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Selective pollination by fungus gnats potentially functions as an alternative reproductive isolation among five *Arisaema* species

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• **Background and Aims** Interspecific difference in pollinators (pollinator isolation) is important for reproductive isolation in flowering plants. Species-specific pollination by fungus gnats has been discovered in several plant taxa, suggesting that they can contribute to reproductive isolation. Nevertheless, their contribution has not been studied in detail, partly because they are too small for field observations during flower visitation. To quantify their flower visitation, we used the genus *Arisaema* (Araceae) because the pitcher-like spathe of *Arisaema* can trap all floral visitors.
• **Methods** We evaluated floral visitor assemblage in an altitudinal gradient including five *Arisaema* species. We also examined interspecific differences in altitudinal distribution (geographic isolation) and flowering phenology (phenological isolation). To exclude the effect of interspecific differences in altitudinal distribution on floral visitor assemblage, we established ten experimental plots including the five *Arisaema* species in high- and low-altitude areas and collected floral visitors. We also collected floral visitors in three additional sites. Finally, we estimated the strength and contribution of these three reproductive barriers using a unified formula for reproductive isolation.
• **Key Results** Each *Arisaema* species selectively attracted different fungus gnats in the altitudinal gradient, experimental plots and additional sites. Altitudinal distribution and flowering phenology differed among the five *Arisaema* species, whereas the strength of geographic and phenological isolations were distinctly weaker than those in pollinator isolation. Nevertheless, the absolute contribution of pollinator isolation to total reproductive isolation was weaker than geographic and phenological isolations, because pollinator isolation functions after the two early-acting barriers in plant life history.
• **Conclusions** Our results suggest that selective pollination by fungus gnats potentially contributes to reproductive isolation. Since geographic and phenological isolations can be disrupted by habitat disturbance and interannual climate change, the strong and stable pollinator isolation might compensate for the weakened early-acting barriers as an alternative reproductive isolation among the five *Arisaema* species.

Key words: Araceae, *Arisaema* section *Pistillata*, coexistence, deceptive pollination, flowering season, habitat elevation, jack-in-the-pulpit, Mycetophilidae, myophily, Sciaridae, speciation.

INTRODUCTION

Reproductive isolation contributes to speciation by reducing gene flow among diverging lineages (Coyne and Orr, 2004), and determines the subsequent persistence of the species generated (Weber and Strauss, 2016) because frequent hybridization can rapidly blur the species boundary (genetic swamping; Todesco *et al.*, 2016). In flowering plants, reproductive isolation includes both pre- and post-pollination barriers: pre-pollination barriers reduce pollen exchange between different species, while post-pollination barriers prevent the formation of a hybrid zygote after the interspecific pollination (Baack *et al.*, 2015). In general, pre-pollination barriers contribute more to total reproductive isolation than post-pollination barriers (Lowry *et al.*, 2008). Although post-pollination barriers (e.g. hybrid inviability and sterility) can cause rapid species exclusion (Buggs and Pannell, 2006; Takakura and Fujii, 2010) because the formation of maladaptive hybrids wastes reproductive effort of parental species and degrades population growth rate

(demographic swamping; Todesco *et al.*, 2016), pre-pollination barriers can mitigate this negative effect (van der Niet *et al.*, 2006). Therefore, pre-pollination barriers should contribute to the stable persistence of sympatric species.

The pre-pollination barrier usually consists of several components, including geographic, phenological and pollinator isolation (Lowry *et al.*, 2008). Among them, pollinator isolation plays an especially important role (Lowry *et al.*, 2008), because ~87.5 % of flowering plants are pollinated by animals (Ollerton *et al.*, 2011). Pollinator isolation by contrasting pollinator types has attracted intense attention (Grant, 1992; Ramsey *et al.*, 2003; Dell'Olivo *et al.*, 2011), and recent studies have reported that subtle differences in pollinator taxa enabled pollinator isolation (Whitehead and Peakall, 2014; Murúa *et al.*, 2017; Cuevas *et al.*, 2018).

Fungus gnats (Mycetophilidae, Sciaridae and allies) are nematoceran flies that inhabit forests (Økland, 1994) and are regarded as decomposers, herbivores or agricultural pests (Binns,

1981; Irmler *et al.*, 1996; Sueyoshi and Yoshimatsu, 2019). Their role as effective pollinators has been overlooked owing to the small body size (Reiter *et al.*, 2019), ephemeral life span at the adult stage (Li *et al.*, 2009) and reduced dispersal ability (Jakovlev, 2012). However, fungus gnat pollination has been found in 12 plant families (Mochizuki and Kawakita, 2018; Guo *et al.*, 2019), some of which employ species-specific fungus gnats (Okuyama *et al.*, 2004; Song *et al*., 2014, 2015; Reiter *et al.*, 2019). Nevertheless, no study has quantified their roles in reproductive isolation, partly because they are too small for field observation (Tremblay and Ackerman, 2007; Mochizuki and Kawakita, 2018). In addition, most studies about species-specific pollination by fungus gnats lacked a comprehensive evaluation of other early-acting barriers (i.e. geographic and phenological isolations), which is needed to calculate the contribution of pollinator isolation to total reproductive isolation (Sobel and Chen, 2014).

The genus *Arisaema* (Araceae) is pollinated by fungus gnats (Vogel and Martens, 2000) and is suitable for the examination of their contribution to reproductive isolation for the following four reasons. First, the quantification of pollinator visits is easy in *Arisaema* because all visitors can be trapped in the pitcher-like spathe (Fig. 1) by artificial manipulation (Barriault *et al*., 2009, 2010). Second, pollinator isolation caused by selective visitation of fungus gnats (Vogel and Martens, 2000) and contrasting fly pollinators (Kakishima *et al.*, 2019; Matsumoto *et al.*, 2019) has been reported in sympatric *Arisaema* species. Third, *Arisaema* section *Pistillata* rapidly diversified in Japan (Ohi-Toma *et al.*, 2016) and several species often coexist in the field (Murata, 1995; Serizawa, 1997). Finally, most Japanese *Arisaema* species have weak post-pollination barriers (Murata

et al., 2018), suggesting the importance of pre-pollination barriers.

In an altitudinal gradient including five *Arisaema* species (Figs 1 and 2), we examined three pre-pollination barriers suggested in *Arisaema*: (1) geographic isolation caused by interspecific differences in altitudinal range (Matsumoto *et al.*, 2018); (2) phenological isolation caused by interspecific differences in flowering phenology (Matsumoto *et al.*, 2019); and (3) pollinator isolation caused by selective pollination by fungus gnats (Vogel and Martens, 2000). We quantified their strength and contribution to the total reproductive isolation using the unified calculation method described by Sobel and Chen (2014).

