

Research Article

Dominant Occurrence of Cleistogamous Flowers of *Lamium amplexicaule* in relation to the Nearby Presence of an Alien Congener *L. purpureum* (Lamiaceae)

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Received 21 March 2013; Accepted 15 April 2013

Academic Editors: P. Ferrandis and P. Rey

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Here we document a novel phenomenon that, based on field observations in central Japan, cleistogamous flowers (or closed flowers) of an annual herb *Lamium amplexicaule* were dominantly expressed near an alien congener *L. purpureum*. The proportion of cleistogamous flowers in an individual *L. amplexicaule* increased with the frequency of *L. purpureum* occurring in the same patches but did not increase with the total density of *Lamium* plants and their own size. To confirm the consistency of the effect of the coexisting alien species, we assessed the cleistogamous frequency at the patch level for three other populations. In these populations as well, the proportion of *L. amplexicaule* producing cleistogamous flowers increased with the frequency of *L. purpureum*. Our transplant experiment at one site found no effect of the nearby presence of *L. purpureum* on the seed set of *L. amplexicaule* and therefore did not support the hypothesis that the adverse effect on the reproduction via interspecific pollination favored cleistogamous flowers that accepted no external pollen. Further studies must be conducted to examine the negative interactions between the related species before and after seed development.

1. Introduction

A cleistogamous (CL) breeding system is a selfing strategy that evolved independently in diverse plant taxa [1]. Cleistogamous flowers possess a reduced structure of nectar or petals and are obligatory self-fertilized without being open, whereas chasmogamous (CH) flowers can be cross-pollinated by another individual with an ordinal flower structure [2]. The cleistogamous breeding system has been regarded as a bet-hedging strategy for unpredictable environments [3–5], in which selfing by producing cleistogamous flowers can ensure reproductive success even in the absence of pollinators [6]. As another function of CL flowers, cost saving in producing flowers has been considered [5, 7] because the CL flowers are usually smaller and require less biomass than CH flowers. Empirically, smaller plants have been known to produce more

CL flowers [8, 9]. Consequently, a few different hypotheses have been advanced for the function of the CL flowers. All have been in intraspecific contexts.

The cleistogamous breeding system can be favored by interspecific interaction although it has been discussed in few reports to date [10–12]. For instance, deposition of heterospecific pollens on stigmas has been known to reduce fruit set in some plants [11, 13, 14]. Such an interspecific interaction in the pollination is a typical mechanism of the reproductive interference, which refers to any sexual interspecific interactions that reduce the reproductive success of either/both of species [15, 16]. This reproductive interference can be an intense force that drives either species to extinction because it acts in the manner of the positive feedback; less abundant species suffer more intense interfering effect in the next generation and therefore reduce their abundance until they

goes to extinction [15]. Reproductive interference by either of them is expected to exclude the other if there sympatrically exist two species that can exert the reproductive interference mutually. However, the reproductive interference can be canceled by switching from CH flowers to CL flowers that received no external pollen. No examination of such an advantage of CL flowers has ever been described in the literature.

Lamium amplexicaule L. (Lamiaceae), a cleistogamous species that is naturally distributed throughout Japan, produces cleistogamous flowers [17], and an individual plant develops either or both CL and CH flowers. The congeneric species, *L. purpureum* L., is an alien species naturalized throughout Japan that produces no CL flowers. This pair of *Lamium* species might present advantages for examining the effects of coexistence with alien species on the expression of CL flowers because the two species coexist in a patch scale. Therefore, we can investigate interactions in the field directly. *Lamium amplexicaule* is expected to increase CL flowers to escape from reproductive interference if the coexistence of these two species in a patch brings about reproductive interference to CH flowers of *L. amplexicaule* individuals.

