



Pollination by biting midges in *Dioscorea tokoro* and *Vincetoxicum aristolochioides* with a secondary contribution of gall midges

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Received: 1 September 2024 / Accepted: 5 March 2025 / Published online: 6 May 2025
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Abstract

Although small flies, such as those of the suborder Nematocera, are abundant and well-known flower visitors, their importance as pollinators is not fully appreciated. In this study, we attempted to identify the pollinators of *Dioscorea tokoro* (Dioscoreaceae) and *Vincetoxicum aristolochioides* (Apocynaceae). Our working hypothesis was that these species are pollinated by gall midges (Diptera: Cecidomyiidae), because gall midges visited flowers and laid eggs in them during our preliminary observations. Flower visitors were investigated using direct observations, time-lapse photography, and sticky traps installed close to the inflorescences. A supplemental pollination experiment demonstrated that *D. tokoro* was not wind pollinated. In both plant species, several genera of nocturnal gall midges were the most frequent flower visitors, followed by diurnal biting midges (Diptera: Ceratopogonidae) belonging to the Forcipomyiinae. Less frequent visits by members of the Sciaridae (Diptera) and Lepidoptera also were observed. Examination of body pollen revealed that biting midges carried pollen on several areas of their bodies, but gall midges were infrequent pollen carriers for either plant species. DNA barcoding showed that gall midges carrying pollen differed from those that engaged in oviposition. Our results suggested that the two plant species were primarily pollinated by biting midges with a smaller contribution from gall midges. This study highlights the importance of biting midges as pollinators. Biting midges also pollinate cacao and several wild plant species, and they may potentially also pollinate crops, such as yam, some of which have floral traits similar to those of *D. tokoro*.

Keywords Apocynaceae · Cecidomyiidae · Ceratopogonidae · Dioscoreaceae · Pollination · Crop wild relatives

Introduction

Pollinator behavior, sensory capacities, and morphology may affect the efficiency and effectiveness of pollen transfer by different pollinator species (Adler and Irwin 2006). Improving our knowledge of pollinator taxonomic diversity

and its consequences for plant–pollinator interactions is important for understanding the ecology and evolution of flowers (Fenster et al. 2004). Many investigations of the relationships between pollinator functional groups and floral traits have been biased toward systems involving well-known pollinator groups such as bees and birds (e.g., Wilson et al. 2004; Smith et al. 2008; Rodrigues et al. 2018). Relatively little effort has been devoted to elucidating relationships between floral traits and less well-investigated pollinator taxa, such as Diptera (Mochizuki et al. 2023).

More recently, however, researchers have realized that pollinator species regarded as less common or unimportant can affect the evolution of unique floral traits. For example, *Aeschynanthus acuminatus* (Gesneriaceae) is pollinated by passerine birds. Its reddish green flowers, with wide-open, short corolla tubes, do not conform to the typical type of bird pollination, which involves red-colored tubular corollas (Chen et al. 2019). Shuttleworth and Johnson (2010) revealed that the plants pollinated by pompilid wasps tend to have dull-colored, dotted petals, and sulfur compounds in

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Handling Editor: Isabel Alves dos Santos.

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the floral scent (Shuttleworth and Johnson 2010, 2012). Fungus gnats have been confirmed as important pollinators of 13 plant families, some of which have acquired unique sets of floral characteristics (pollination syndromes) in response to pollination by fungus gnats (Mochizuki and Kawakita 2018; Lu et al. 2021; Mochizuki et al. 2023). These examples suggest that an improved understanding of floral trait diversity could be acquired by studying poorly known pollinators, highlighting the importance of uncovering unnoticed pollination systems in pollination ecology.

One of the pollinator groups whose role in pollination is poorly understood is the suborder Nematocera, a paraphyletic group of Diptera with long antennae containing craneflies, mosquitoes, biting midges, gall midges, and fungus gnats (Marshall 2012). Species from most Nematoceran families are known to visit flowers (Larson et al. 2001; Raguso 2020), and their importance as pollinators has recently been noticed for gall midges (Luo et al. 2018; Kawakita et al. 2022; Matsumoto et al. 2023; Sunakawa et al. 2024) and fungus gnats (Mochizuki and Kawakita 2018; Lin et al. 2022). Among the Nematocera, fungus gnats from the families Mycetophilidae and Sciaridae are known pollinators. Some fungus-gnat-pollinated plants, such as the genera *Aucuba* (Garryaceae), *Euonymus* (Celastraceae), and *Mitella* (Saxifragaceae), share similar floral traits, i.e., size (approximately 7 mm in diameter), dark red or greenish color, flattened shape with exposed nectary disks, and short stamens (Okuyama et al. 2008; Mochizuki and Kawakita 2018). In fungus gnat-pollinated plants, the pollen generally adheres to the frontal coxa of fungus gnats when they forage for nectar from the nectary. Pollinator size and behavior fit the floral morphology (Mochizuki and Kawakita 2018).

Plant species that share similar floral traits to the above-described fungus gnat-pollinated plants may be pollinated by fungus gnats. We have also noticed some plant species whose flowers are similar to fungus gnat-pollinated plants but smaller in size (approximately 4 mm in diameter), e.g., *Dioscorea tokoro* Makino (Dioscoreaceae) and *Vincetoxicum aristolochioides* (Miq.) Franch. et Sav. (*Tylophora aristolochioides* Miq., Apocynaceae) distributed in Japan (Fig. 1). These plants may be pollinated by small fungus gnats. Alternatively, other nematoceran flies with body structures similar to fungus gnats, such as gall midges (Cecidomyiidae) and biting midges (Ceratopogonidae), could also serve as pollinators (Larson et al. 2001). Gall midges are known pollinators of 44 species of 11 plant families, including some plants with brood site rewards (Luo et al. 2018; Kawakita et al. 2022; Sunakawa et al. 2024). Biting midges are lesser known as pollinators with the reports from six plant families (Bogarín et al. 2018) but known as important pollinators of cacao, an important crop (Tarmadja 2015). Revealing the pollinators of *D. tokoro* and *V. aristolochioides* may shed

light on the hitherto unclear role of these micro-dipteran insects as pollinators and their relationship with floral traits.

During the preliminary observations, we observed several gall midges visit *D. tokoro* flowers in Hakusan-shi, Ishikawa prefecture in 2016 and 2017, and oviposition behavior and the presence of gall midge larvae in male flower buds were confirmed in 2019 (Fig. S1a,b; Elsayed et al. 2025). Likewise, gall midges were observed ovipositing on the flowers, and larvae were discovered in flowers of *V. aristolochioides* during subsequent field research in 2019 (Fig. S1c,d). This gall midge was later described as a new species, *Ametrodiplosis adetos* Elsayed, Yukawa & Tokuda, whose larvae do not transform flowers into galls but feed on the nectar externally (Elsayed et al. 2021). These observations prompted us to hypothesize that *D. tokoro* and *V. aristolochioides* are pollinated by gall midges that use buds and flowers as brood sites.