MATERIALS AND METHODS

Plant materials

The genus *Arisaema* consists of dioecious perennial herbs that mainly grow at deciduous forests (Murata *et al.*, 2018). They have a pitfall-trap pollination system (Barnes, 1935; Vogel and Martens, 2000). Their pitcher-like inflorescences (Fig. 1) mainly attract fungus gnats through an odour emitted from the spadix appendage and/or spathe (Barnes, 1935; Vogel and Martens, 2000; Kakishima *et al.*, 2019). Most *Arisaema* species lack any floral rewards except for some species with nectaries (Vogel and Martens, 2000). Attracted fungus gnats slip down to the bottom of the pitcher-like spathe because the spadix appendage and inner surface of the spathe are covered with wax (Vogel and Martens, 2000). An exit hole opens at the bottom of the male spathe, and the captured fungus gnats can escape with pollen. In contrast, the
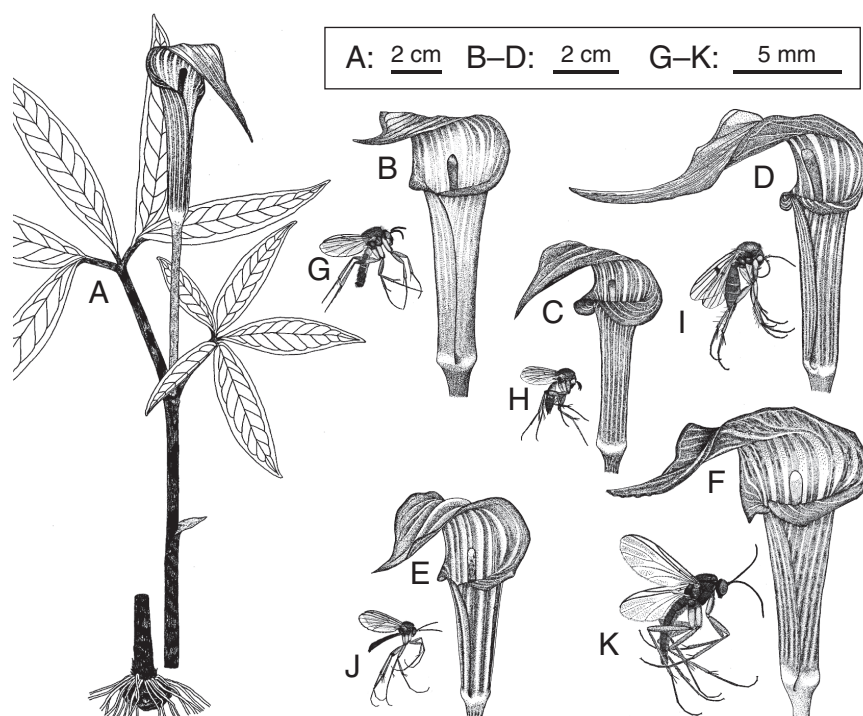

FIG. 1. Morphology of the genus *Arisaema*. (A) Habit. (B–F) Inflorescence of the five *Arisaema* species: (B) *A. maekawae*, (C) *A. angustatum*, (D) *A. peninsulae*, (E) *A. ovale* and (F) *A. pseudoangustatum* var. *pseudoangustatum*. (G–K) The main floral visitors of the five *Arisaema* species in the altitudinal gradient of Wakasugi: (G) *Megophthalmidia* sp., (H) *Cordyla* sp., (I) *Mycetophila* sp., (J) *Anatella* sp. and (K) *Trichosia* sp.
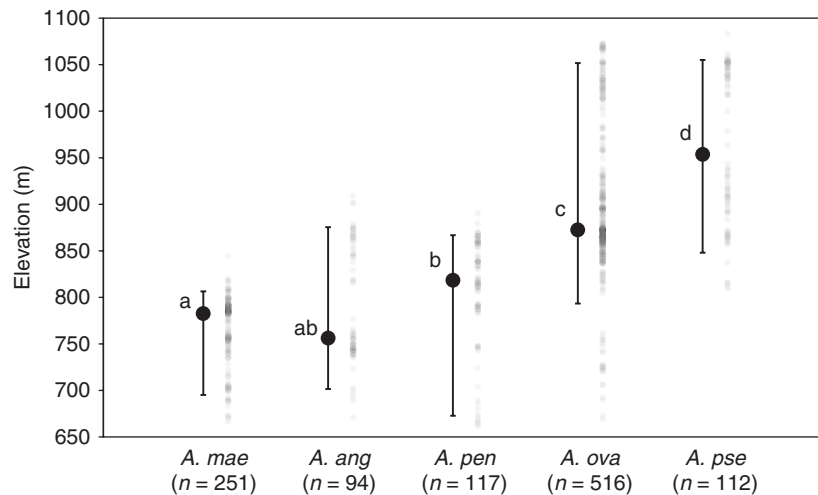
FIG. 2. Altitudinal ranges of the five examined species in the altitudinal gradient of Wakasugi. Black circles are radians of habitat elevation. Error bars represent the 5th and 95th percentiles. Small grey circles indicate raw values. Different letters mean significant differences (Dunn's test) at $P ≤ 0.05$. Species abbreviations: *mae*, *maekawae*; *ang*, *angustatum*; *pen*, *peninsulae*; *ova*, *ovale*; *pse*, *pseudoangustatum*.

female spathe has no exit hole, and the captured fungus gnats move around the female spadix, depositing pollen grains on the stigma, and then die. Therefore, all floral visitors of male and female inflorescences can be collected by closing the exit hole of male inflorescence (Barriault *et al.*, 2010).

In this study, we focused on five *Arisaema* species from section *Pistillata* (Murata *et al.*, 2018): *A. maekawae* (Fig. 1B), *A. angustatum* (Fig. 1C), *A. peninsulae* (Fig. 1D), and *A. pseudoangustatum* var. *pseudoangustatum* (Fig. 1F, hereafter *A. pseudoangustatum*) from the *Arisaema serratum* group (Matsumoto *et al.*, 2018) and *A. ovale* (Fig. 1E) from the *A. ovale* group (Kobayashi *et al.*, 2008). The chromosome numbers of the first four species are $2n = 28$ (Murata *et al.*, 2018; Y. Hayase, Botanic Gardens of Toyama, Japan, unpubl. res.). *Arisaema ovale* shows heteroploidy ($2n = 26, 39, 52, 65, 78$), with tetraploid populations ($2n = 52$) widely distributed across western Japan, including the study site (Murata *et al.*, 2018). Previous studies reported that F_1 hybrids generally showed high germination rates and high pollen fertility in artificial crossing experiments among several species in section *Pistillata* (Murata and Ohno, 1989; Murata *et al.*, 2018), and that the pollen stainability of the putative hybrid of *Arisaema limbatum* ($2n = 26$) and *Arisaema ringens* ($2n = 28$) was considerably lower (32.6 %) compared with those of each parental species (88.8 and 91.8 %) (Kobayashi *et al.*, 2005). In addition, the natural putative hybrid between *Arisaema monophyllum* ($2n = 28$) and *A. ovale* ($2n = 52$) showed an intermediate chromosome number ($2n ~40$) (Murata *et al.*, 2018). Therefore, homoploid hybrids among the four species belonging to the *A. serratum* group may be as fertile as purebreds, while hybrids between *A. ovale* and the other four species are expected to show intrinsic inviability and/or sterility (Matsumoto *et al.*, 2018).