First, we showed that the proportion of CH flowers in *L. amplexicaule* individuals decreased continuously with increasing frequency of *L. purpureum* based on detailed field surveys in a natural population located in Japan. This detailed survey also showed that the size of *L. amplexicaule* and the total density of the two *Lamium* species had no marked effect. Subsequently, to test the universality of the effect of *L. purpureum*, we conducted additional patch-level field surveys at three different sites. Results show that the frequency of *L. amplexicaule* individuals with CH flower decreased continuously with increasing frequency of *L. purpureum* in the patches. Finally, to examine the existence of reproductive interference, we performed a field experiment in which *L. amplexicaule* individuals were transplanted into *L. purpureum* patches.

2. Materials and Methods

2.1. Study Species and Sites. The present study specifically addresses the cleistogamy in *Lamium amplexicaule*, a native plant distributed throughout Japan except in the northernmost parts. This annual plant, which usually inhabits cultivated land, wasteland, and roadsides in lowland areas, consists of one to several stems with opposite leaves [18, 19]. Flowering usually starts in March and ends in May in western Japan. This species has a cleistogamous breeding system. Sometimes both the CL and CH flowers occur within an individual. The CL flowers of *L. amplexicaule* can produce seeds without being open and thus have no opportunity for outcrossing. This type of CL flowers thus refers to “true” cleistogamy according to a previous review [2].

Lamium purpureum, an alien congener, was first discovered at the end of the 19th century. It is now naturalized across Japan [20]. This alien species inhabits cultivated lands, wastelands, and roadsides in lowland areas [19]: the habitats

overlap mostly with that of the native species. The life-cycle and phenology of *L. purpureum* is also similar to *L. amplexicaule*, but it produces no cleistogamous flowers. Both *Lamium* species have the same chromosome number ($2n = 18$) [21], but no hybrid has been recorded to date.

We conducted field studies at four sites. The first was a roadside in the North Campus of Kyoto University, Kyoto ($35^{\circ}01'54''\text{N}$, $135^{\circ}47'14''\text{E}$; referred to hereinafter as “Kyoto”). This long and narrow site has patch sizes of weeds including *Lamium* species smaller than 1.0 m radius. The second site was a grassland near a plantation in Tsurumi Park, Osaka ($34^{\circ}42'43''\text{N}$, $135^{\circ}34'25''\text{E}$; “Tsurumi”), located in a well-developed urban area. The third site, a roadside area, was surrounded by rice fields at Omihachiman, Shiga ($35^{\circ}10'05''\text{N}$, $136^{\circ}7'32''\text{E}$; “Omi”). The fourth site was located in a suburb of a rural town, Uchiko, in Ehime Prefecture, Shikoku Island ($33^{\circ}32'54''\text{N}$, $132^{\circ}39'0''\text{E}$; “Uchiko”), which is a sunny farmland site adjacent to a road, a vegetable field, and a fruit garden with sparse tree cover. At these latter three sites, *Lamium* species formed patchy vegetation. The patch size was approximately 1.5 m radius.

2.2. Proportion of CH Flowers at an Individual Level. At the Kyoto site, we conducted a detailed field survey of the frequency of CH flowers on each individual and its relation with the frequency of *L. purpureum*, the total number of *Lamium* plants in each patch, and the plant size (stem length) of *L. amplexicaule*. The *L. purpureum* frequency was intended to reflect the intensity of the reproductive interference because it depends on the relative frequency of two species [15]. The total number of *Lamium* plants was presumed to represent the habitat quality and the intraspecific and interspecific resource competition between the two species. The plant size was used as an index of the physiological state of *L. amplexicaule*. We arbitrarily chose patches and counted flowering individuals of *L. amplexicaule* and *L. purpureum* within each patch. Subsequently, we collected an individual of *L. amplexicaule* from the center of each patch, counted the CH and CL flowers, and measured the length of the longest stem (referred to hereinafter as stem length) with a scale. Small buds of collected individuals were identified either as CH or CL flowers under a binocular microscope in the laboratory. The number of plants collected was 15 per study on April 3, 13, and 25, 2010.