To test the hypothesis that *D. tokoro* and *V. aristolochioides* are pollinated by gall midges that use buds and flowers as brood sites, we performed observation on floral visitors, measured the visitation frequencies of each pollinator functional group, and examined pollen attachment. Based on the data obtained, we evaluated the relative importance of pollinators. We also performed a pollination experiment to test the possibility that *D. tokoro* could be wind pollinated. Additionally, we investigated pollen transfer efficiency (PTE) in *V. aristolochioides*.

Because the studied plants produce tiny flowers, direct observation was challenging. To eliminate the possibility of human observers missing visitors, we monitored *D. tokoro* flowers using time-lapse filming. For *V. aristolochioides*, we developed a cost-effective, easy-to-make sticky trap installed near flowers to conduct an unbiased investigation of flower visitors. Based on the floral visitors that were observed and captured and an examination of pollen carried on the body of each insect, we discuss the pollination systems of *D. tokoro* and *V. aristolochioides*.

Methods

Study plants

The yam genus *Dioscorea* consists of 635 species of twining herbs with a global distribution (POWO 2024). The genus is part of the family Dioscoreaceae, along with *Tacca* and the other small-membered genera *Stenomerris* and *Trichopus* (Viruel et al. 2016). Most *Dioscorea* species are dioecious. Their tubers are used as food in Africa, South America, the Caribbean and Pacific islands, and Japan (Asiedu and Sartie 2010). Yams are recognized as the fourth most important tuber crop after potatoes, cassava, and sweet potatoes (Asiedu and Sartie 2010).

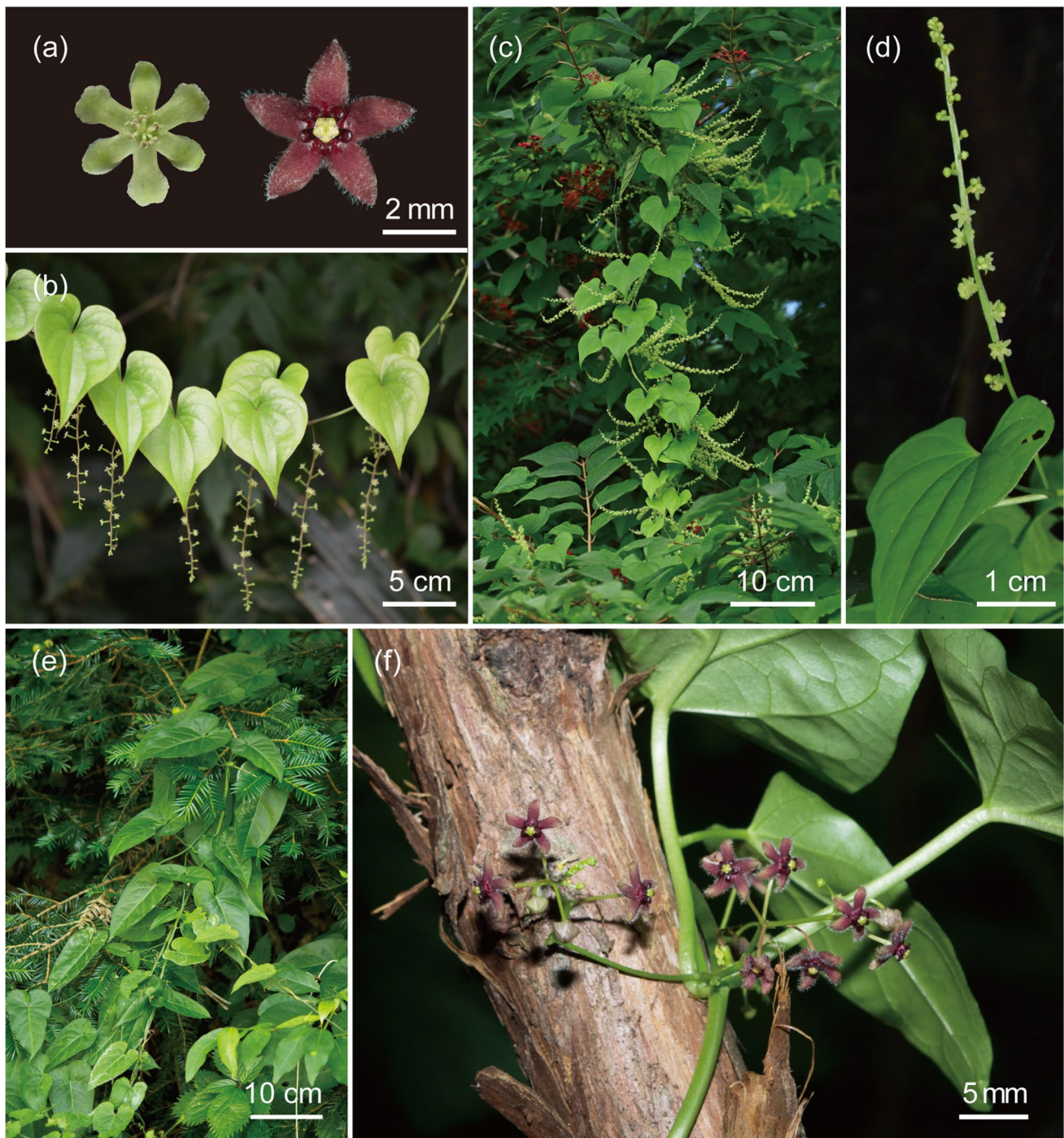


Fig. 1 Images of the studied plants. **a** Flowers of *Dioscorea tokoro* (left) and *Vincetoxicum aristolochioides* (right). **b** Pendant female inflorescences of *D. tokoro*. **c** Flowering male *D. tokoro* individual

d Close-up view of a male inflorescence **e** *V. aristolochioides* hanging on branches of *Cephalotaxus harringtonia* var. *nana*. **f** Flowering inflorescences

Despite their economic importance, the pollination system of *Dioscorea*, which is crucial for sustainable cultivation, is poorly understood. Their flowers are generally small (about 4 mm in diameter, Noda et al. 2020) and are believed to be entomophilous due to the sticky surface

of their pollen (Sadik and Okereke 1975). Segnou et al. (1992) reported that a wide range of insects, including members of the Coleoptera, Diptera, Hymenoptera, Hemiptera, and Thysanoptera visit *Dioscorea rotundata*. These authors regarded thrips as the most important

pollinators because their small bodies provided a good fit to the flowers. Similarly, Mizuki et al. (2005) observed thrips on both male and female flowers of *D. japonica* and suspected that they acted as pollinators. Thrips appear to be the only insects suspected of pollinating *Dioscorea* species. *Dioscorea tokoro* is a perennial climbing herb distributed in Japan, Korea, and China. This plant produces numerous greenish, saucer-shaped flowers with diameters of approximately 4 mm from July to August (Fig. 1b–d; eFloras 2008). The inflorescences are erect in male plants and pendant in female plants.

