 observed values with the expectations of null models. Null model-based analyses are
241 one of the most general approaches for assessing niche use trends (Gotelli 2001;
242 Albrecht & Gotelli 2001). As a well-known example, Lawlor (1980) tested the patterns
243 of niche use among 10 North American lizards using four types of null model (the
244 algorithms RA1–4) and confirmed significantly low overlap in resource use, suggesting
245 that interspecific competition plays an important role in constructing community
246 structure. We used the R package EcoSimR (Gotelli *et al*. 2013) for the enemy niche
247 and the program TimeOverlap, which is based on the algorithm ROSARIO
248 (Castro-Arellano *et al.* 2010), for the temporal niche. Because of the sequential and
249 continuous nature of time, a different kind of randomization model is required for the
250 temporal niche (Castro-Arellano *et al.* 2010). Samples that did not host parasitoid wasps
251 were excluded from the analysis of enemy niche. In all tests, the two-tailed probability
252 of the observed value was calculated based on 10,000 randomizations. We employed
253 Lawlor's (1980) algorithm RA3 for constructing null models. Additionally, we
254 calculated the standardized effect size (SES) as the observed test statistic minus the
255 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, we 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, we 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; we 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, we 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

303 partitioning might not be necessary for closely related herbivores to coexist on shared
304 hosts.

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

318 Alternatively, niche partitioning may genuinely be absent, and species
319 coexistence may be facilitated by other mechanisms. For example, shared natural
320 enemies enhance species coexistence, either if random predation eases interspecific
321 competition among herbivores (Strong *et al*. 1982) or if negative frequency-dependent
322 predation decreases the population of the more abundant species (Ishii & Shimada
323 2012). Strong (1982) found resource partitioning to be virtually absent among hispine
324 beetles (Chrysomelidae), which commonly coexist as adults in the rolled leaves of
325 *Heliconia* plants, suggesting that pressure from predators and parasites has a stronger
326 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, our 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. We 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

375 explained, improved knowledge of the mechanisms that enable such coexistence is
376 ultimately necessary to our understanding of the processes that generate diversity in
377 herbivorous insects.
378
379

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385

386 **References**

387 Albrecht M, Gotelli NJ (2001) Spatial and temporal niche partitioning in grassland ants.
388 *Oecologia*, **126**, 134–141.
389 Andrew RL, Bernatchez L, Bonin A *et al.* (2013) A road map for molecular ecology.
390 *Molecular ecology*, **22**, 2605–2626.
391 Field Science Education and Research Center, Kyoto University, Ashiu Forest Research
392 Station (2015) (in Japanese) http://fserc.kyoto-u.ac.jp/asiu/gaiyou/gaiyou2015.pdf
393 Bailey R, Schönrogge K, Cook JM *et al.* (2009) Host niches and defensive extended
394 phenotypes structure parasitoid wasp communities. *PLoS Biology*, **7**, e1000179
395 Benson WW (1978) Resource partitioning in passion vine butterflies. *Evolution*, **32**, 493–
396 518.
397 Borer ET, Seabloom EW, Tilman D (2012) Plant diversity controls arthropod biomass
398 and temporal stability. *Ecology Letters*, **15**, 1457–1464.

399 Brandon-Mong G-J, Gan H-M, Sing K-W *et al.* (2015) DNA metabarcoding of insects
400 and allies: an evaluation of primers and pipelines. *Bulletin of Entomological*
401 *Research*, 1–11.
402 Camacho C, Coulouris G, Avagyan V *et al.* (2009) BLAST+: architecture and
403 applications. *BMC bioinformatics*, **10**, 1.
404 Castro-Arellano I, Lacher TE, Willig MR, Rangel TF (2010) Assessment of
405 assemblage-wide temporal niche segregation using null models. *Methods in Ecology*
406 *and Evolution*, **1**, 311–318.
407 Condon M a, Scheffer SJ, Lewis ML *et al.* (2014) Lethal interactions between parasites
408 and prey increase niche diversity in a tropical community. *Science*, **343**, 1240–1244.
409 De Prins J, De Prins W (2015) Global taxonomic database of Gracillariidae
410 (Lepidoptera). Available at http://www.gracillariidae.net. (accessed 6 February
411 2016).
412 Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST.
413 *Bioinformatics*, **26**, 2460–2461.
414 Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon
415 reads. *Nature methods*, **10**, 996–998.
416 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves
417 sensitivity and speed of chimera detection. *Bioinformatics*, **27**, 2194–2200.
418 Farrell BDD (1998) "Inordinate fondness" explained: why are there so many beetles?
419 *Science*, **281**, 555–559.
420 Feder JL, Chilcote CA, Bush GL (1988) Genetic differentiation between sympatric host
421 races of the apple maggot fly *Rhagoletis pomonella*. *Nature* **336**, 61–64.
422 Feinsinger P, Spears EE, Poole RW (1981) A simple measure of niche breadth. *Ecology*,