Study site

This study was conducted on an altitudinal gradient including the five *Arisaema* species in an area on both sides of a road (total width of area, 50 m) ranging from Ohgaya Skiing Resort (35°13′ N, 134°21′E; 650 m a.s.l.) to the Wakasugi Pass (35°15′ N, 134°23′ E; 1050 m a.s.l.) in the northern Okayama Prefecture, western Japan (hereafter, Wakasugi). The limited width of study area was due to the mountain stream and cliffs along the road. The mean annual precipitation and temperature were ~2500 mm and 8.2 °C, respectively (Mizunaga and Nakashima, 1997). The low-altitude area (from 650 to 930 m a.s.l.) comprises a conifer plantation with *Cryptomeria japonica* and *Chamaecyparis obtusa*. The forest floor of the conifer plantation is dominated by shrubs, including *Edgeworthia chrysantha*, *Symplocos sawafutagi* and *Weigela hortensis*, and herbaceous species, including *Caulophyllum robustum*, *Chloranthus quadrifolius*, *Macleaya cordata*, *Miscanthus sinensis* and *Oxalis acetosella* var. *longicapsula*. The high-altitude area (from 930 to 1050 m a.s.l.) comprises an old-growth beech forest with *Fagus crenata*, *Magnolia obovata* and *Betula grossa* as the main tree species and *Sasa kurilensis* in the understorey. The five *Arisaema* species are mainly distributed in the forest edge along the road; *A. pseudoangustatum* is endemic in high-altitude areas (≥ 850 m a.s.l.) and three species (*A. maekawae*, *A. angustatum* and *A. peninsulae*) are distributed in low-altitude areas (≤850 m a.s.l.) of Wakasugi (Matsumoto *et al.*, 2018). *Arisaema ovale* mainly grows in the mid-altitude areas of Wakasugi (Matsumoto *et al.*, 2018).

Geographic isolation

To examine the interspecific difference in altitudinal distribution, the habitat elevation of the five *Arisaema* species was recorded using a portable GPS (GPSMAP® 64SJ; Garmin International, Olathe, KS, USA) from 21 June 2018 to 16 July 2018. We examined all flowering *Arisaema* plants growing within 25 m of both sides of the road, ranging from 650 to 1050 m a.s.l. We found 251 *A. maekawae*, 94 *A. angustatum*, 117 *A. peninsulae*, 516 *A. ovale* and 112 *A. pseudoangustatum* plants (Fig. 2).

Phenological isolation

To evaluate the interspecific difference in flowering phenology, 61 *A. maekawae* (21 female, 40 male), 53 *A. angustatum* (9 female, 44 male), 44 *A. peninsulae* (13 female, 31 male), 67 *A. ovale* (16 female, 51 male) and 58 *A. pseudoangustatum* (9 female, 49 male) were observed every day from 24 April 2018 to 7 July 2018. The flowering period started when the spathe tube opened and ended when it faded and/or withered (Barriault *et al.*, 2009). We examined different numbers of females and males because the sex ratio of *Arisaema* populations is usually biased towards males (Richardson and Clay, 2001).

Pollinator isolation

To investigate interspecific differences in floral visitor assemblage, we collected all floral visitors in the spathe using a hand-made aspirator simultaneously with the investigation of flowering phenology, and identified the arthropods captured by inflorescences of the five *Arisaema* species. The exit holes of male spathes were closed with Teflon tape, before they opened, to prevent the escape of attracted insects (Matsumoto *et al.*, 2019). In 2018 we collected 429 arthropods from *A. maekawae*, 292 from *A. angustatum*, 383 from *A. peninsulae*, 630 from *A. ovale* and 837 from *A. pseudoangustatum* (Supplementary Data Table S1). The collected insects were fixed in a freezer overnight and dried at room temperature (~10–30 °C). Subsequently, the samples were observed under a stereomicroscope (SZX7; Olympus, Tokyo, Japan) and identified to family for dipterans and to genus for fungus gnats, using identification manuals (McAlpine *et al.*, 1981, 1987; Papp and Darvas, 1997) and articles (Menzel and Mohrig, 1997*a*, *b*, 1999; Saigusa, 2018), if possible. For the exact identification of sciarid genera, some specimens were mounted on a glass slide using

Euparal after maceration with 30 % KOH overnight. Other arthropods were identified to order.

Since the altitudinal distribution range scarcely overlapped between *A. maekawae* and *A. pseudoangustatum* in Wakasugi (Matsumoto *et al.*, 2018) (Fig. 2), in 2019 we established ten 1 × 1 m plots (hereafter, experimental plots), each consisting of five potted individuals of each of the five *Arisaema* species in Wakasugi in order to compare the floral visitor assemblage between low- and high-altitude habitats. Of the ten plots, five were set on low-altitude areas (650–750 m a.s.l.) and five were set on high-altitude areas (800–900 m a.s.l.), based on the altitudinal boundary (~820 m a.s.l.) between *A. maekawae* and *A. pseudoangustatum* (Fig. 2). We avoided plot establishments on higher-altitude areas (>900 m a.s.l.), including a special protection area of the Quasi-National Park. Plants of the experimental plots were collected around Wakasugi on 16 July 2018. Then we collected females for the experimental plots when this was possible because males sometimes fail to produce inflorescences in the following year. We replaced 12 plants on 7 May 2019 due to deer herbivory immediately after plot establishment. All collected plants were grown in plastic pots with sieved pumice and the pots were arranged at the centre and four corners of each plot on 3 May 2019. Of the 50 plants that were introduced, eight *A. maekawae* (5 female, 3 male), eight *A. angustatum* (5 female, 3 male), six *A. peninsulae* (2 female, 4 male), nine *A. ovale* (all female) and eight *A. pseudoangustatum* (2 female, 6 male) avoided deer herbivory. We collected 93 arthropods from *A. maekawae*, 110 from *A. angustatum*, 49 from *A. peninsulae*, 33 from *A. ovale* and 167 from *A. pseudoangustatum* (Supplementary Data Table S2) from 12 May 2019 to 21 July 2019. We identified all floral visitors in the same way as for the individuals along the altitudinal gradient. In the experimental plots the early-flowering species (especially *A. ovale*) delayed
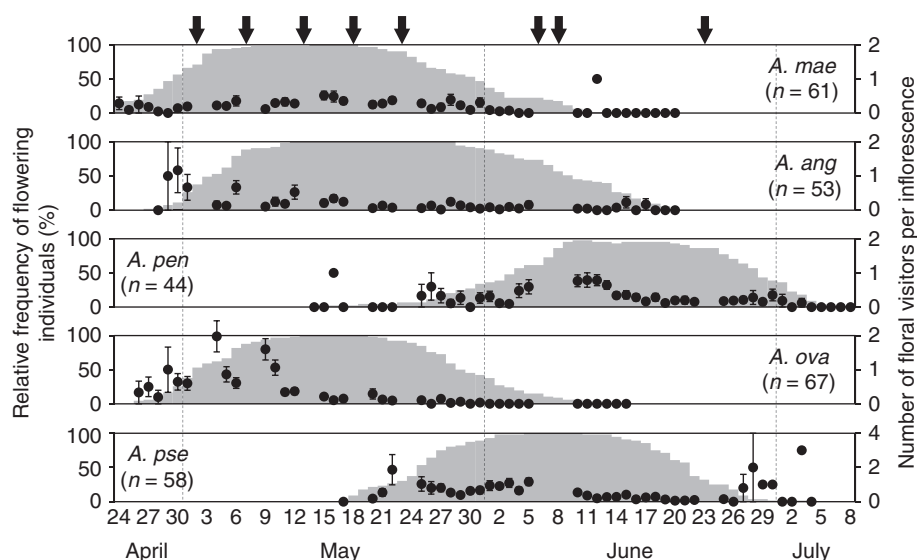