The genus *Vincetoxicum* Wolf comprises ca. 250 species and belongs to the subfamily Asclepiadoideae in the family Apocynaceae (Liede-Schumann 2012; 2016), which is one of the plant groups with the highest accumulation of information on pollinators among flowering plants (Ollerton et al. 2019). *Vincetoxicum* species exhibit diverse pollination systems across species, involving moths, butterflies, wasps, several groups of Diptera, beetles, and cockroaches (Xiong et al. 2020). *Vincetoxicum aristolochioides* is a perennial twining herb endemic to Japan with a distribution from Hokkaido to the southernmost part of Kyushu (Yamashiro 2017; Fig. 1e). It produces 3–10 flowered inflorescence on each leaf axil of the growing twine from June to September. The flowers are about 4 mm in diameter and are generally dark red but sometimes greenish white (Fig. 1f). *Vincetoxicum aristolochioides* is positioned at the base of the *Vincetoxicum* sensu stricto clade, where pollination by Diptera predominates (Xiong et al. 2020), thus its pollination system is important in the evolution of pollination in this clade. Yamashiro et al. (2008) reported that the flowers of *V. aristolochioides* were visited by unidentified gall midges, one individual of which was confirmed to carry pollinarium, suggesting that gall midges act as pollinators.

The pollination mechanism of Asclepiadoideae is highly specialized. Their anthers are modified to surround the stigma, forming a structure known as the gynostegium, with pollen compacted into pollinia (Endress 2018; Fig. S2a). The stigmas are hidden behind staminal guide rails that form slits which morphologically restrict pollinators (Endress 2018; Fig. S2b). A flower is equipped with five pollinaria and each pollinium is formed by two pollinia connected by the corpusculum (clip) and the caudicle (translator arms) (Endress 2018; Fig. S2c). The pollinia are located at the narrower distal end of the slits, which are picked up by the pollinator body that passes through the slit. When the body of a pollinator with pollinia attached passes through the slit, the pollinium or the whole pollinarium will be left in the stigmatic chamber, completing pollen transfer. Because of this specialized flower morphology, pollination of

Asclepiadoideae is performed by a few functional groups of pollinators (Ollerton and Liede-Schumann 1997; Shuttleworth and Johnson 2017; Mochizuki et al. 2017; Ollerton et al. 2019). Animals that carry a pollinarium on their body are likely to contribute to pollen deposition (Wiemer et al. 2012).

Study sites

Field observations of *V. aristolochioides* were conducted in Ozoe, Hakusan-shi, Ishikawa Prefecture (36°16'17"N, 136°42'37"E) in a deciduous forest dominated by Japanese beech. The study was conducted on plants growing between 600 and 900 m.a.s.l. For *D. tokoro*, observation was conducted in Ozoe (same location to above) and Shiramine, Hakusan-shi, Ishikawa Prefecture (36°10'14"N 136°37'17"E) and the Nikko Botanical Garden, Nikko City, Tochigi Prefecture (36°45'02"N, 139°35'11"E). Shiramine is located approximately 600 m.a.s.l. and has vegetation similar to that of Ozoe. The Nikko Botanical Garden is located 600 m.a.s.l. in the Nikko National Park and is designated as a special reserve. The field research was conducted from 2019 to 2021 (see Supplementary tables S1, S2, and S3 for detailed information regarding the field observations).

Monitoring of pollinators

To identify pollinators, we conducted field observations and collections on flower visitors employing observations by human observers in both species, photography using time-lapse cameras in *D. tokoro*, and sticky traps in *V. aristolochioides*. Direct observations were made from morning (0900) to until after midnight (2500), with occasional rest periods. At night, we used a headlight covered with red film to avoid disturbing insect visitors. First, we chose ten flowering individuals of both plant species for direct observation. Each individual was carefully checked for the presence of flower visitors over 5–10 min. To effectively observe the behavior of small flower-visiting insects, we used a Nikon D7200 camera (Nikon Tokyo, Japan) equipped with either an AF-S Micro NIKKOR 60 mm f/2.8G ED lens or a TAMRON SP AF 90 mm f/2.8 Di MACRO 1:1 lens (Tamron, Saitama, Japan).

For *D. tokoro*, the flower visitors were recorded for their taxa and frequency to minimize the number of insects killed, but the gall midge visitors were captured to perform detailed identification. Some of the visitors were photographed to check for body pollen to complement the evaluation of pollinators in addition to the comparison of visitation frequency. For *V. aristolochioides*, visitors were captured whenever possible for the detailed examination of pollinarium attachment. Flower visitors were collected using an aspirator and immediately preserved in 99% ethanol.

Note that the pollinarium attached to insect bodies did not become detached when the insect was soaked in ethanol. The detailed placement of the pollinarium was studied using SEM (JFC-3000FC: JEOL, Ltd., Tokyo, Japan).

Time-lapse photography was used to supplement the direct observations of *D. tokoro*. WG-50 cameras (Ricoh Co., Ltd., Tokyo, Japan) were set to include one inflorescence in the field of view, and images were taken at 1-min intervals. Because the study sites Ozoe and Shiramine are close to each other and have similar environments, we pooled the data obtained from these locations (hereafter, pooled populations are indicated as “Hakusan”). Further details of the observations are summarized in Supplementary tables S1, S2, and S3.

For *V. aristolochioides*, flowers were usually visited by tiny insects (1–3 mm). Thus, there was a risk that human observers would miss these small visitors. Hence, we crafted a sticky trap to capture them. A laminate film of 100- μ m thickness (IRIS OHYAMA Inc., Miyagi, Japan) was heated with a laminator to obtain a transparent sheet. Disks of 7 cm diameter were cut out of the sheets. A small hole was made in the center of the disk and a notch was cut from the hole toward the outer circumference. Kinryu sticky glue (IRIS OHYAMA Inc., Miyagi, Japan) was sprayed on the disk; once the solvent dried, the disks were set behind the inflorescences by sliding the plant stem through the notch until it passed through the hole. Disks also were set to stem nodes that did not bear flowers as a control to test disk capture rates in the absence of flowers. The sticky traps were left in place for 24 h. In all, 62 traps (45 on floral nodes and 17 on non-floral nodes) were set on 29 flowering individuals, with some individuals used repeatedly. Arthropods caught by the traps were removed using either kerosene or hexane, after which they were identified and inspected for pollinia attachment.

Taxonomic identification of visitors

Morphological identification of insects was first performed at the order level. Most dipterans were identified to the family level, and frequent visitors from the Cecidomyiidae and Ceratopogonidae were identified to the genus level. Ceratopogonidae that visited *D. tokoro* also were identified by Dr. Art Borknet (The American Museum of Natural History, British Columbia, Canada) through photographs. All collected gall midge visitors were identified morphologically to subfamily Cecidomyiinae and then assigned to supertribes. Because the gall midge flower visitors were always female, molecular phylogenetic analysis was used to assign them to tribes. Larvae of gall midges found in flower buds were reared to adults following Elsayed et al. (2018) and were identified to the genus level based on morphological characteristics of larvae, pupae, and adults.

Total DNA was extracted and a 472-bp fragment of the mitochondrial COI gene was sequenced using the method described by Elsayed et al. (2020). The resulting sequences were deposited in the DNA Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank (www.ncbi.nlm.nih.gov/genbank) under the accession numbers shown in Supplementary Table S4.