2.3. Proportion of CH Flowers at Patch Level. In additional field surveys at three sites (Tsurumi, Omi, and Uchiko), we sought to confirm the consistency of the tendency that the CH flower decreased with increasing *L. purpureum* frequency. To cover the multiple populations, we estimated the frequency of CH flowers at the patch level, namely, the proportion of individuals with one or more CH flowers in all individuals of *L. amplexicaule* in a patch. We arbitrarily chose patches and counted flowering individuals of *L. amplexicaule* and *L. purpureum* in each patch. Then we checked all individuals of *L. amplexicaule* to ascertain whether they had one or more CH flower(s). All surveys were conducted during the peak

of flowering season of the two *Lamium* species (from mid-March to late April). At the Tsurumi site, surveys were done three times on April 4, 2006, April 6, 2006, and March 17, 2009, and 21, 30, and 43 patches were surveyed, respectively. At the Omi site, 26 patches were surveyed on April 17, 2006. At the Uchiko site, 19 patches were surveyed on March 23, 2007.

2.4. Transplant Experiment of *Lamium amplexicaule*. We conducted a transplant experiment of *L. amplexicaule* individuals to patches of *L. purpureum* to test the effect of the latter species on the reproductive success of the former. The transplantation was conducted on April 3, 2006 at the Tsurumi site. In this experiment, we used potted plants with an aim to separate effects of resource competition via soils from interfering effects during reproduction. We dug 19 *L. amplexicaule* individuals up and set them into plastic pots (90 mm diameter, 90 mm height). All blooming flowers and fruits were removed with precision scissors, and buds of CH flowers were left intact. Of them, 15 pots (treatment) were arbitrarily chosen; each was buried individually at the center of the patch of *L. purpureum* so that the top of the pot matched the ground surface. The diameter of each *L. purpureum* patch was greater than 1 m. The remaining four pots (control) were buried outside patches at places more than 5 m distant from any *L. purpureum* patch. During the five days following transplantation, pollinator insects were allowed to access these flowers freely. Then, we covered all CH flowers during the five days with thin papers to inhibit subsequent pollination and seed loss and to mark flowers that opened during the five days. CL flowers and buds were removed. The developed and undeveloped seeds were counted in the laboratory on April 27, 2006.

2.5. Statistical Analysis. All datasets in this study exhibited similar structures. The response variables were binomial: whether each flower was CH or not in the individual-level analysis for the Kyoto site, whether each had CH flower(s) or not in the patch level analyses for the other sites, and whether each seed developed or not in the *L. amplexicaule* transplants into *L. purpureum* patches. For the dataset from the Kyoto site, the explanatory variables were the frequency of *L. purpureum*, the total number of the two *Lamium* species in each patch, and the stem length. The *L. purpureum* frequency was defined as the proportion of the *L. purpureum* individuals to the total number of individuals of the two *Lamium* species in each patch. For the dataset from the other three sites, the explanatory variables were the frequency of *L. purpureum* and the total number of individuals of the two *Lamium* species in each patch. For the dataset from transplant experiment at the Tsurumi site, the explanatory variable was the treatment or control (i.e., whether pots were put within or outside of patches of *L. purpureum*).

There were clustering factors that should be assigned to random effects such as individuals or patches, dates, or sites. Although they were not a main concern in this study, it was necessary to regulate the interblock (sites, dates, patches, and individuals) variances for fair analyses [22]. The two or three

levels of random effect were nested hierarchically for the field data. For the dataset from the Kyoto site, random effects were two levels: individuals within dates. For the datasets from the other sites, there were three levels of random effects: individuals within dates, dates within sites. For the dataset from the transplant experiment, the clustering factor of individuals was regarded as a random effect.

We used R version 2.15.1 [23] for all statistical analyses. The lmer function implemented in R was used for GLMM analyses. Significance of fixed effects was tested using Wald tests.