FIG. 3. Flowering phenology of the five examined species in the altitudinal gradient of Wakasugi. Circles and error bars represent the mean number of floral visitors and standard errors. Shaded bars show the relative frequency of flowering individuals. Broken lines separate months. Black arrows denote rainy days. In the case of a rainy day, we collected pollinators on the following day. However, these data were excluded from this figure because they did not represent the number of pollinators per day. Species abbreviations as in Fig. 2 legend.

their flowering by 3–5 weeks (Supplementary Data Fig. S1), unlike those under the natural condition (Matsumoto *et al.*, 2018) (Fig. 3). This was partly because each corm was preserved in a refrigerator (~6 °C) until transfer to plots. Therefore, the flowering phenology largely overlapped among the five *Arisaema* species in June 2019 (Supplementary Data Fig. S1).

To evaluate whether the five *Arisaema* species consistently employ the same pollinators outside Wakasugi, we collected floral visitors from about ten individuals per *Arisaema* species in three additional sites in 2019 (Supplementary Data Table S3). We used only female *Arisaema* plants that do not need the exit hole to be closed before the collection of floral visitors, because floral visitor assemblages did not differ between sexes at Wakasugi (Supplementary Data Table S1). In the additional sites, we collected 148 arthropods from *A. maekawae*, 206 from *A. angustatum*, 74 from *A. peninsulae*, 114 from *A. ovale* and 69 from *A. pseudoangustatum* (Supplementary Data Table S4).

Statistical analysis

Interspecific differences in altitudinal distribution range were examined using the Kruskal–Wallis test followed by an *a posteriori* Dunn's test (Zar, 1998), because elevation did not follow a Gaussian distribution (Shapiro–Wilk test, $P < 0.05$).

Interspecific differences in the apparent flowering phenology were also examined using the Kruskal–Wallis test and Dunn's test. We compared flowering start and end dates among the five *Arisaema* species.

The interspecific differences in the floral visitor assemblage were examined using two-way permutational multivariate analysis of variance (PERMANOVA) with 10 000 permutations. The distance matrix was obtained using the Bray–Curtis dissimilarity index. When the floral visitor assemblage was significantly different among species, we conducted pairwise PERMANOVA with Bonferroni correction for all species pairs. For the altitudinal gradient of Wakasugi, we considered species (*A. maekawae*, *A. angustatum*, *A. peninsulae*, *A. ovale* and *A. pseudoangustatum*), sex expression of *Arisaema* plants (female or male) and their interaction as factors. For the experimental plots, we considered species, elevation (high or low) and their interaction as factors. For the additional sites, we considered species, sites (A–G) and their interaction as factors. We also separately compared the floral visitor assemblage of each species among three sites using one-way PERMANOVA. As the resolution of identification varied among arthropod taxa (Supplementary Data Tables S1, S2 and S4), differences in floral visitor assemblage might be underestimated in these analyses.

The statistical significance was determined at $P < 0.05$ in this study. All analyses were performed with the free software R (version 3.5.1.; R Development Core Team, 2018). The Kruskal–Wallis test and Dunn's test were performed with the dunn.test function using the package dunn.test (Dinno, 2017). PERMANOVA and pairwise PERMANOVA were performed with the adonis2 function in the package vegan (Oksanen *et al.*, 2018) and the pairwise.perm.manova function in the package RVAideMemoire (Hervé, 2019), respectively.

Strength of each reproductive barrier

We calculated the strength of each pre-pollination barrier among all species pairs using the equation RI_{4C} in Sobel and Chen (2014) as follows:

$$RI_{4C} = 1 - \left(\frac{S}{S + U} \right) \tag{1}$$

where RI_{4C} is an index of the strength of individual pre-pollination barrier, S and U denote shared and unshared reproductive opportunities (i.e. habitat elevation, flowering days and pollinator) between different species, respectively. RI_{4C} can range from 0 (all reproductive opportunities are shared) to 1 (no reproductive opportunity is shared).

The strength of geographic isolation was estimated based on the interspecific differences in habitat elevation. Although there is no accurate information on the dispersal ability of fungus gnats, Nishizawa *et al.* (2005) estimated that the pollen movement of *A. serratum*, conducted mainly by fungus gnats, was not strongly dependent on the distance between females and potential pollen donors in an 11 × 11 m plot, based on paternity analysis using microsatellite markers. The distance of pollen movement might exceed 50 m in *A. serratum* populations due to the long-distance migration of fungus gnats blown off by wind (T. Nishizawa, University of Fukui, Japan, pers. comm.). Ohara (2015) reported that the maximum distance between a maternal plant and pollen donors was 44.3 m in a population of *A. peninsulae* subsp. *boreale*, comb. nud. Thus, we assumed that pollen movement might occur when the inter-individual difference in habitat elevation is <100 m a.s.l. This criterion might be very conservative because the actual (i.e. slope or horizontal) distance between individual plants exceeded the vertical distance (i.e. difference in elevation). Based on this assumption, we calculated the total number of heterospecific plants within ±100 m a.s.l. for all plants of each *Arisaema* species, and the mean number was used as a representative value for the shared reproductive opportunity. Unshared reproductive opportunity was also estimated using the mean total number of heterospecific plants outside ±100 m a.s.l. We did not distinguish between male and female plants while calculating the strength of geographic isolation. This is because all *Arisaema* plants can reproduce as males and females in the long term because of their size-dependent sex lability (Atkinson, 1898; Schaffner, 1922; Maekawa, 1924).