Only two gall midge individuals carried pollinia of *V. aristolochioides*; one of these individuals was morphologically similar to *A. adetos*, whereas the other was morphologically dissimilar to *A. adetos* (see Results). The specimen similar to *A. adetos* was subjected to phylogenetic analysis.

Nine gall midge flower visitors to *D. tokoro* and seven larvae developing in *D. tokoro* male flower buds were analyzed. Sequence data were analyzed with GenBank sequences of COI, 16S, ITS, CAD, and 28S genes of in-group species representing several tribes of the subfamily Cecidomyiinae, as well as three outgroup species representing basal subfamilies Catotrichinae, Micromyinae, and Porricondyliinae (provided in Supplementary tables S4 and S5, Dorchin et al. 2019; Elsayed et al. 2019, 2021, 2023, 2025; Elsayed and Mille 2022). The sequences of COI were aligned using the *ClustalW* algorithm in MEGA 11 (ver. 11.0.10, see <https://www.megasoftware.net/>, Tamura et al. 2021). Sequences of other genes were aligned separately using the E-INS-i algorithm in *MAFFT* online servers (ver. 7, <https://mafft.cbrc.jp/alignment/server/>). The final combined and aligned sequence lengths, including gaps, were 5218 bp. Phylogenetic relationships were inferred using the maximum likelihood method in RAXML GU (ver. 2.0.6; Edler et al. 2021) based on 1000 bootstrap replications, and using the GTR + G + I model determined by *modeltest* in the same software.

Further, to investigate whether flower visitors were identical to the gall midges reproducing in the flowers, we compared the sequences of flower visitors to those of *A. adetos* and the gall midge larvae inhabiting the flower buds of *D. tokoro*, as reported by Elsayed et al. (2021) and Elsayed et al. (2025) (Supplementary tables S4 and S5).

Pollination experiments and pollination success

To test the dependency of each plant species on insect pollination, we conducted pollination experiments in Nikko Botanical Garden for *D. tokoro* and investigated pollen transfer efficiency and fruit set in *V. aristolochioides* at Ozoe.

For *D. tokoro*, 30 female inflorescences from five plants were enclosed in fine mesh bags (mesh size = 0.2 mm) before flowering to prevent insects from accessing the developing flowers. After flowering, the bagged inflorescences were processed using either one of three treatments: 1) the bags

were retained on the inflorescence (negative control); 2) the bags were removed, allowing flowers to be freely pollinated (positive control); 3) the bags were removed and flowers were hand-pollinated using pollen from the flowers of male individuals, after which the inflorescences were bagged again until they set fruit (hand pollination treatment). The mesh bags were placed over inflorescences on August 3, 2020. From August 5 to 10, the mesh bags were removed from the inflorescences for hand pollination. Male inflorescences were cut; male flowers were applied to female flowers using forceps and a magnifying glass to ensure that anthers had contacted stigmas. The number of flowers subjected to each treatment was recorded for each individual. During the assessment of the fruit set on September 9, 2020, the number of fruits was recorded at the individual level rather than the inflorescence level, and the fruit set was calculated for each individual. The final value was the average of individual calculations.

We did not conduct pollination experiments on *V. aristolochioides*, because it is known that the morphologically complex flowers of this species are not wind-pollinated (see Study plants and sites). Instead, pollination success was investigated by quantifying fruit sets under natural conditions and by calculating PTE (Wiemer et al. 2012). To investigate fruit set, 456 flowers from 16 plants were tagged on July 25, 2020, and the tagged flowers were checked for fruit set on September 3, 2020. The fruit set ratio was calculated by averaging the fruit set ratio of individual plants. To investigate the PTE, the numbers of pollinaria removed and deposited were counted using flowers that were close to dropping, as indicated by the closing corolla.

Sampling of the flowers was performed from July 24 to 26, 2020. Flowers were dissected under a stereomicroscope to count the number of pollinaria removed from anthers and inserted into the stigmatic chamber. In total, 45 flowers from seven individuals were assessed. Flowers that had fallen beneath the individual or were about to fall were collected. Then the PTE value was calculated by dividing the total number of pollinia inserted by the total number of pollinia removed (Wiemer et al. 2012). The total number of pollinia removed was determined as twice the number of pollinaria removed, because each pollinarium contains two pollinia (Wiemer et al. 2012).

Statistics

To compare the number of insects captured by sticky traps placed on inflorescence nodes and non-inflorescence nodes, we conducted analyses for each insect taxonomic group using a Generalized Linear Mixed Model (GLMM) implemented with the *glmer* function from the *lme4* package in R (Bates et al. 2015). Because each plant was used multiple times

for trap placement, the position of the trap was included as a fixed effect, while the identity of individual plants was included as a random effect.

Differences in fruit set among the treatments were also analyzed using GLMM with a binomial distribution. This analysis was performed using the *glmer* function from the *lme4* package in R (Bates et al. 2015). The number of fruits set by each individual plant was used as the response variable, with treatments as the explanatory variable and identity of individual plants as a random effect. Post hoc comparisons were conducted using the Tukey test, calculated with the *glht* function from the *multcomp* package (Hothorn et al., 2008). All analyses were performed using R version 3.3.3 (R Core Team 2017).

Results

Flower visitors and pollinators

Observers spent 37.5- and 62.5-h watching flowers of *D. tokoro* in Hakusan and Nikko, respectively. Additionally, 2,808 (35 h) time-lapse photographs were taken in Hakusan and 23,718 (282.5 h) in Nikko. These combined observations recorded members of the Diptera, Lepidoptera, Coleoptera, Hymenoptera, and Hemiptera in a quantitative order (Table 1, Fig. 2). Thrips were not recorded. Insect visitors were observed to push their mouthparts against the base of flowers, from which they appeared to forage for nectar. Hoverflies were observed to feed on pollen from male inflorescences.

Nocturnally foraging gall midges (Cecidomyiidae) were the most frequent visitors recorded by direct visual observation and by cameras in both male and female flowers. Gall midge visitors comprised taxa belonging to the tribes Lestodiplosini, Cecidomyiini, Clinodiplosini, and Mycodiplosini (Fig. 3). Unlike *V. aristolochioides*, flower visitors to *D. tokoro* did not include flower-inhabiting gall midges (Fig. 3). Daytime visiting biting midges (Ceratopogonidae) were the second most frequent visitors overall based on the total number of records (Table 1, Fig. 2a). Pollen-dusted Ceratopogonidae were frequently observed, with at least 7 out of 10 successfully photographed individuals among the 38 total visitation events observed (Table 1), likely because their small size enabled them to access male flowers while foraging for nectar (Fig. 2b). Based on photographs, we inferred that other insect visitors generally carried a few pollen grains with only each two Coleoptera and Hymenoptera carried pollen (Fig. S4).

