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Thrips as a supplementary pollinator in an orchid with granular pollinia: is this mutualism?

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The very high floral diversity of Orchidaceae has often been attributed to the intimate relationships between orchids and their pollinators, with 60–70% of all orchid species being dependent on discrete pollinator lineages or even single species (Cozzolino and Widmer 2005). Bees pollinate nearly 60% of orchid species and their secondary important pollinators include wasps (9.9%), flies (6.9%), birds (5.5%), settling moths (3.6%), and sphingid moths (3.1%) (Peter 2009). Despite the long history of orchid pollination studies, the identity and specificity of most orchid pollinators remain inadequately explored, and even recent studies have reported novel interactions in orchid pollination systems (Micheneau et al. 2010).

Among the many potential pollinators of orchid species, thrips remain one of the most controversial ones. These tiny insects possess piercing-sucking mouthparts and are often observed on flowers, where they feed on nectar, pollen, or cell content in various plant tissues (Moog et al. 2002). Darwin (1862) hypothesized that thrips may pollinate certain orchid species by crawling over the flowers, thereby transferring pollinia. However, until now, the contribution of thrips to orchid pollination has never been investigated experimentally.

We have occasionally observed that thrips often inhabit *Epipactis thunbergii* flowers and that these thrips carry dozens of the orchid's pollen grains. These observations led us to hypothesize that thrips function as a supplemental pollinator of *E. thunbergii*, while it is mainly pollinated by the hoverflies (Sugiura 1996). Therefore, we attempted to investigate whether thrips contribute to fruit and seed set in *E. thunbergii* by observing the behaviors of thrips and conducting differential pollinator-exclusion experiments. The study was conducted in a sunny, damp area in Sumada, Sanda City, Hyogo Prefecture, Japan.

From 2013 to 2015, we documented the behavior of *E. thunbergii* floral visitors from early to late June, a period that coincides with the peak flowering period of *E. thunbergii* by walking around the study plant populations or sitting near the flowering individuals. During the peak of diurnal insect activity (09:00 to 16:00 h), the behaviors of individual floral visitors were carefully observed for a total of ~20 h. In addition, floral visitors other than thrips were captured for identification immediately after they had left a flower. However, because it was somewhat difficult to observe the behavior of thrips under natural conditions, we often removed the sepals of the orchid flowers to facilitate observation. During the study period, we captured 66 adult thrips for the species-level identification. In the laboratory, we prepared microscope specimen slides using conventional procedures (Okajima 2006).

During June 2015, we also conducted pollination-exclusion experiments to determine the role of thrips in the pollination of *E. thunbergii*. The experiments included five treatments. For the pollinator-exclusion treatment, each bud was completely covered using paper bags (Daiichi Vinyl, Fukui, Japan; six inflorescences, 47 flowers). For the thrips-only treatment, each bud was completely covered using polyethylene netting (pore size 1.8 mm; Howdy, Tokyo, Japan) to exclude all floral visitors, except thrips (six inflorescences, 71 flowers). For the manual-autogamy treatment, the flowers were artificially self-pollinated by transferring pollinia to the stigmas of the same flowers (five inflorescences, 10 flowers). For the manual-allogamy treatment, the flowers were artificially cross-pollinated by transferring pollinia to the stigmas of different flowers (five inflorescences, 10 flowers). For the open-pollinated treatment, fruit set was monitored under natural conditions (12 inflorescences, 106 flowers). Then, in mid-August 2015, all mature but undehisced fruits were picked from the artificially self- and cross-pollinated individuals. After the fruits (capsules) were silica dried, the mass of the dry seeds from each capsule was measured to the nearest 0.1 mg. The seeds from all capsules on each plant were mixed, and 100 randomly selected seeds from each plant were examined under a dissecting microscope to assess the number of seeds with an embryo. The effects of pollination treatment on fruit set, seed mass, and proportion of seeds with an embryo were tested using ANOVA, followed by Fisher's multiple comparisons test.