The strength of phenological isolation was estimated based on the apparent flowering phenology (i.e. the duration of opening of the inflorescences). Although the functional flowering phenology (i.e. seasonal variation in pollinator visitation frequency) might be more accurate (Matsumoto *et al.*, 2019), the intermittent pollinator visitation throughout the flowering period (data not shown) prevented the identification of the duration of pollinator attractivity for each *Arisaema* individual. We calculated the number of flowering days of overlap with that of a heterospecific male plant for all female plants of each *Arisaema* species, and the mean value was used as a representative value of shared reproductive opportunity. Unshared reproductive opportunity was also estimated using the mean number of flowering days not overlapping with that of a heterospecific male.

The strength of pollinator isolation was estimated based on the visitation frequency of each arthropod taxon. We calculated

the total number of floral visitors shared with a heterospecific male plant for all female plants of each *Arisaema* species, and the mean number was used as a representative value of shared reproductive opportunity. Unshared reproductive opportunity was also estimated using the mean total number of floral visitors unshared with the heterospecific male plant.

The cumulative strength of all pre-pollination barriers was calculated based on the equation RI_{4E} in Sobel and Chen (2014), as follows:

$$RI_{4E} = 1 - 2 \times \left(\frac{S_{\text{total}} \times P(H|S) + U_{\text{total}} \times P(H|U)}{\begin{array}{c} S_{\text{total}} \times P(H|S) + U_{\text{total}} \times P(H|U) \\ + S_{\text{total}} \times P(C|S) + U_{\text{total}} \times P(C|U) \end{array}} \right)$$ (2)

where RI_{4E} is an index of the strength of total reproductive isolation, S_{total} and U_{total} denote total shared and unshared reproductive opportunities between different species, $P(H|S)$ and $P(H|U)$ denote the probability of heterospecific gene flow when the reproductive opportunity was shared and unshared, $P(C|S)$ and $P(C|U)$ denote the probability of conspecific gene flow when reproductive opportunity was shared and unshared, respectively. We calculated S_{total} by multiplying all the shared reproductive opportunity, and subtracted S_{total} from 1 to estimate the U_{total} value (Sobel and Chen, 2014). As we did not examine any post-pollination barriers, we assumed that the probability of fecundation for conspecific and heterospecific pollen was similar if reproductive opportunity was shared (Sobel and Chen, 2014). Therefore, we assigned an equal value of 0.5 to $P(H|S)$ and $P(C|S)$. As only conspecific gene flow occurred when reproductive opportunity was unshared between different species (Sobel and Chen, 2014), we assigned values of 0 and 1 to $P(H|U)$ and $P(C|U)$, respectively.

To compare the contribution of each pre-pollination barrier with the total reproductive isolation, we calculated their absolute contributions using equation AC_i from Sobel and Chen (2014) as follows:

$$AC_i = RI_{[1,i]} - RI_{[1,i-1]}$$ (3)

where AC_i is an index of the contribution of individual barrier to the total reproductive isolation, $RI_{[1,i]}$ denotes the combined isolation calculated by RI_{4E}, including all barriers from the first to act [1] through the focal barrier [i], and $RI_{[1,i-1]}$ denotes the same calculation omitting the focal barrier. In this study, we assumed the following order of barriers: (1) geographic isolation; (2) phenological isolation; and (3) pollinator isolation (Cuevas *et al.*, 2018; Christie and Strauss, 2019).

RESULTS

Geographic isolation

Altitudinal range varied significantly among the five *Arisaema* species in Wakasugi (Kruskal–Wallis test, $\chi^2 = 626.66$, d.f. = 4, $P < 0.001$, Fig. 2). Based on an *a posteriori* test, *A. maekawae* (666–844 m a.s.l.), *A. angustatum* (670–908 m a.s.l.) and *A. peninsulae* (661–890 m a.s.l.) were mainly distributed in the low-altitude area, while *A. pseudoangustatum* was endemic to high-altitude areas (810–1083 m a.s.l.) (Fig. 2). *Arisaema ovale* was distributed throughout the study area of Wakasugi (669–1072 m a.s.l.) and was aggregated around intermediate altitude areas (800–950 m a.s.l.) (Fig. 2).

Phenological isolation

The date of initial flowering was significantly different among the five *Arisaema* species in Wakasugi (Kruskal–Wallis test, $\chi^2 = 211.59$, d.f. = 4, $P < 0.001$) (Fig. 3, Supplementary Data Fig. S2A). Based on an *a posteriori* test, *A. maekawae* began flowering ahead of the other four *Arisaema* species (day of year, 114–134), a few days before *A. angustatum* (117–132) and *A. ovale* (116–133), and several weeks before *A. peninsulae* (134–166) and *A. pseudoangustatum* (137–156) (Fig. 3, Supplementary Data Fig. S2A).
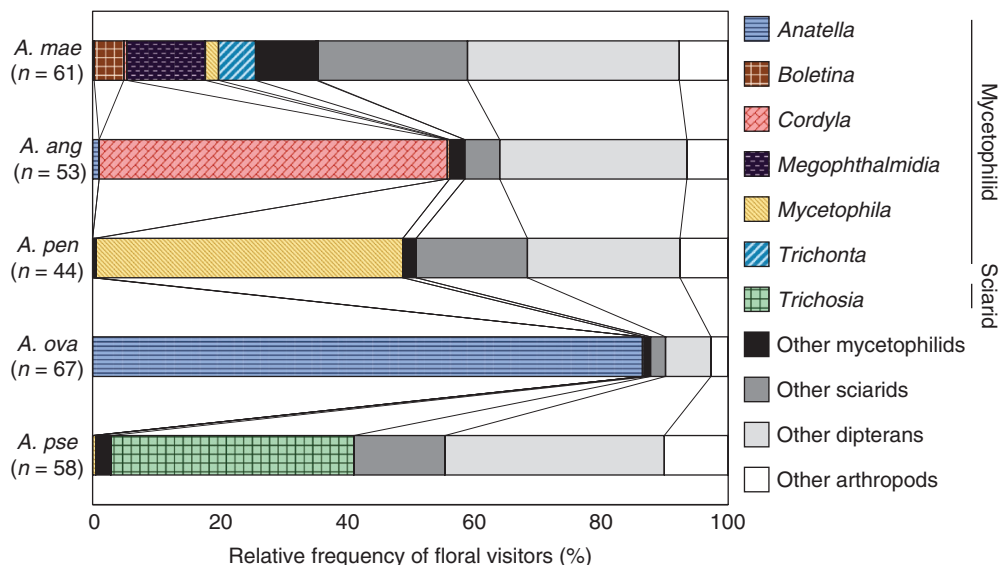

FIG. 4. Floral visitor assemblages of the five examined species in the altitudinal gradient of Wakasugi. 'Other arthropods' includes some arthropods that we failed to collect from inflorescences of the five *Arisaema* species. Species abbreviations as in Fig. 2 legend.