During 165 h of direct observations of *V. aristolochioides*, we observed 46 biting midges (Ceratopogonidae) during the daytime (Fig. 2d, e), 42 gall midges (Cecidomyiidae) during the night (Fig. 2f, g), and occasional fungus gnats

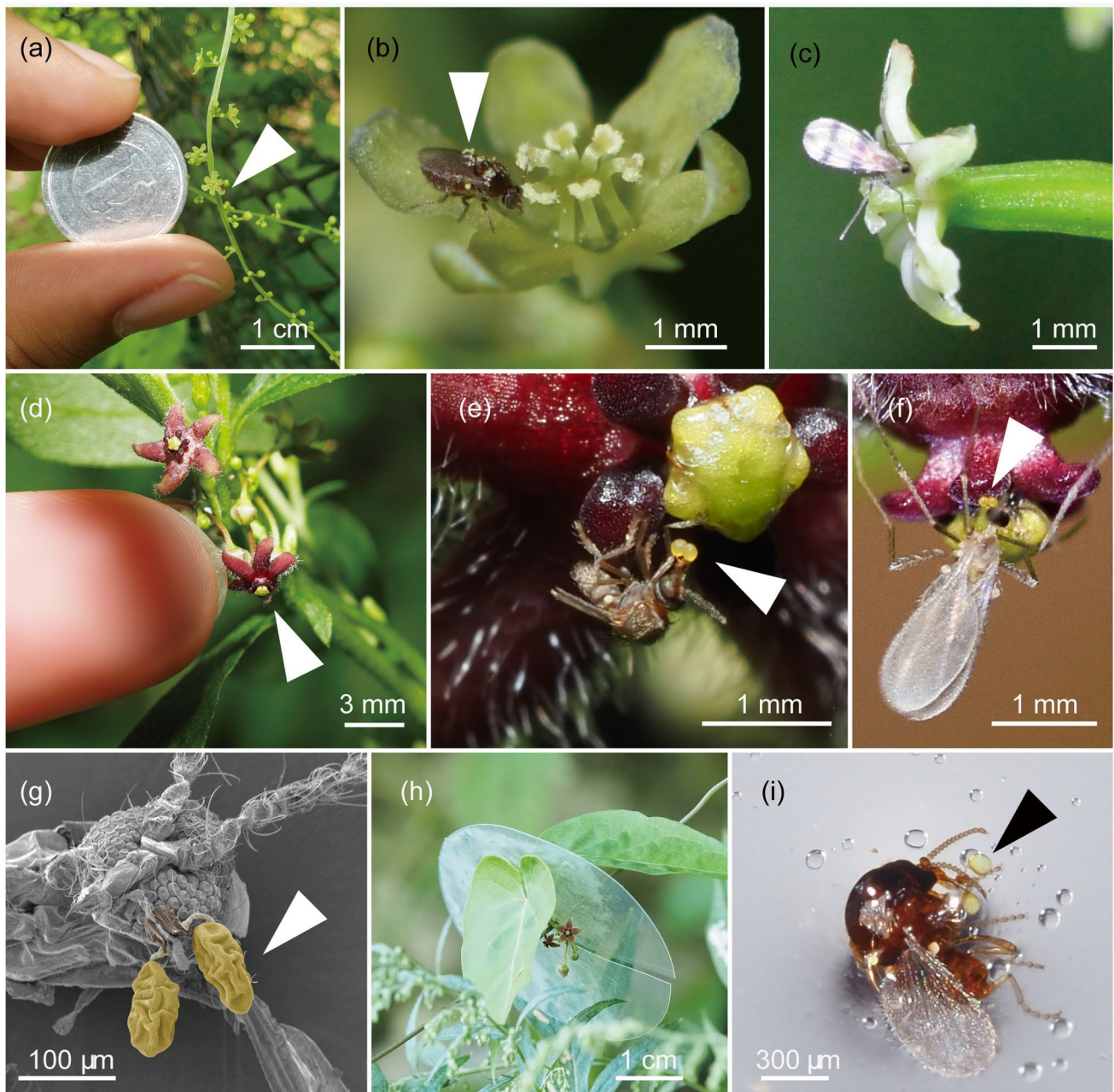
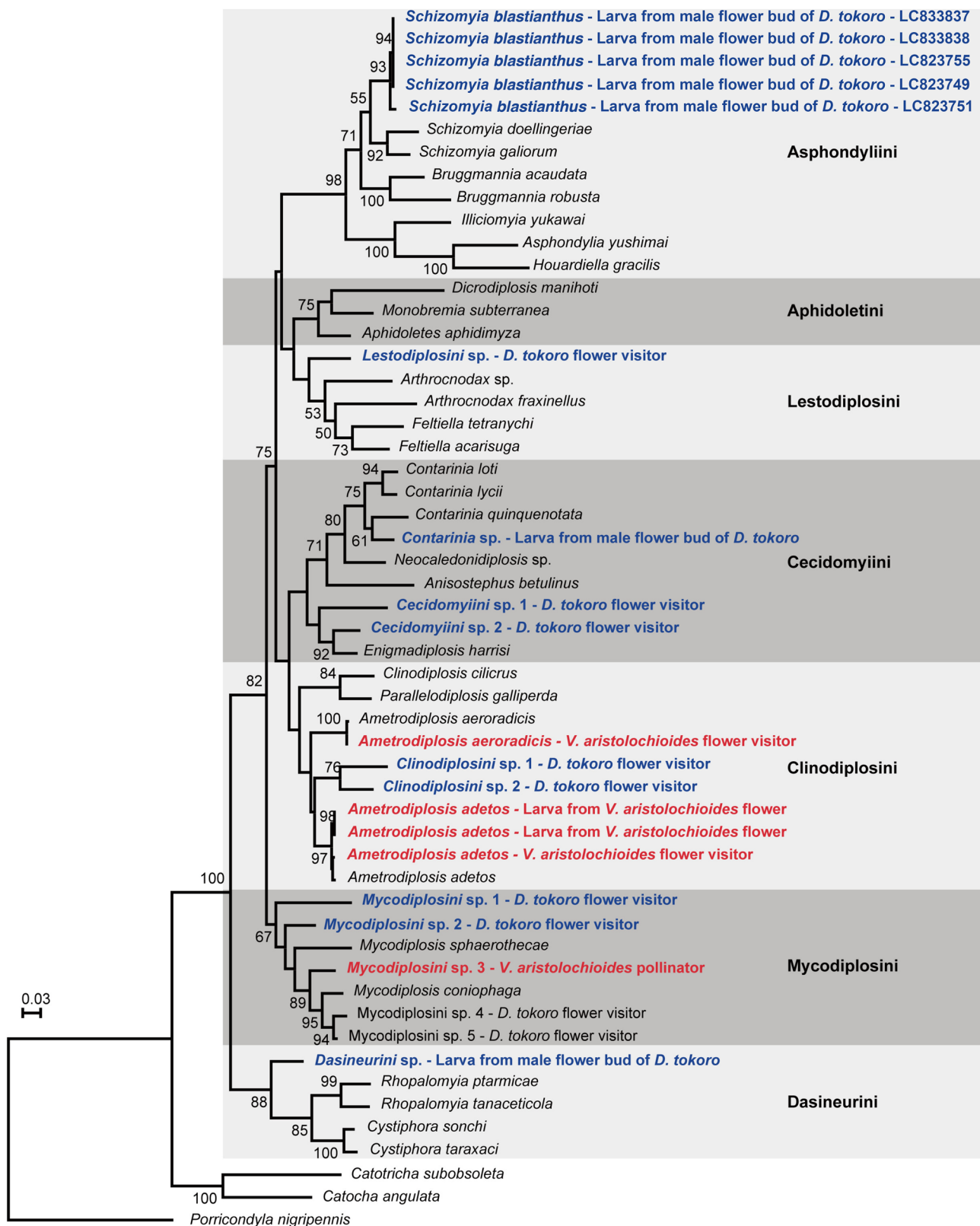