62, 27–32.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology*, **3**, 294–299.

van Gelderen DM, de Jong PC, Oterdoom HJ (1994) Maples of the world. Timber Press, Portland, OR.

Germain RM, Weir JT, Gilbert B (2016) Species coexistence: macroevolutionary relationships and the contingency of historical interactions. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20160047.

Gordon A, Hannon GJ (2010) Fastx-toolkit. FASTQ/A short-reads preprocessing tools (unpublished) http://hannonlab. cshl. edu/fastx_toolkit.

Gotelli NJ (2001) Research frontiers in null model analysis. *Global Ecology and Biogeography*, **10**, 337–343.

Gotelli NJ, Ellison AM. (2013) EcoSimR 1.00. http://www.uvm.edu/~ngotelli/EcoSim/EcoSim.html

Hairston N, Smith F, Slobodkin L (1960) Community structure, population control, and competition. *The American Naturalist*, **94**, 421–425.

Hawthorne DJ, Via S (2001) Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature*, **412**, 904–907.

Hebert PDN, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 313–321.

Ishii Y, Shimada M (2012) Learning predator promotes coexistence of prey species in host-parasitoid systems. *Proceedings of the National Academy of Sciences of the*

United States of America, **109**, 5116–5120.

Imada Y, Kawakita A, Kato M (2011) Allopatric distribution and diversification without niche shift in a bryophyte-feeding basal moth lineage (Lepidoptera: Micropterigidae). *Proceedings of the Royal Society B: Biological Sciences*. **278**, 3026–3033.

Joshi NA, Fass JN (2011) Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33). *Available at* https://github.com/najoshi/sickle

Kartzinel TR, Chen P a., Coverdale TC *et al.* (2015) DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Sciences*, **112**, 8019–8024.

Kawahara AY, Plotkin D, Ohshima I *et al.* (2016) A molecular phylogeny and revised higher-level classification for the leaf-mining moth family Gracillariidae and its implications for larval host-use evolution. *Systematic Entomology*,

Kisel Y, McInnes L, Toomey NH, Orme CDL (2011). How diversification rates and diversity limits combine to create large-scale species–area relationships. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **366**, 2514-2525.

Kumata T. (1982) A taxonomic revision of the *Gracillaria* group occurring in Japan (Lepidoptera: Gracillariidae). Insecta Matsumurana **26,** 1–186.

Kumata T, Kobayashi S, Hirowatari T (2013) Gracillariidae. Pp. 91–155 in Nasu Y, Hirowatari T, Kisida Y, eds. The standard of moths in Japan IV. Gakken Education Publishing, Tokyo, Japan (in Japanese).

Lawlor LR (1980) Overlap, similarity, and competition coefficients. *Ecology*, **61**, 245–251.