The end date of flowering also differed among the five *Arisaema* species (Kruskal–Wallis test, χ^2 = 214.17, d.f. = 4, $P < 0.001$) (Fig. 3, Supplementary Data Fig. S2B). Based on an *a posteriori* test, *A. maekawae* (138–171) and *A. ovale* (139–166) finished earliest (Fig. 3, Supplementary Data Fig. S2B), followed ~10 d later by *A. angustatum* (148–171) and several weeks later by *A. peninsulae* (162–189) and *A. pseudoangustatum* (161–185) (Fig. 3, Supplementary Data Fig. S2B).

The frequency of insect visitation was uniformly low (mostly fewer than two visitors per day) throughout the flowering periods for all *Arisaema* species (Fig. 3). Insect visitation gradually decreased at the later stage of the flowering season in *A. angustatum*, *A. ovale* and *A. pseudoangustatum* (Fig. 3).

Pollinator isolation

In the altitudinal gradient of Wakasugi in 2018 the floral visitor assemblage differed significantly among species (PERMANOVA, R^2 = 0.38, $P < 0.001$), but not between sexes (R^2 = 0.003, P = 0.18). The interaction between species and sex was not significant (R^2 = 0.007, P = 0.93). All pairs of the five *Arisaema* species were significantly different in the floral visitor assemblage (pairwise PERMANOVA with Bonferroni correction, $P < 0.001$). Although all *Arisaema* species mainly attracted fungus gnats (55.44–90.16 %), the assemblage of fungus gnats differed considerably among them (Supplementary Data Table S1, Fig. 4). *Arisaema maekawae* attracted various genera of fungus gnats, including *Megophthalmidia* spp. (Fig. 1G, 12.35 %), *Trichonta* spp. (5.83 %) and *Boletina* spp. (4.66 %) (Fig. 4). *Arisaema angustatum*, *A. peninsulae* and *A. ovale* attracted the species-specific mycetophilid genera *Cordyla* spp.

(Fig. 1H, 54.79 %), *Mycetophila* spp. (Fig. 1I, 48.30 %) and *Anatella* spp. (Fig. 1J, 86.51 %), respectively (Fig. 4). *Arisaema pseudoangustatum* mainly attracted *Trichosia* spp. (Sciaridae) (Fig. 1K, 38.23 %) (Fig. 4).

In the experimental plots of Wakasugi, in 2019, floral visitor assemblage differed significantly among the five *Arisaema* species (PERMANOVA, R^2 = 0.43, $P < 0.001$) but not between the high- and low-altitude areas (R^2 = 0.02, P = 0.13) (Supplementary Data Table S2, Fig. 5). The interaction between species and elevation was not significant (R^2 = 0.08, P = 0.16). All pairs of the five *Arisaema* species had significantly different floral visitor assemblages (pairwise PERMANOVA with Bonferroni correction, $P \leq 0.01$). Overall, the floral visitor assemblage in the experimental plots was similar to that in the altitudinal gradient of Wakasugi, showing only minor differences in the composition of each insect taxon (Supplementary Data Table S2, Fig. 5).

Outside Wakasugi, in 2019, floral visitor assemblage differed significantly among the five *Arisaema* species (PERMANOVA, R^2 = 0.32, $P < 0.001$) and among sites (R^2 = 0.06, $P < 0.001$). An interaction between species and site was detected (R^2 = 0.03, $P < 0.001$). All combinations of the five *Arisaema* species had significantly different floral visitor assemblages (pairwise PERMANOVA with Bonferroni correction, $P < 0.001$). Differences among sites in floral visitor assemblage were found for *A. maekawae* (PERMANOVA, R^2 = 0.25, $P < 0.001$) but not for *A. angustatum* (R^2 = 0.11, P = 0.09), *A. peninsulae* (R^2 = 0.06, P = 0.53), *A. ovale* (R^2 = 0.10, P = 0.23) and *A. pseudoangustatum* (R^2 = 0.11, P = 0.09) (Supplementary Data Table S4, Fig. 6). *Boletina* spp. (50.85 %) and *Tetragoneura* spp. (16.95 %) exclusively dominated the floral visitors of *A. maekawae* at site B, *Docosia* sp. (31.25 %) at site C, and *Megophthalmidia* spp. (19.51 %) and *Boletina* spp. (17.07 %) at site G (Supplementary Data Table S4, Fig. 6). Contrary to *A. maekawae*, the other four *Arisaema* species consistently
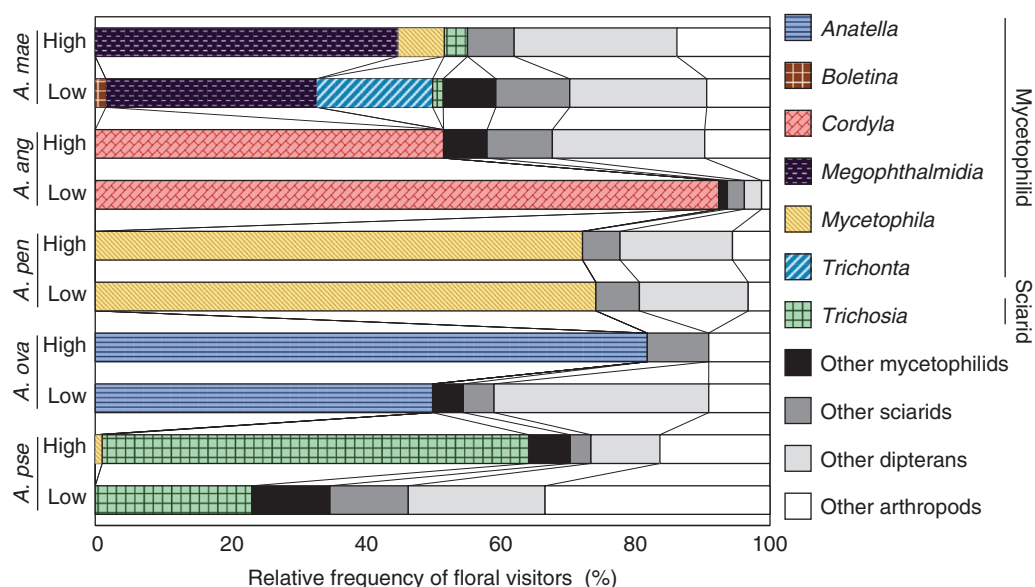