Fig. 2 Flower visitors and pollinators of *D. tokoro* (a–c) and *V. aristolochioides* (d–i). **a** Flower-visiting biting midge (triangle) in *D. tokoro* male flower. **b** A biting midge visiting a male flower and carrying pollen on its thorax and wings (triangle). **c** A gall midge visiting female flowers without carrying pollen. **d** Flower-visiting biting midge (triangle) in *V. aristolochioides*. **e** A biting midge carrying pol-

linarium (triangle) on its proboscis. **f** A gall midge visiting the flower and removing the pollinarium (triangle). **g** Scanning electron microscope image of the pollinarium-carrying gall midge. **h** A laminate sticky trap installed on the flowering node. **i** A biting midge captured in a sticky trap carrying pollinarium (triangle)

(Mycetophilidae and Sciaridae), ants, and Miridae (Table 2). Of the 42 observed Cecidomyiidae, 33 individuals were identified as *A. adetos* based on morphological characteristics and their oviposition behavior at the flowers (Elsayed et al. 2021). However, no individuals were confirmed to carry pollinia. Two gall midges were confirmed to carry pollinaria on their palpus (Fig. 2f,g; Table 2). One

was captured and identified to be Mycodiplosini based on DNA (Fig. 3). The other individual could not be captured but was identified as Clinodiplosini or Lestopterini based on morphology. Eight Forcipomyiinae biting midges carried at least one pollinarium each on their mouthparts (Table 2: Fig. 2e). No other insects carried pollinaria.



Gall midges (Cecidomyiidae) were the most abundant insects captured in sticky traps attached to inflorescence nodes, followed by flies belonging to the families Sciaridae,

Ceratopogonidae, and Mycetophilidae and other insects (Fig. S3). Only gall midges and Mycetophilidae were captured more frequently in traps set at floral nodes (Fig. S3:

Fig. 3 A maximum likelihood phylogenetic tree based on the alignment of partial sequences of COI, 16S, ITS, CAD, and 28S genes (5218 bp including gaps) of gall midges representing major tribes of Cecidomyiinae and including sequences of gall midges inhabiting and visiting the flowers of *V. aristolochioides* (highlighted in red) and *D. tokoro* (highlighted in blue). Samples not collected in this study were retrieved from the database and an unpublished manuscript; they are summarized in Supplementary tables S4 and S5. The DDBJ accession numbers are added to the larval samples of *Schizomyia blasianthus*. Support of less than 50% bootstrap value is not shown. The scale bar indicates 0.03 nucleotide changes per site

Table S6). Overall, 45 traps installed at floral nodes (Fig. 2h) captured 50 gall midges and 42 biting midges. Of these, four individuals of Forcipomyiinae sp. carried pollinaria (Table 2; Fig. 2i).

Overall, the results suggest that biting midges were the most effective pollinator of *V. aristolochioides* with 12 out of 88 individuals carrying pollinaria (13.6%) and gall midges were the secondary pollinator with 2 out of 92 individuals (2.17%), and no other insects carried pollinaria.

Pollination experiment and pollination success

The fruit set of *D. tokoro* was observed in $37.9 \pm 2.5\%$ (mean $\pm$ standard error) of naturally pollinated flowers (positive control, $n = 5$ individuals), $1.3 \pm 0.9\%$ (mean $\pm$ standard error) of flowers covered with mesh bags (negative control, $n = 5$ individuals), and $32.7 \pm 13.8\%$ (mean $\pm$ standard error) of hand-pollinated flowers ($n = 5$ individuals). Both the hand-pollinated and positive control treatments had significantly higher fruit set compared to the negative control treatment. Specifically, the fruit set was significantly higher in the hand-pollinated treatment compared to the negative control treatment (Estimate = 4.00059, SE = 0.43487, $z = 9.199$,

Table 2 Identity and numbers of recorded floral visitors to *Vincetoxicum aristolochioides*. Numbers within parentheses and brackets indicate the number of captured individuals and the number of individuals carrying at least one pollinarium, respectively

Order	Family	Observation	Sticky trap	Total
Diptera	Ceratopogonidae	46 (33) [8]	42 [4]	88 [12]
	Cecidomyiidae	42 (27) [2]	50	59 [2]
	Mycetophilidae	1	34	35
	Sciaridae	1	45	46
	Psychodidae		3	3
	Phoridae		16	16
	Drosophilidae		6	6
Hymenoptera	Formicidae	5	3	8
	Others		31	31
Lepidoptera	Crambidae		2	2
Thysanoptera			1	1
Hemiptera	Miridae	3	7	10
Araneae			3	3

$p < 0.001$) and in the positive control treatment compared to the negative control treatment (Estimate = 4.08332, SE = 0.42154, $z = 9.687$, $p < 0.001$). However, there was no significant difference in fruit set between the positive control and hand-pollinated treatments (Estimate = 0.08273, SE = 0.17237, $z = 0.480$, $p = 0.874$).

Among the 45 dropped or nearly dropped flowers of *V. aristolochioides*, eight pollinaria (4.71%) had been removed and two pollinia had been deposited. Therefore, the pollinia arrival rate, PTE, was 12.5%. Among 456 flowers from 16 individuals, 6 flowers produced fruits, resulting in a fruit set of 1.3%.

Table 1 Identity and number of recorded floral visitors to flowers of *Dioscorea tokoro* for each location and observer. Hakusan (data pooled from Ozoe and Shiramine populations) and Nikko indicate the study locations

Order	Family	Hakusan		Nikko		Total
		Hunam observers	Cameras	Hunam observers	Cameras	
Diptera	Ceratopogonidae	13	2	25	5	45
	Cecidomyiidae	65	44	39	74	222
	Culicidae	7	1	6	4	18
	Sciaridae	2	0	1	0	3
	Syrphidae	5	0	5	2	12
	Chloropidae	1	0	0	0	1
	Tephritidae	3	0	0	0	3
	Muscidae	3	1	0	2	6
	Unidentified spp.	0	0	6	3	9
Hymenoptera		6	0	11	2	19
Lepidoptera		5	2	8	14	29
Coleoptera		9	0	14	4	27
Hemiptera		0	0	4	0	4

Discussion

Pollination by small Nematoceran flies is uncommon, but recent studies suggest a certain relationship between floral traits. The flowers of some species pollinated by fungus gnats have similar floral traits, i.e., size (approximately 7 mm in diameter), dark red or green color, flattened shape with exposed nectary disks and short stamens (Mochizuki and Kawakita 2018). In this study, we investigated pollinators and reproductive success in *V. aristolochioides* and *D. tokoro* whose flowers are similar to those of the fungus gnat pollination syndrome, but which are substantially smaller (approximately 4 mm in diameter). Our working hypothesis was that the primary pollinators of these plants were gall midges. As a result of our research, we discovered two novel cases of pollination by biting midges.