3. Results

3.1. Proportion of CH Flowers at Individual Level. At the Kyoto site, the proportion of CH flowers in an individual *L. amplexicaule* plant decreased concomitantly with increasing frequency of the *L. purpureum* individuals (Figure 1). The effect was significant (GLMM and Wald test, coefficient \pm SE = -9.09 ± 2.38 , $Z = -3.8$, and $P < 0.001$). Regarding other explanatory variables, the total number of individuals of the two *Lamium* species and the stem length of *L. amplexicaule* showed no significant effect on the individual-level CH proportion of *L. amplexicaule* (total number; GLMM and Wald test, coefficient \pm SE = 0.003 ± 0.004 , $Z = 0.8$, and $P = 0.43$; Figure 1) and the stem length (stem length; GLMM and Wald test, coefficient \pm SE = 0.064 ± 0.040 , $Z = -1.6$, and $P = 0.12$; Figure 1).

3.2. Proportion of CH Flowers at Patch Level. At all three sites, the proportion of *L. amplexicaule* with CH flowers decreased concomitantly with increasing frequency of *L. purpureum* (Figure 2). A significant negative effect of the frequency of *L. purpureum* was found (GLMM and Wald test, coefficient \pm SE = -3.01 ± 0.60 , $Z = -5.0$, and $P < 0.0001$). The effect of the total number of *Lamium* plants was not significant (GLMM and Wald test, coefficient \pm SE = 0.0001 ± 0.0011 , $Z = 0.15$, and $P = 0.88$; Figure 2).

3.3. Transplant Experiment of *Lamium amplexicaule*. Seed sets of *L. amplexicaule* were consistently high irrespective of the treatment (Figure 3). Statistical analyses supported this tendency: no significant effect of the transplanting treatment on the seed set was found (GLMM and Wald test, coefficient \pm SE = -0.034 ± 0.349 , $Z = -0.090$, and $P = 0.928$; Figure 3).

4. Discussion

Our field surveys revealed that the proportion of CH flowers in *L. amplexicaule* declined markedly with the frequency of an alien congener occurring in the same patches. At the Kyoto site, the proportion of CH flowers in an individual *L. amplexicaule* decreased concomitantly with increasing frequency of *L. purpureum*. This tendency was also found in the additional patch-level surveys at three different study sites. This field evidence suggests that interactions with the

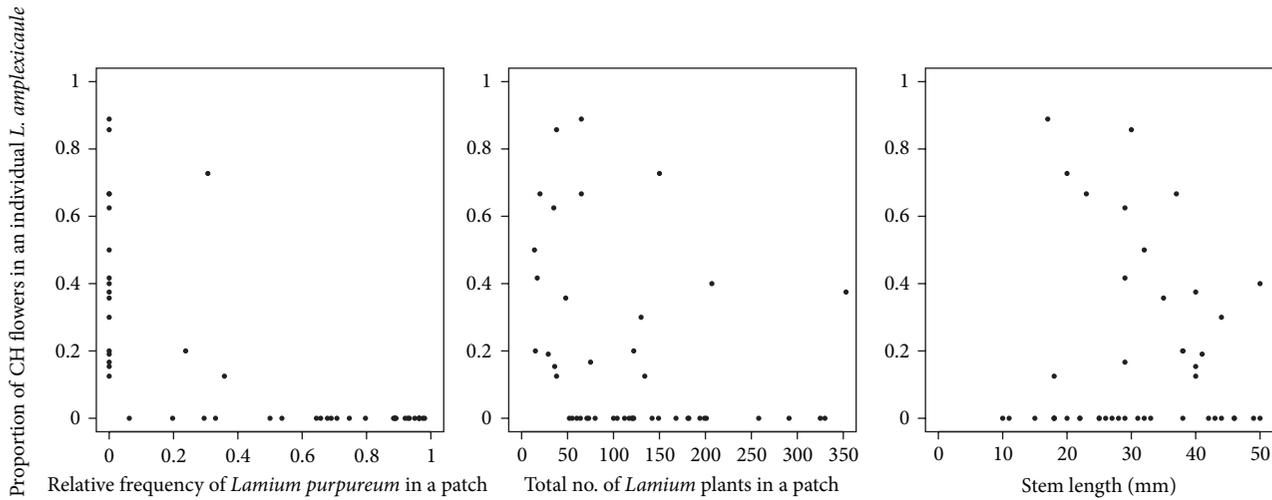


FIGURE 1: Relations between the proportion of CH flowers in an individual *L. amplexicaule* and the frequency of *L. purpureum* in a patch (left), the total number of *Lamium* plants (center), and the stem length (mm) of *L. amplexicaule* (right) at the Kyoto site. Data collected during different dates are pooled in this figure, although the study dates were incorporated as a random effect in the statistical analysis (see the text).