FIG. 5. Floral visitor assemblages of the five examined *Arisaema* species in ten experimental plots established on high- (n = 5) and low-altitude areas (n = 5) of Wakasugi. The number of examined *Arisaema* plants is shown in Table S3. 'Other arthropods' includes some arthropods that we failed to collect from inflorescences of the five *Arisaema* species. Species abbreviations as in Fig. 2 legend.
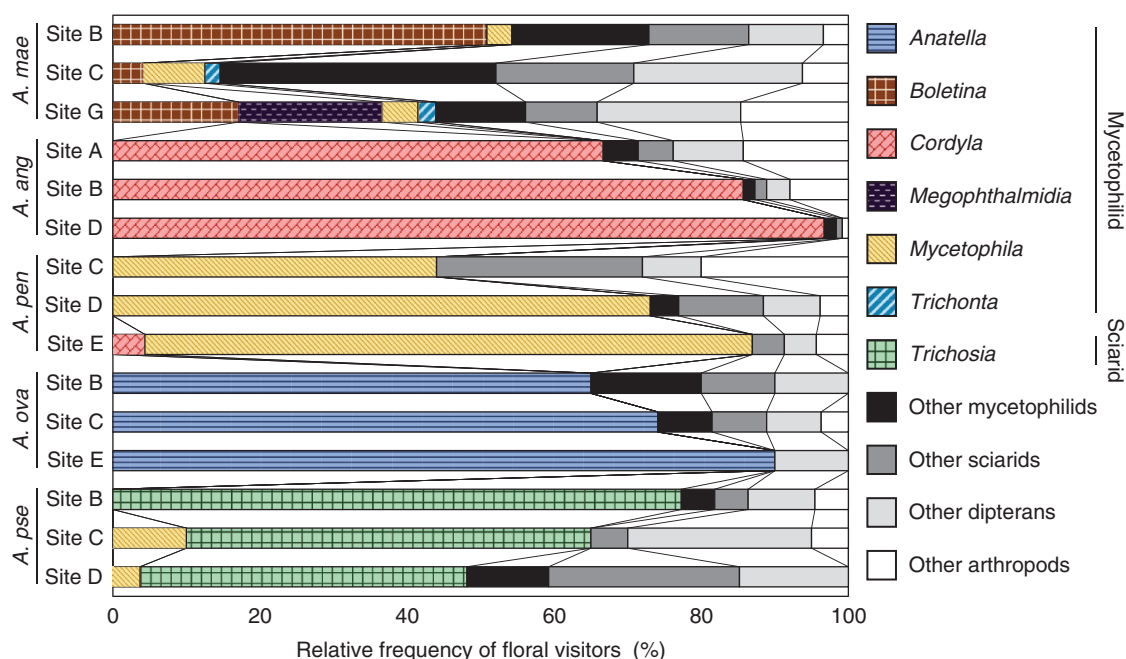
FIG. 6. Floral visitor assemblages of the five examined *Arisaema* species in additional study sites (A–G). 'Other mycetophilids' include some genera (*Docosia* and *Tetragoneura*) mentioned in the text. Detailed information about these sites and the number of examined *Arisaema* plants and floral visitors are shown in Table S3. Species abbreviations as in Fig. 2 legend.

attracted similar fungus gnats in the altitudinal gradient and the experimental plots of Wakasugi (Supplementary Data Table S4, Fig. 6).

Strength of pre-pollination barrier

The strength of geographic isolation (mean ± s.d., 0.50 ± 0.23) ranged from 0.16 to 0.84 among the five *Arisaema* species (Supplementary Data Table S5, Fig. 7A). Geographic isolation was clearly weaker for pairs between lowland species (0.16–0.26) than for pairs between lowland and highland species (0.65–0.84) (Supplementary Data Table S5, Fig. 7A). The absolute contribution of geographic isolation was equal to its strength because we assumed that it occurred before the two other pre-pollination barriers. Geographic isolation contributed more to total reproductive isolation than the other two barriers for twelve of the 20 pairs (Supplementary Data Table S5).

The strength of phenological isolation (mean ± s.d., 0.58 ± 0.29) varied from 0.08 to 0.99 among the five *Arisaema* species (Supplementary Data Table S5, Fig. 7B). Phenological isolation relatively declined among similar phenological groups (i.e. early- or late-flowering) (0.08–0.47) compared with pairs between early- and late-flowering species (0.45–0.99) (Supplementary Data Table S5, Fig. 7B). Although the absolute contribution of phenological isolation (0.30 ± 0.23) varied drastically from 0.03 to 0.77, phenological isolation was the most effective barrier for seven of the 20 pairs (Supplementary Data Table S5).

The strength of pollinator isolation (mean ± s.d., 0.86 ± 0.07) was consistently high (0.74–0.96) in all pairs of the five *Arisaema* species (Supplementary Data Table S5, Fig. 7C). The absolute contribution of pollinator isolation (0.17 ± 0.15)

was limited in almost all pairs, although pollinator isolation amounted to 72 % of the strength of the total pre-pollination reproductive isolation from male *A. angustatum* to female *A. maekawae* (Supplementary Data Table S5).

In 18 of the 20 pairs examined, the strength of total pre-pollination reproductive isolation (mean ± s.d., 0.97 ± 0.03) exceeded 0.96 (Supplementary Data Table S5). The lowest values for reproductive isolation (0.89 and 0.90) were found in the two pairs that consisted of *A. maekawae* and *A. angustatum* (Supplementary Data Table S5).