In agreement with previous studies (Sugiura 1996), we found that *E. thunbergii* flowers were frequently visited by several species of hoverflies, including *Sphaerophoria macrogaster*, *S. indiana*, *Melanostoma orientale*, and *M. scalare*, and that *S. macrogaster* and *M. orientale* were the primary pollinators of *E. thunbergii* at the study site (Table 1; Fig. 1a). These hoverflies usually oriented themselves to a flower by hovering for a few seconds, landed on the distal part of the floral lip (i.e., epichile), and then walked toward the basal part of the floral lip (hypochile) to feed on nectar in the hypochile. After feeding on nectar, the flies retreated to the epichile but were thrown onto the stigma by the sudden upward movement of the epichile. As reported by Sugiura (1996), during this process, pollinia were deposited onto the dorsal thorax of each fly, or if the flies were already carrying pollinia on their thorax, pollinia were transferred to the stigmatic surface.

We also observed that these hoverflies occasionally oviposited on and near the flowers of *E. thunbergii*, where aphids were not found, as noted by Sugiura (1996). Female hoverflies, the pollinator of *Epipactis veratrifolia*, have similarly been reported to oviposit on the flowers as oviposition sites (Stökl et al. 2011). Stökl et al. (2011) also reported that *E. veratrifolia* flowers produce α- and β-pinene, β-myrcene, and β-phellandrene, which mimic alarm pheromones released by several aphid species, and that these compounds induce oviposition behavior in female hoverflies. Therefore, it is possible that a similar mimicry system is at play in *E. thunbergii.* Chemical analyses of floral volatiles may help to reveal the mechanism by which hoverflies are attracted as pollinators.

In the present study, we also noticed that some *E. thunbergii* flowers were not visited by hoverflies and that often, even if hoverflies visited the flower, pollinia were neither removed nor deposited. Indeed, the deposition of pollinia was only possible when the hoverflies entered deep into the lip. However, in most cases, the hoverflies only visited the distal part of the lip. Furthermore, even when the hoverflies entered deep into the lip, the insects were not always successfully manipulated by the epichile to make contact with the pollinia or stigma. Overall, while we could observe the detailed behavior of the hoverflies visiting *Epipacits* flowers 47 times, only 11 hoverflies were involved in pollen removal and/or pollen deposition. Instead, in the flowers from which the hoverflies failed to remove pollinia, we frequently observed pollinia to be spread by the crawling thrips (Fig. 1b). Of the 66 thrips specimens captured, *Frankliniella intonsa*, known as the common flower-living thrip, was the most frequent floral visitor of the investigated *E. thunbergii* population (Table 1; Kirk 1985).

Table 1. Composition of pollinators in *Epipactis thunbergii.*

Order and species	Number of individuals carrying pollen grains observed on the flowers
Diptera	
Sphaerophoria macrogaster	18
Melanostoma orientale	10
Melanostoma scalare	3
Sphaerophoria indiana	2
Hymenoptera	
Lasioglossum spp.	3
Thysanoptera†	
Frankliniella intonsa	56
Haplothrips aculeatus	5
Haplothrips brevitubus	3
Thrips sp.	2

†The number of thrips individuals carrying pollen grains observed on the flowers is underestimated because (1) we could not capture all the thrips carrying pollinia and (2) could identify adult thrips only, due to difficult identification of the larval stage.

Our pollinator-exclusion experiments clearly demonstrated that at least some *E. thunbergii* flowers developed fruits when non-thrips visitors were excluded. Our result also showed that fruit set was much lower when all visitors were excluded (pollinator-exclusion treatment) than when only the non-thrips visitors were excluded (thrips-only treatment; Table 2). Therefore, the present study clearly demonstrates that thrips can contribute to the pollination of *E. thunbergii.* The low fruit set (only one inflorescence developed fruits) in the pollinator-exclusion treatment seems to indicate experimental error, such as accidental pollinator intrusion.

While Darwin (1862) forwarded the hypothesis that thrips are capable of pollinating certain orchids, little is known about the pollinating ability of thrips (Williams et al. 2001), and virtually nothing is known about orchid pollination by thrips (Matsui et al. 2001). Any hypothesis put forth by Darwin, which has escaped experimental examination for over 150 yr, including this one, will be of special interest to evolutionary ecologists.