471 Leray M, Meyer CP, Mills SC (2015) Metabarcoding dietary analysis of coral dwelling
472 predatory fish demonstrates the minor contribution of coral mutualists to their
473 highly partitioned, generalist diet. *PeerJ*, **3**, e1047.
474 Leray M, Yang JY, Meyer CP *et al.* (2013) A new versatile primer set targeting a short
475 fragment of the mitochondrial COI region for metabarcoding metazoan diversity:
476 application for characterizing coral reef fish gut contents. *Frontiers in zoology*, **10**,
477 34.
478 MacArthur R, Levins R (1967) The limiting similarity, convergence, and divergence of
479 coexisting species. *The American Naturalist*, **101**, 377–385.
480 Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve
481 genome assemblies. *Bioinformatics*, **27**, 2957–2963.
482 Malausa T, Bethenod M-T, Bontemps A *et al.* (2005) Assortative mating in sympatric
483 host races of the European corn borer. *Science* **308**, 258–260.
484 McCabe DJ, Hayes-Pontius EM, Canepa A, Berry KS, Levine BC (2012) Measuring
485 standardized effect size improves interpretation of biomonitoring studies and
486 facilitates meta-analysis. *Freshwater Science*, **31**, 800–812.
487 Nakadai R, Kawakita A (2016) Phylogenetic test of speciation by host shift in leaf cone
488 moths (*Caloptilia*) feeding on maples (*Acer*). *Ecology and Evolution*, **6**, 4958–4970.
489 Nakadai R, Murakami M (2015) Patterns of host utilisation by herbivore assemblages of
490 the genus *Caloptilia* (Lepidoptera; Gracillariidae) on congeneric maple tree (*Acer*)
491 species. *Ecological Entomology*, **40**, 14–21.
492 Nakadai R, Murakami M, Hirao T (2014) Effects of phylogeny, leaf traits, and the
493 altitudinal distribution of host plants on herbivore assemblages on congeneric *Acer*
494 species. *Oecologia*, **175**, 1237–1245.

495 Nosil P, Crespi BJ, Sandoval CP (2002) Host-plant adaptation drives the parallel
496 evolution of reproductive isolation. *Nature* **417**, 440–443.
497 Novotny V, Drozd P, Miller SE *et al.* (2006) Why are there so many species of
498 herbivorous insects in tropical rainforests? *Science*, **313**, 1115–1118.
499 Nyman T, Linder HP, Peña C, Malm T, Wahlberg N (2012) Climate-driven diversity
500 dynamics in plants and plant-feeding insects. *Ecology letters*, **15**, 889–898.
501 Nyman T, Vikberg V, Smith DR, Boeve J-L (2010) How common is ecological
502 speciation in plant-feeding insects? A "higher" Nematinae perspective. *BMC*
503 *Evolutionary Biology*, **10**, 266.
504 Pianka ER (1973) The structure of lizard communities. *Annual Review of Ecology and*
505 *Systematics*, 53–74.
506 Pompanon F, Deagle BE, Symondson WOC *et al.* (2012) Who is eating what: diet
507 assessment using next generation sequencing. *Molecular ecology*, **21**, 1931–1950.
508 Prinzing A, Powrie LW, Hennekens SM, Bartish IV, Ozinga WA (2016)
509 "High-co-occurrence genera": Weak but consistent relationships with global
510 richness, Niche partitioning, hybridization and decline. *Global Ecology and*
511 *Biogeography*, **25**, 55–64.
512 Rabosky DL (2009) Ecological limits and diversification rate: alternative paradigms to
513 explain the variation in species richness among clades and regions. *Ecology Letters*,
514 **12**, 735–743.
515 Rougerie R, Smith MA, Fernandez-Triana J *et al.* (2011) Molecular analysis of parasitoid
516 linkages (MAPL): gut contents of adult parasitoid wasps reveal larval host.
517 *Molecular Ecology*, **20**, 179–186.
518 Siemann E, Tilman D, Haarstad J, Ritchie M (1998) Experimental tests of the