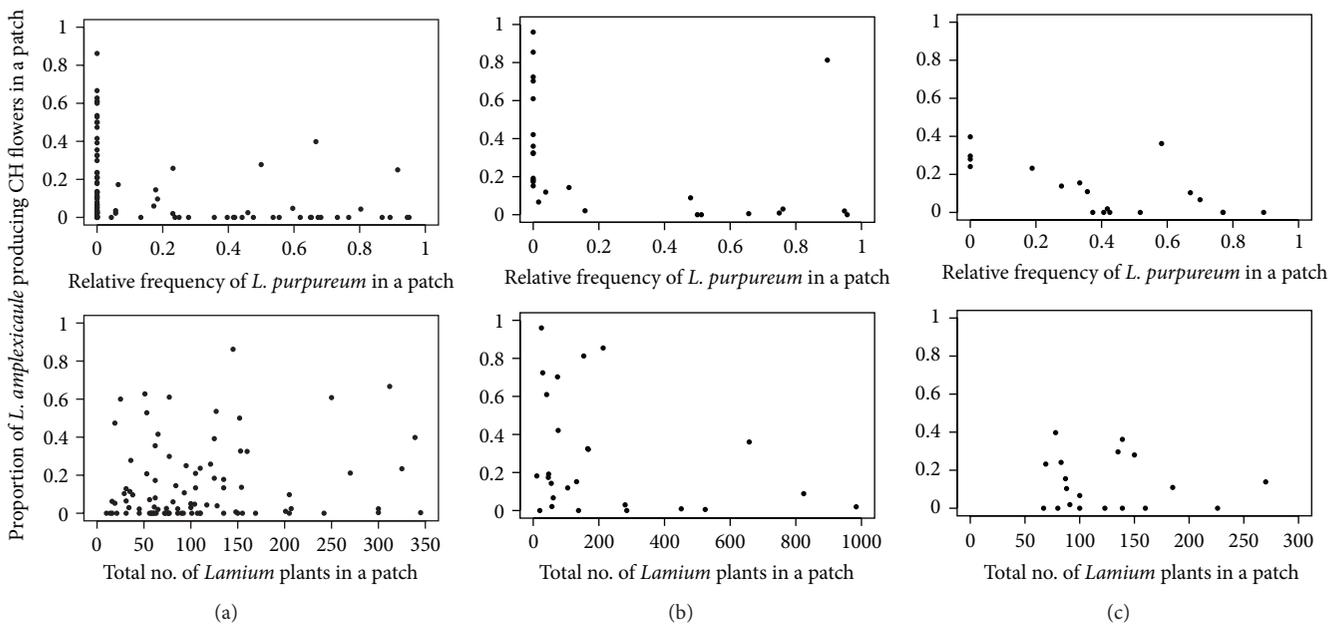


FIGURE 2: Relations between the proportion of *L. amplexicaule* producing CH flowers and the frequency of *L. purpureum* in a patch (top) and the total number of *Lamium* plants (bottom) at the three sites: (a) Tsurumi, (b) Omi, and (c) Uchiko. Data collected from different years and dates are pooled for each study site, although the study dates and sites were incorporated as random effects in statistical analyses (see the text).

alien congener can reduce the proportion of CH flowers in the native species.