It has been suggested that thrips often utilize tiny, hidden, and globose or urn-like flowers, rather than medium or large flowers (Williams et al. 2001). Interestingly, *E. thunbergii* flowers do not possess these traits. Yet, there is growing evidence that the actual pollinators of plant species are determined by complex factors that include not only floral syndromes, but also local pollinator availability (Huang and Fenster 2007, Ollerton et al. 2009, Xie et al. 2013). In addition, specific pollination syndromes do not necessarily preclude visitation by less-

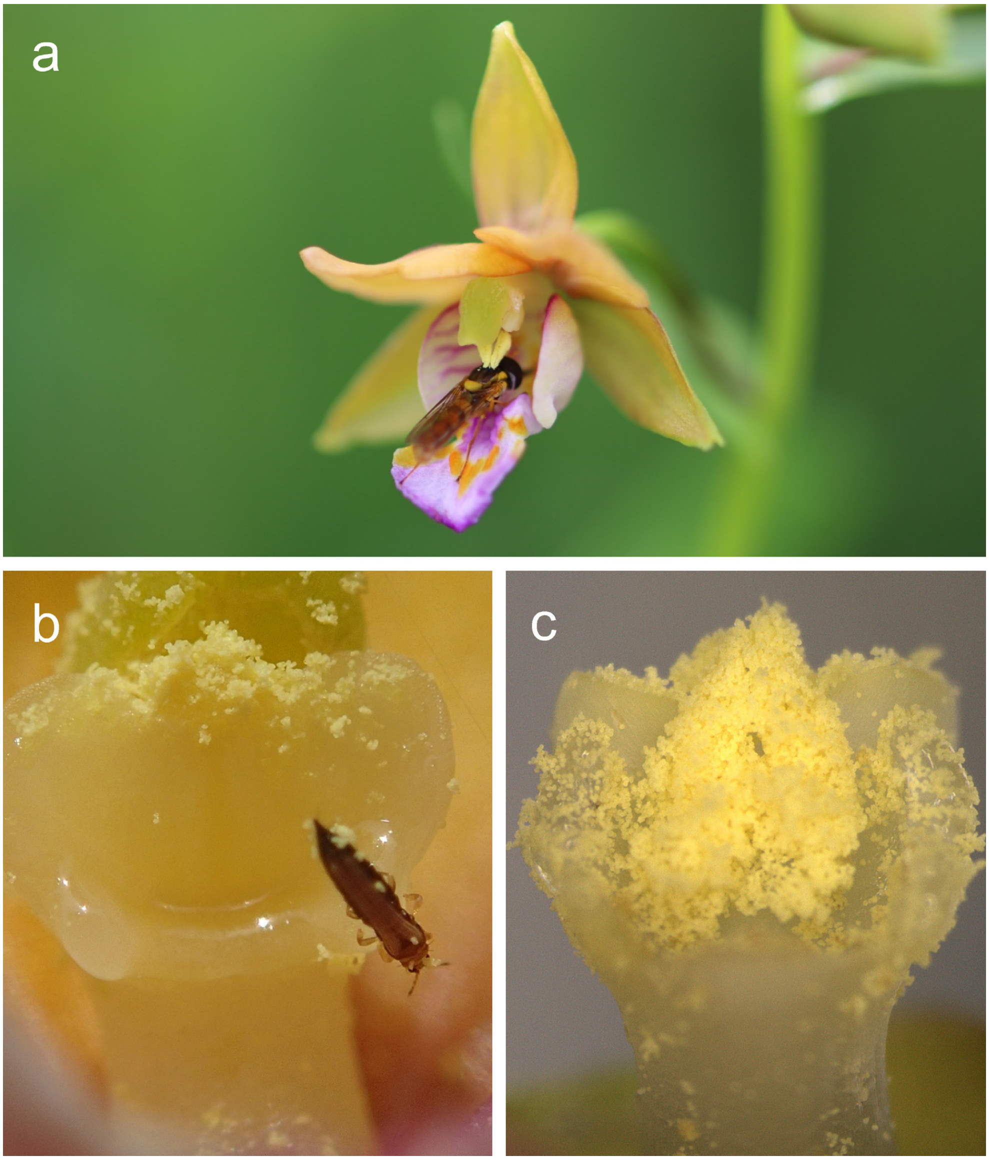

FIG. 1. Pollination biology of *Epipactis thunbergii*. (a) The hoverfly, *Sphaerophoria macrogaster*, carrying pollinia. (b) The thrips, *Frankliniella intonsa*, transferring pollen grains to an *E. thunbergii* stigma. (c) *E. thunbergii* pollinia that have been partially consumed by thrips and therefore exhibit weakened cohesiveness.