519 dependence of arthropod diversity on plant diversity. *The American Naturalist*, **152**,
520 738–750.
521 Strong DR (1982) Harmonious coexistence of hispine beetles on *Heliconia* In
522 experimental and natural communities. *Ecology*, **63**, 1039–1049.
523 Strong DR, Lawton J H, Southwood SR (1984) Insects on plants. Community patterns
524 and mechanisms. Blackwell Scientific Publicatons.
525 Storch D, Keil P, Jetz W (2012) Universal species–area and endemics–area relationships
526 at continental scales. *Nature*, **488**, 78–81.
527 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular
528 evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, **30**,
529 2725–2729.
530 Tobias JA, Cornwallis CK, Derryberry EP *et al.* (2013) Species coexistence and the
531 dynamics of phenotypic evolution in adaptive radiation. *Nature*, **506**, 359–363.
532 Vestheim H, Jarman SN (2008) Blocking primers to enhance PCR amplification of rare
533 sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs.
534 *Frontiers in Zoology*, **5**, 12.
535 Wirta HK, Hebert PDN, Kaartinen R *et al.* (2014) Complementary molecular information
536 changes our perception of food web structure. *Proceedings of the National Academy*
537 *of Sciences of the United States of America*, **111**, 1885–1890.
538 Yamamoto S, Sota T (2009) Incipient allochronic speciation by climatic disruption of the
539 reproductive period. *Proceedings of the Royal Society B: Biological Sciences* **276**,
540 2711– 2719.
541 Yasuda S, Nagamasu H (1995) Flora of Ashiu, Japan. Contributions from the Biological
542 Laboratory, Kyoto University, **28**, 367– 486.

Data Accessibility

Obtained DNA sequences have been deposited in the DDBJ database under accession numbers DRX072707–DRX073080 (BioProject: PRJDB5369) and LC201483–LC201501.

Author Contributions

R.N and A.K conceived the study and wrote the manuscript.

R.N. designed the study and performed sampling, laboratory work, and data analysis.

552 **Legends**

553 Figure 1 Leaves rolled by leaf cone moths on Japanese maples; moth species are very

554 difficult to identify based solely on the morphology of rolled leaves and larvae. (a) *Acer*

555 *japonicum*, (b) *A. crataegifolium*, (c) *A. pictum*, (d) *A. palmatum*, (e) *A. rufinerve*, (f) *A.*

556 *maximowiczianum*.

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

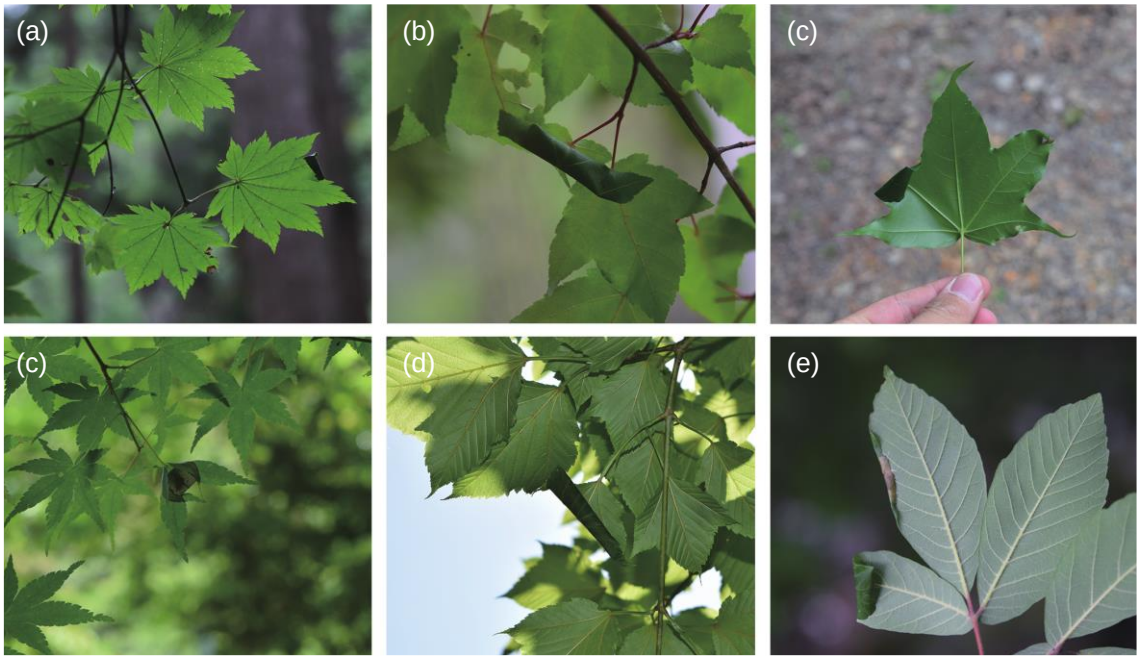
558 Figure 3 Phenology of 10 *Caloptilia* moths feeding on maples in this study area.