Pollinators of *D. tokoro*

We considered it unlikely that *D. tokoro* relies on wind pollination, as the flowers covered with mesh bags (mesh size: 0.2 mm), which allowed the passage of free-blown pollen (13.8 μ m in diameter; Jeong et al. 2022), produced almost no fruits, suggesting that opportunities for wind pollination are extremely limited. Flower visitors push their mouthparts toward the basal part of the sepals, which is considered nectar-feeding behavior (Fig. 2b, c), thus it is likely that *D. tokoro* secretes nectar from the floral disk. These observations and the fact that *D. tokoro* flowers set fruit under natural conditions led us to conclude that nectar-feeding insects have a role in pollination. Caution is needed when interpreting the result of fruit set in *D. tokoro*. Because different treatments were applied to individual bags, ideally, the data should have been grouped by bag and analyzed statistically. However, we pooled the data and the fruit set was calculated at the individual plant level, ignoring the bag-level structure. Nonetheless, given the clear differences in fruit set among the three treatments, this is unlikely to have significantly impacted the conclusions.

Although we did not quantitatively compare the effectiveness of pollen transfer among the insects, biting midges likely make a greater contribution to pollination than gall midges. We arrived at this conclusion because 1) gall midges were the most frequent visitors followed by biting midges in each population, 2) biting midges and gall midges were the only insects observed to visit both female and male inflorescences, and 3) although biting midges were often dusted in pollen, gall midges were not (Fig. 2b, c). Behavioral differences between biting midges and gall midges may contribute to differential pollen attachment. Biting midges crawl inside the space between the sepals and anthers, which results in pollen attachment to the back

of the head, and to the thorax and wings (Fig. 2b). Such behavior and the remarkable pollen load observed on biting midges make them strong candidates as primary pollinators of *D. tokoro*. Gall midges that visit flowers of *D. tokoro* have larger bodies than biting midges, which may prevent them from crawling inside the flowers while they forage for nectar. Instead, their legs and the ventral side of their abdomens make superficial contact with anthers and stigmas (Fig. S4a). Such minimal contact with the anthers does not result in a noticeable pollen load. However, their frequent visits to flowers may enable gall midges to make a minor contribution to pollination.

Some gall midge species have been observed to lay eggs in the male flower buds (Fig. Sa), but neither oviposition nor the presence of larvae was observed in female flower buds (Elsayed et al. 2025). Although one might simply consider gall midges to be parasitic, the development of gall midge larvae in male flower buds raises questions about their relationship with *D. tokoro*. For example, larvae of mutualistic gall midges that pollinate *Phyllanthus flexuosus* were discovered only in male flower buds (Kawakita et al. 2022; Elsayed and Kawakita 2022). Therefore, further investigations into the interactions between gall midges and *D. tokoro* are required.

We also note that the pollination biology of the genus *Dioscorea* and its family, the Dioscoreaceae, is poorly understood despite their importance in agriculture and botany. Many members of the genus *Dioscorea*, which includes both wild and cultivated species, share similar floral traits to *D. tokoro* (Sadik and Okereke 1975; Segnou et al. 1992; Noda et al. 2020). The biting midges may therefore be widely shared pollinators of this genus. Moreover, because biting midges are the dominant pollinators of the genus *Tacca*, which is another major member of the Dioscoreaceae (Viruel et al. 2016), our findings suggest the possibility that biting midges may be ancestral pollinators in the Dioscoreaceae.

Pollinators of *V. aristolochioides*

Field observations indicated that the nocturnal gall midge *A. adetos* was the most frequent visitor to *V. aristolochioides*. This species repeated oviposition and nectar-feeding behavior two or three times, but no individuals carried pollinaria. Therefore, we considered it a floral parasite that both adults and larvae presumably consumed nectar (Fig. S1d; Elsayed et al. 2025), although it was unclear whether this behavior had a negative impact on the plant's reproductive success. Pollen transfer was observed in only two individuals of two species among 92 observed gall midges (2.17%; Table 2), suggesting that the contribution of gall midges to pollination is rare. Pulling out the pollinarium requires physical robustness in the mouthparts, so it may

be difficult for gall midges, which lack the long, tough mouthparts like biting midges (Mochizuki pers. obs.).

In contrast, biting midges, which were the second most frequently observed and trapped insect group, carried pollinaria more frequently (13.6% of observed individuals) and thus may be more effective pollinators. In the sticky trap experiments, biting midges (Forcipomyiinae) were captured at both floral and non-floral nodes (Fig. 2i; Fig. S3). Although slightly more biting midges were caught at floral nodes, the difference was not statistically significant (Fig. S3; Table S6). This does not necessarily suggest that biting midges exhibit opportunistic behavior and are not attracted to flowers. One possible explanation is that the observed Forcipomyiinae biting midges in this study are haemolymph-feeding and are quickly attracted to prey captured by predators, such as spiders, mantises, and Asilidae robber flies (Marshall et al. 2015). Therefore, it cannot be ruled out that the biting midges were drawn to other insects already caught in the sticky traps. Further research on the ecology of pollinator attraction of *V. aristolochioides*, including identifying and characterizing the composition and function of floral scent, would provide insights into how the flowers attract biting midges.

The contribution of biting midges to successful pollination is likely underestimated because we could not reliably find them during 2020. Only two individuals were observed during this period; they were difficult to see because they are tiny and similar in color to the dark-colored flowers of *V. aristolochioides*. The strategy was modified slightly in 2021, where flowers were checked from every angle to spot any unusual changes in the floral silhouettes that might indicate the presence of biting midges. This revised observation technique led to more frequent observations of biting midges in that year.

The 1.3% fruit set under natural conditions in the Ozoe population was not exceptionally low, given that the rate of fruit set is generally 1–5% in members of the Asclepiadoideae (Wyatt and Broyles 1994; Chirango et al. 2019; Domingos-Melo et al. 2019). Furthermore, the 12.5% PTE value demonstrated successful pollen transfer, because it fell within the range reported for other Asclepiadoideae (Ollerton et al. 2003; Shuttleworth and Johnson 2009, 2012; Shuttleworth et al. 2017). Therefore, the Ozoe population of *V. aristolochioides* inhabits an environment with effective pollinators. Therefore, we conclude that the biting midges are the primary pollinator of *V. aristolochioides* at least at the study site.