TABLE 2. Effect of pollination treatment on fruit set and proportion of seeds with an embryo.

Treatment	Pollinator exclusion	Manual autogamy	Manual allogamy	Thrips only	Open pollination
Fruit set	$5/47^{a}$	$9/10^{b}$	$9/10^{b}$	$24/71^{c}$	$64/106^{b}$
Seed mass (g)	3.1 ± 1.2^{a}	18.4 ± 5.7^{b}	18.7 ± 6.3^{b}	2.8 ± 1.3^{a}	13.4 ± 7.9^{c}
Seeds with embryo (%)	$91.2 \pm 6.0^{a,b}$	$92.3 \pm 3.1^{a,b}$	$91.8 \pm 2.1^{a,b}$	90.3 ± 4.2^{b}	94.0 ± 3.9^{a}

Notes: Fruit set is indicated by number of developed fruits/number of examined flowers; seed mass and seeds with embryo values represent means ± SD. Pollination treatments that had significant differences ($P \leq 0.05$) are indicated by different superscript letters. Because four fruit capsules in open treatments had already dehisced when collecting samples, data from these four fruits were excluded from seed mass and seeds with embryo.

efficient visitors (i.e., secondary pollinators; Stebbins 1970, Rosas-Guerrero et al. 2014). Therefore, although *E. thunbergii* is categorized as a hoverfly-pollinated flower, its adaptation to supplementary pollination by thrips may not be completely surprising. Moreover, it is important to note that, unlike the compact pollinia of typical orchid species, the pollinia of *E. thunbergii* are mealy and friable and the pollen grains can be transferred in small clumps by thrips. We suggest that supplemental thrips pollination may be more common than previously recognized among orchids with granular pollinia.

Nevertheless, it should be noted that seed mass was significantly lower under the thrips-only treatment than under the open-pollination, manual-autogamy, or manual-allogamy treatments, possibly owing to the lower number of pollen grains deposited by thrips than by hoverflies (Table 2). This result suggests that thrips are inferior to hoverflies as pollinators of *E. thunbergii*. In addition, in comparison with vegetative herbivory, the consumption of reproductive organs can greatly affect plant fitness (McCall and Irwin 2006). For example, in pollination–seed-predation systems in which insects function as both pollinators (as adults) and seed predators (as larvae), the overall interactions can be mutualistic, commensal, or antagonistic, depending on the presence and abundance of effective co-pollinators (Thompson and Pellmyr 1992, Thompson and Cunningham 2002).

Therefore, while thrips contribute to pollination, they have the potential to inflict heavy costs in terms of plant fitness. If the cost of "mutualism" is substantially high for the plants, abundant co-pollinators can swamp the mutualistic effect of pollination. In fact, pollinia that have been partially consumed by thrips lose their cohesiveness and are less likely to attach to hoverflies than do intact pollinia, even when the epichiles place hoverflies in contact with pollinia (Fig. 1c). Therefore, even if thrips deposit pollen grains on stigmatic surfaces, it is possible that the thrips also ruin most of the remaining pollen grains, which could have been transferred by more efficient pollinators (i.e., hoverflies), thereby negatively affecting plant fitness.

Overall, we demonstrated that thrips, which are usually considered antagonists, can contribute to orchid reproduction. Several recent studies have also endorsed the idea of a mutualism–parasitism continuum (i.e., symbiotic associations are not always simply defined as "mutualism" or "parasitism" and can be context dependent) in many biological interactions (Frederickson 2017). Our results also suggested that positive, neutral, and negative effects of thrips can be detected in *E. thunbergii*, depending on the effectiveness and abundance of other pollinators. Additional studies are needed to determine whether thrips are mutualists or otherwise of *E. thunbergii* under a variety of conditions.

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