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

560 Figure 5 The relationship between the overlaps of temporal and enemy niches in the

561 Pianka index (Mantel $r = 0.41$, $P = 0.046$).

562 **Figure 1**



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565 **Figure 2**

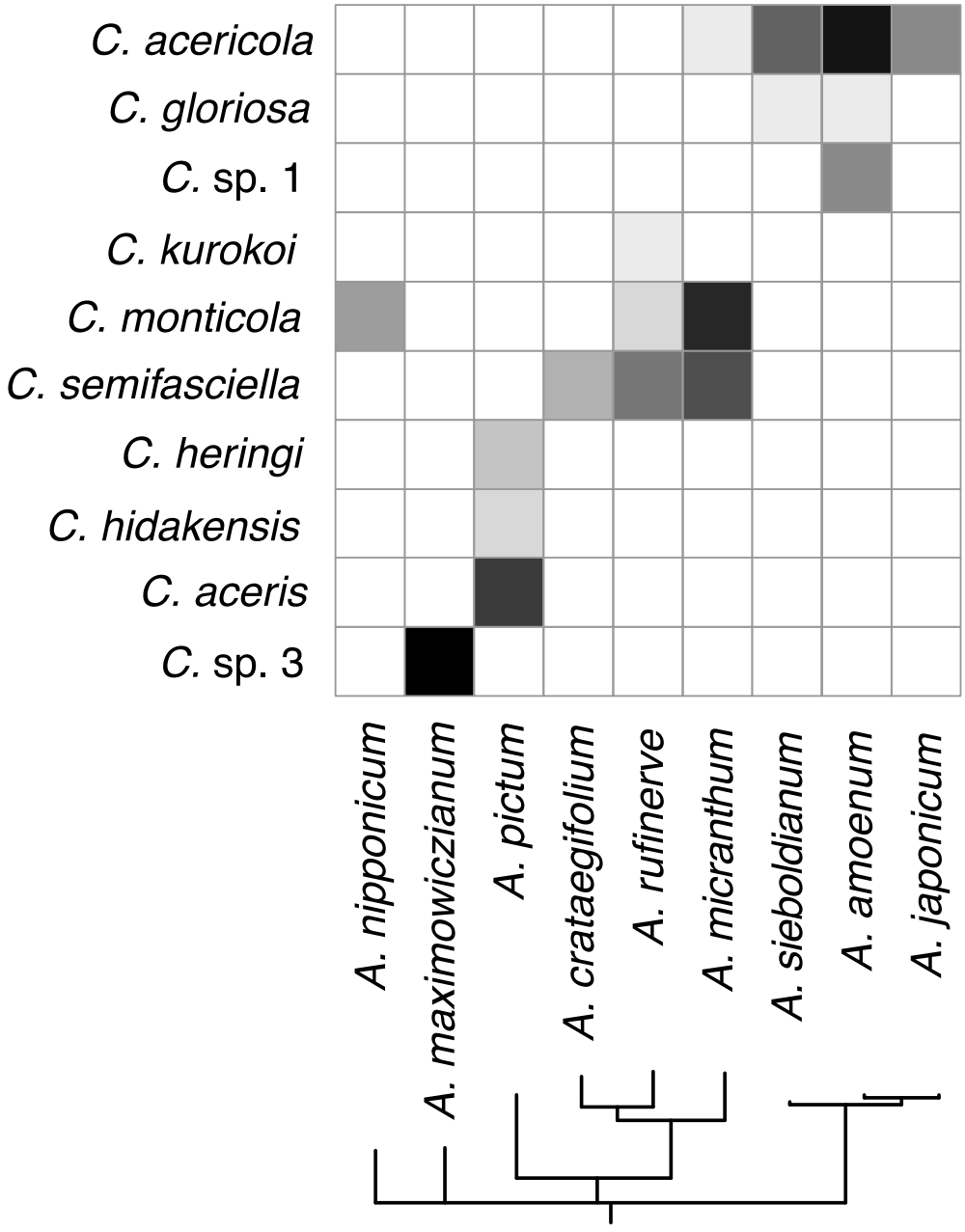

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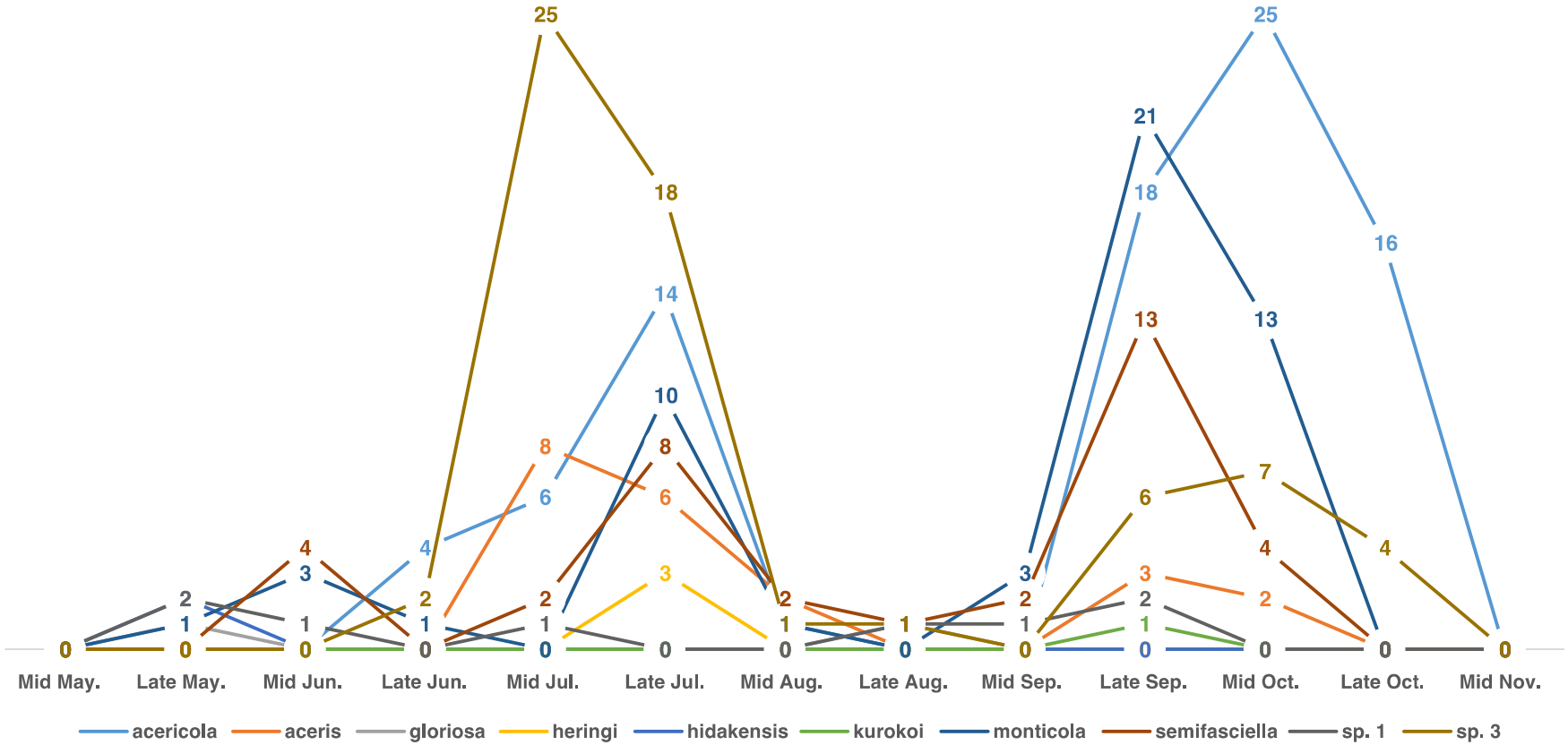
Figure 3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Late May.