Pollination by biting midges and floral characteristics

Pollination by biting midges (Ceratopogonidae) is an uncommon type of pollination with reports from plants of eight genera of six families (Bogarín et al. 2018). Biting

midges are a group of Diptera belonging to Culicomorpha in the suborder Nematocera and are closely related to the Chironomidae (Narayanan et al. 2018). Globally, there are 6206 known species of biting midge, and some of these are known pests of humans (Borkent and Dominiak 2020). The genera *Forcipomyia* and *Atripogon*, observed as pollinators in this study, are known as ectoparasites that feed on insect blood and kleptoparasites that feed on the exudates of insects hunted by predators (e.g., spiders and mantids, Marshall et al. 2015).

According to Bogarín et al. (2018), *Trichosalpinx* species may attract kleptoparasitic biting midge pollinators via mimicry, because the flowers secrete exudate containing protein. Although the detailed ecology of pollinator attraction in plants pollinated by biting midges is unclear, kleptoparasitic biting midges pollinate several other plants, such as *Aristolochia* spp. (Aristolochiaceae, Berjano et al. 2009), *Arum conophalloides* (Araceae, Gibernau et al. 2004), *Tacca* spp. (Dioscoreaceae, Chua et al. 2020), *Bulbophyllum macphersonii* (Orchidaceae, Bartareau 1994), *Theobroma cacao* (Malvaceae: Tarmadja 2015; Arnold et al. 2019), and *Ceropegia* spp. (Apocynaceae, Ollerton et al. 2017). Among these plants, *Trichosalpinx*, *Bulbophyllum*, *Ceropegia*, and *Theobroma* share several characteristics, such as dark purple, motile floral appendages, suggesting convergent evolution in the attraction of biting midges (Bogarín, 2019). However, the floral characteristics of *D. tokoro* and *V. aristolochioides* do not fit the previously hypothesized traits associated with biting midge pollination. This may suggest the differential strategies of pollinator attraction of *D. tokoro* and *V. aristolochioides* compared to previous examples. Currently, how plants including the studied plants attract biting midges is unclear, but may be worth studying given the kleptoparasitic life of biting midges. Further detailed studies are needed to elucidate the ecology of attraction and the role of floral traits in attracting biting midges.

At the beginning of this study, we hypothesized that *D. tokoro* and *V. aristolochioides* would exhibit a gall midge pollination syndrome. However, our results suggest that biting midges are the principal pollinators with gall midges acting as additional pollinators. Considering that similar floral traits are shared by gall midge-pollinated plants, such as *Phyllanthus flexuosus* (Kawakita et al. 2022) and *Vincetoxicum* species (Yamashiro et al. 2008; Kidyoo et al. 2024), it appears that pollination by biting midges, gall midges, or a mix of both cannot be clearly distinguished based on floral traits at present. Investigations into the pollination of plants with floral traits similar to those of *D. tokoro* and *V. aristolochioides*, such as other species of *Vincetoxicum* and *Dioscorea*, as well as *Galium*, *Rubia*, *Scleromitron* (Rubiaceae), and *Actinostemma* (Cucurbitaceae), may provide a more precise understanding

of the relationships between floral traits and midge pollinators.

Application of sticky traps in revealing tiny pollinators

One of the challenges in studying pollination systems with tiny pollinators is to observe and catch the insects, particularly under conditions where the timing of pollinator visits is unknown. The sticky traps that we employed successfully captured biting midges that carried pollinaria (Fig. 2i), a result which demonstrated their value for studying tiny pollinators. Although direct observations enabled us to capture a larger number of biting midges, sticky traps can be used to enrich the dataset, because they do not require special skills or experience. Lim and Raguso (2017) also set sticky glass traps in front of the inflorescences of *Tacca integrifolia*, which resulted in the successful collection of biting midge pollinators.

Some caution in the use of sticky traps is needed. First, they do not provide temporal information or insights into the behavior of flower visitors. To address this limitation, it is important to combine the sticky traps with direct observations or camera traps capable of capturing flower visitors. However, to the best of our knowledge, camera traps designed to capture small insects are not currently available. Developing such equipment would significantly advance our understanding of the role of small insects as pollinators.

Second, inappropriate installation of these traps may result in the capture of non-floral visitors. For example, if the traps are too far from flowers, they may catch more insects unrelated to pollination than flower visitors (Fig. S3). The duration of trap installation should also be adjusted (24 h in this study), because captured insects attract scavengers, such as opilions (Mochizuki, pers. obs.). Our sticky trap was made from laminate films, which are cost-effective, easy to process, and convenient to carry. They can be formed into any shape, which may help identify the tiny pollinators. Because both gall midges and biting midges are abundant in many natural habitats (Borkent and Dominiak 2020; Yukawa and Tokuda 2021), their role in pollination may never be fully described. Further investigations that focus on floral traits, when combined with techniques employed in this study, may further refine our knowledge of the ecological roles played by midges and their interactions with plants.

Conclusion

Although biting midges are relatively uncommon as pollinators, previous studies have shown that they play a critical role in pollinating cacao, an agriculturally significant crop, with pollination having been documented in eight genera across six families. Our

study expands this knowledge by identifying biting midges as pollinators in two additional plant families, Apocynaceae (specifically *D. tokoro*) and Dioscoreaceae (*V. aristolochioides*), highlighting their broader ecological significance.

Based on our findings, biting midges may serve as pollinators for some yam species, which would make them more ecologically and agriculturally significant than previously recognized. The shared floral morphology of *D. tokoro* and *V. aristolochioides* appears to be adapted for pollination by biting midges. This raises the possibility that other plants with similar floral morphology might also rely on biting midges for pollination.

Further research, including behavioral studies of insect visitors and chemical analyses of floral traits, is necessary to establish a clearer relationship between floral morphology and biting midge pollination. Additionally, detailed investigations into the pollination biology of *D. tokoro* and *V. aristolochioides*, such as the composition of floral scents and their role in attracting biting midges, could provide valuable insights into the ecology and evolution of this rare yet significant pollination system.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11829-025-10142-4>.

Acknowledgements We thank Mr. Sumio Miyano for kindly hosting the authors during the research at Ozoe. We are grateful to Dr. Art Borkent for the identification of Ceratopogonidae. We also express our gratitude to Ms. Ayako Watanabe-Taneda for her help in obtaining a SEM image. This work was financially supported by the JSPS Grant-in-Aid for Early-Career Scientists [20K15859] and [24K18176] to KM, the 30th Botanical Research Grant of ICHIMURA Foundation For New Technology (<http://www.sgkz.or.jp/project/plant/>) to KM, the JSPS Standard Postdoctoral Fellowship [P19087], and the JSPS Grant-in-Aid for Research Activity Start-up [22K20582] to AKE.

Funding Open Access funding provided by The University of Tokyo, Japan Society for the Promotion of Science, 20K15859, Ko Mochizuki, 24K18176, Ko Mochizuki, P19087, Ayman Khamis Elsayed, 22K20582, Ayman Khamis Elsayed, Ichimura Foundation of New Technology, and 30th Botanical Research Grant, Ko Mochizuki.

Data availability Data regarding the fruit set and PTE have been uploaded to figshare: <https://doi.org/https://doi.org/10.6084/m9.figshare.26826034>

Declarations

Conflict of interest Not applicable.

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