The proportion of CH flowers among *L. amplexicaule* was not affected markedly by the total number of individuals of the two *Lamium* species or the size of *L. amplexicaule* plants. The high density of the individuals of the same and alien species can affect the expression of CL flowers in at least two pathways: the resource competition and the consequent size reduction. High density of individuals tends to degrade the

light condition and the nutrient salts uptakes. Indeed, the past study showed that the proportion of CH flowers of *L. amplexicaule* becomes small under low light intensity [17]. Furthermore, the intraspecific and interspecific competition in the high-density patches might engender the size reduction of plants [24, 25]. Such a size reduction can be another trigger to increase the CH flowers as suggested by some studies [8, 9]. Consequently, these mechanisms can be generally regarded as reducing the proportion of CH flowers in cleistogamous

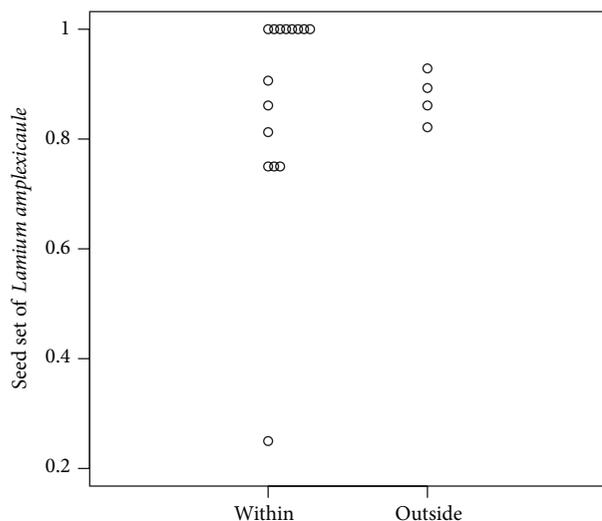


FIGURE 3: Seed set of *L. amplexicaule* transplanted within (treatment) or outside of the patches of *L. purpureum* (control). Seed set was defined as the proportion of developed seeds in the total number of seeds produced by an individual plant. A dot represents an individual of *L. amplexicaule*. The same values of seed sets are stacked in a row.

species. However, our field surveys revealed no evidence of the effects of the total number of *Lamium* species or the stem length on the proportion of CH flowers in *L. amplexicaule*. Effects of the density and the plant size were probably weak relative to the effect of the coexistence with the alien congener, *L. purpureum*.

Our transplant experiment found no effects of nearby presence of *L. purpureum* on the seed set of *L. amplexicaule*. The deposition of heterospecific pollen has been regarded as a pivotal cause of reproductive interference [14, 26], but we did not study the pollinator fauna and behavior at this site. At the Kyoto site, a common long-tongued bee *Tetralonia nipponensis* was observed to visit *L. amplexicaule* and *L. purpureum* (Y. Sato, pers. obs.). Thus, some opportunities for interspecific pollination exist between these two species. The fauna and behavior of pollinators for these plants remain an issue for future study.

Even if the reduction of seed set via the interspecific pollination is not apparent at the stage of seed production, some possible mechanisms remain which might alter the reproductive tactics of *L. amplexicaule*. First, allelochemicals via air or soils might inhibit the development of CH flowers in *L. amplexicaule*. Second, because this study did not examine the processes after the seed set, it is possible that the viability of *L. amplexicaule* offspring decreases after the deposition of heterospecific pollen. The existence of these factors must be tested by exclusion of *L. purpureum*, reciprocal transplant experiments, and artificial pollination experiments. Third, the possibility exists that the reproductive tactics of *L. amplexicaule* in our study sites have already been altered through interactions with the alien congener. For example, CH flowers in these sites might produce seeds mainly by selfing, irrespective of morphology, although these hypotheses are not mutually exclusive.

Overall, this paper described the potential effects of inter-specific interaction on the cleistogamous breeding system. Furthermore, the dominant occurrence of CL flowers had little relation to the plant density or the size of cleistogamous species. Our field evidence provides the first step in the examination of the role of cleistogamy to mitigate interfering interactions between related species. Additional experimental studies are necessary to elucidate causal mechanisms for this phenomenon.

Acknowledgments

The authors thank two anonymous reviewers for helpful comments on the paper. This work was partly supported by a Grant-in-Aid for Challenging Exploratory Research (no. 20657005 to Takayoshi Nishida) and for Young Scientists (B) (no. 19770023 to Koh-Ichi Takakura) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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