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Mid Nov.
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heringi
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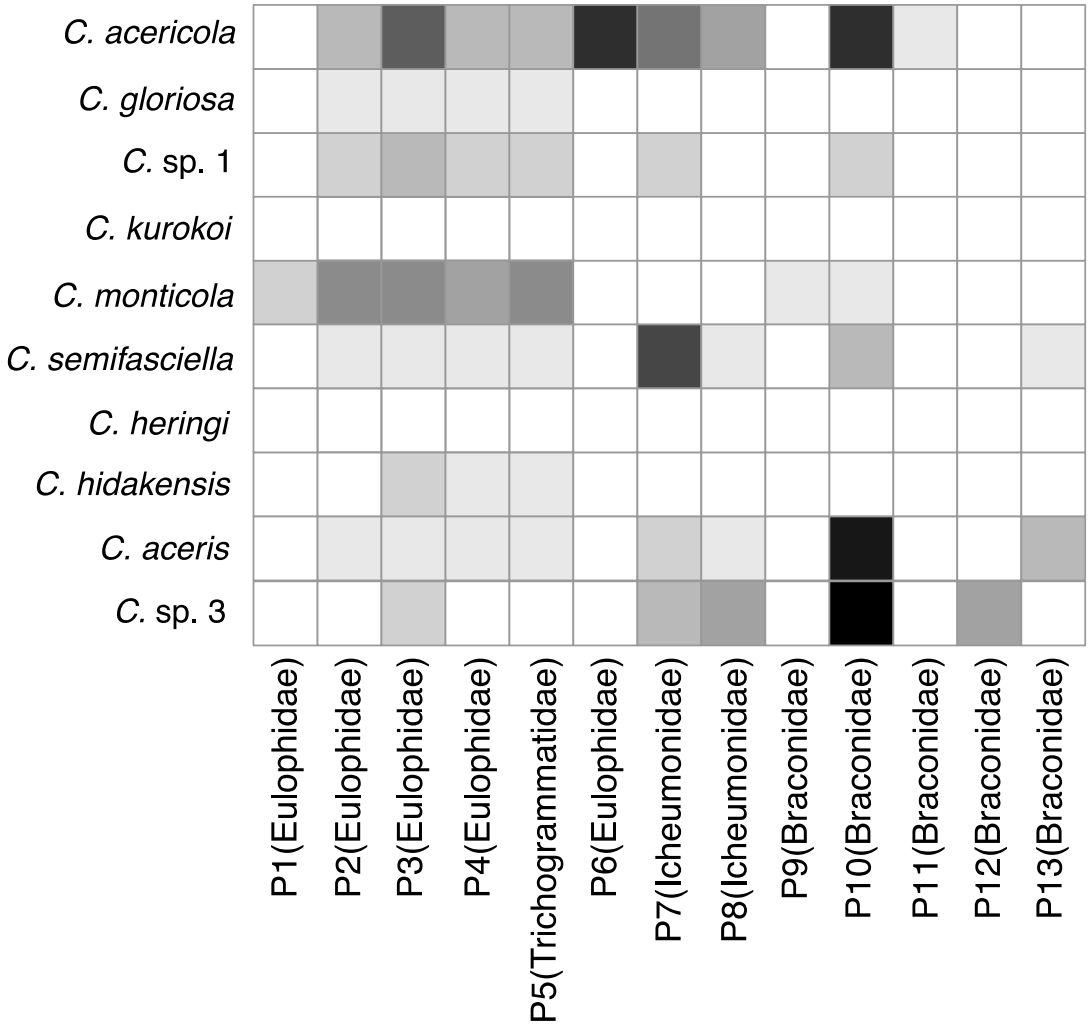
monticola
semifasciella
sp. 1
sp. 3

569 **Figure 4**



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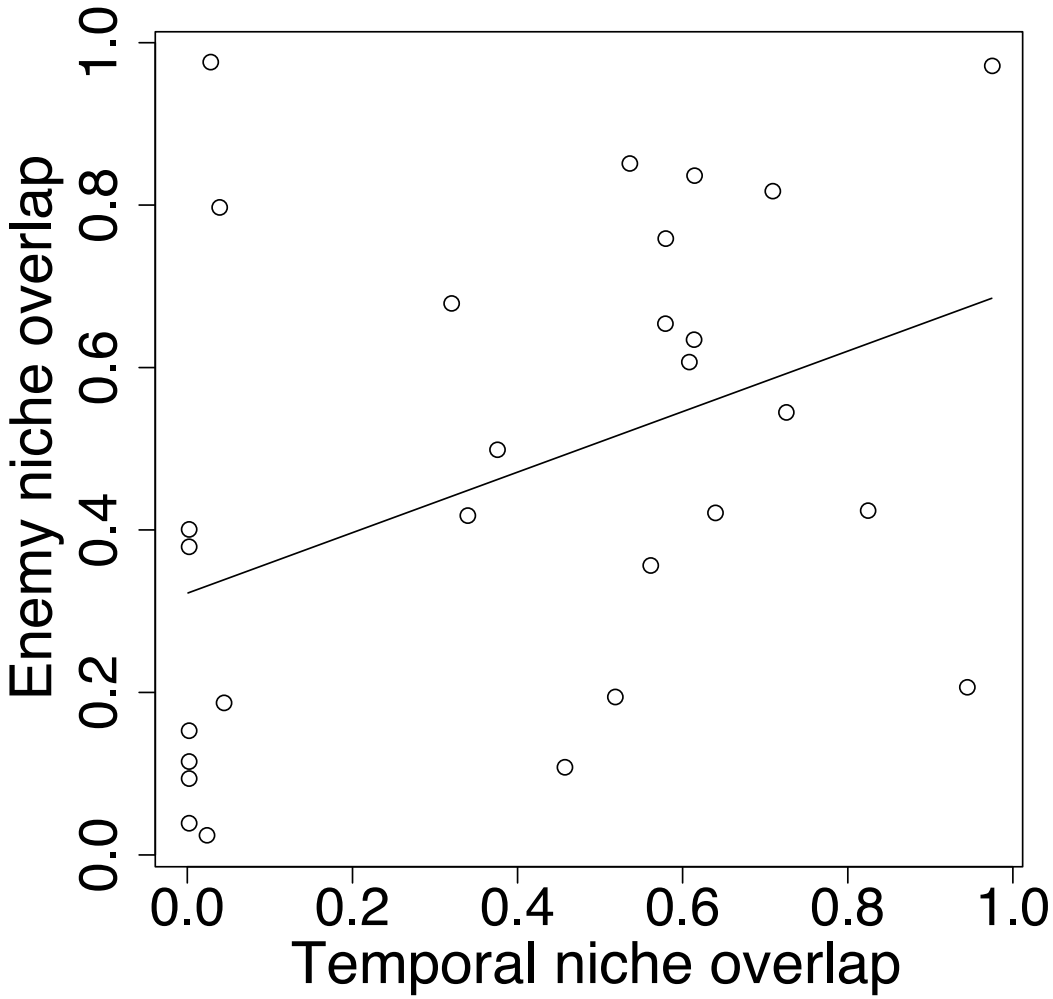
572 **Figure 5**



573

574 **Table 1** The results of comparison with the null model based on 10,000 randomizations.

575 These tests employed Lawlor's (1980) algorithm RA3, using the R package EcoSimR

576 (Gotelli *et al*. 2013) for enemy niche and the program TimeOverlap, based on the

577 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**

578 Bold letters indicate significant results in two-tailed randomization tests

579 **Table 2** Results of Mantel tests of the correlations among resource niche overlap,
580 temporal niche overlap, enemy niche overlap, and phylogenetic distance between
581 *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

582 Bold letters indicate significant results in Mantel tests

583 **Supporting information**

584 Table S1 OTUs used in the analysis.

585 Table S2 DDBJ accession numbers.

586 Table S3 Abundance and parasitoid rate for each species.

587 Table S4 Information on parasitoid wasps that were used for constructing the blocking

588 primer for *Caloptilia* moths.

589 Figure S1 Phylogeny of *Caloptilia* moths and their related groups. The phylogeny was

590 constructed by the maximum-likelihood method using four genomic regions (COI,

591 ArgK, CAD, and EF-1a) of 71 species (Nakadai & Kawakita 2016). Red indicates the

592 lineage of *Caloptilia* moths associated with maples.

593 Supplementary file 1 Fasta-formatted representative sequences of each OTU, created by

594 the UPARSE pipeline.

595 Supplementary file 2 Performance assessment of the blocking primer created in this

596 study for *Caloptilia* moths associated with maples.

597

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