DISCUSSION

Pollinator specificity of Japanese Arisaema *species*

Fungus gnats were the main visitors to the five *Arisaema* species, as reported for 20 other species (Matsumoto and Senda, 2020), although other dipterans and arthropods might partly contribute to the pollination. Selective visitation by different fungus gnats did not derive from the spatial and/or regional heterogeneity of the fungus gnat fauna (Økland, 1996; Toft *et al.*, 2001; Kurina and Grootaert, 2016), except for *A. maekawae*. Despite the regional pollinator variation of *A. maekawae*, pollinator segregations were observed among all pairs outside Wakasugi. Vogel and Martens (2000) also reported no overlap in floral visitor assemblages between *Arisaema griffithii* (attracts a species-specific mycetophilid) and *Arisaema nepenthoides* (employs various sciarids) in Nepal. *Arisaema* species with low pollinator specificity may selectively attract multiple fungus gnats that are not employed by sympatric congeners, resulting in the pollinator segregation between generalist and specialist *Arisaema* species.
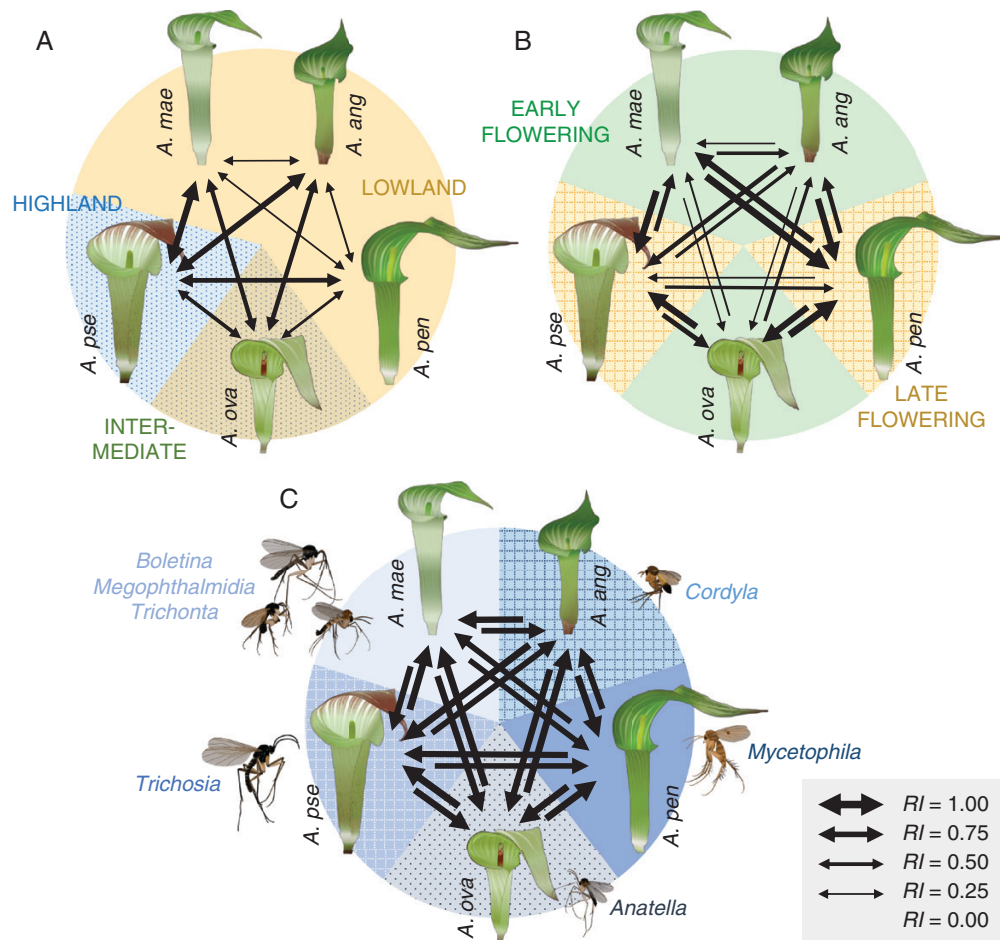
FIG. 7. The strength of each reproductive isolation (*RI*) in the altitudinal gradient of Wakasugi. The arrows indicate the strength of (A) geographic isolation, (B) phenological isolation and (C) pollinator isolation. Shaded areas denote each group of the five *Arisaema* species [e.g. yellow, blue and green areas show lowland, highland and intermediate species, respectively, in (A)]. Since we did not distinguish males and females for the evaluation of geographic isolation, only one arrow is drawn for each species pair in (A). Species abbreviations as in Fig. 2 legend.

Pollinator segregation may prevail among Japanese *Arisaema* species, because selective fungus gnat visitation was also found in three other species (Sasakawa, 1993; Matsumoto *et al.*, 2019; Kakishima *et al.*, 2020). It is tempting to speculate that the pollinator specificity partly accounts for the rapid diversification of Japanese *Arisaema* species (Murata and Kawahara, 1995; Ohi-Toma *et al.*, 2016), as suggested in various plant taxa (Kato *et al.*, 2003; Cosacov *et al.*, 2009; Peakall *et al.*, 2010). In *Arisaema*, the peculiar pollination system, in which the deceived pollinators are died when they visit female flower (Vogel and Martens, 2000), might facilitate the evolution of avoidance behaviour. This will bring about severe pollen limitation especially for specialist *Arisaema* species. High species diversity of fungus gnats across the forest (Økland, 1996; Toft *et al.*, 2001) might provide a substantial pool of alternative pollinators for them and allow rapid diversification through pollinator shift.

Pollinator shift often results from different spathe colour and floral odour in aroids (Gibernau, 2016; Díaz Jiménez *et al.*, 2019). In fungus gnat pollination, flower appearance might not be important for pollinator attraction (Phillips *et al.*, 2014; Katsuhara *et al.*, 2017) but floral odour can be more relevant in selective pollinator visitation (Song *et al.*, 2014; Okamoto

et al., 2015). Also, several deceptive orchids seem to attract species-specific fungus gnats via sexual pheromone mimicry (Blanco and Barboza, 2005; Phillips *et al.*, 2014; Reiter *et al.*, 2019). Selective visitation by fungus gnats might be determined by particular odours emitted from the spadix appendage and/or the spathe (Barnes, 1935; Vogel and Martens, 2000; Kakishima *et al.*, 2019), although scent has not been investigated in any species included in this study. To clarify the relationship between the rapid diversification and fungus gnat pollination, pollinator specificity and floral odour should be comprehensively examined for all Japanese *Arisaema* species.

The role of fungus gnats in reproductive isolation

As a result of the pollinator segregation, pollinator isolation was consistently strong between all pairs, although the strength of geographic and phenological isolations varied greatly depending on species combination. The pollinator isolation in this study (mean ± s.d., 0.86 ± 0.07) was as strong as the reproductive isolation caused by differences in pollinator guilds (0.88–1.00; Ramsey *et al.*, 2003; Dell'Olivo *et al.*, 2011; Sobel

and Chen, 2014) or pollinator taxa (0.83–1.00; Whitehead and Peakall, 2014; Murúa *et al.*, 2017; Cuevas *et al.*, 2018). These results clearly supported the expectation in previous studies that selective flower visitation by fungus gnats can contribute to pollinator isolation (Okamoto *et al.*, 2015; Thalwitzer *et al.*, 2018).

Despite the high strength of pollinator isolation, its absolute contribution to total reproductive isolation was smaller than that of geographic and phenological isolations. The reproductive barriers function sequentially in life history (Ramsey *et al.*, 2003; Sobel and Chen, 2014), and early-acting barriers (i.e. geographic and phenological isolations in this study) can weaken the contribution of late-acting barriers (i.e. pollinator isolation) to total reproductive isolation even when the latter is considerably stronger than the former (Ramsey *et al.*, 2003; Christie and Strauss, 2019). On the other hand, habitat disturbance can disrupt spatial or geographic isolation between parapatric species (Levin *et al.*, 1996; Mitsui *et al.*, 2011; López-Caamal *et al.*, 2014), and the strength of phenological isolation can fluctuate greatly due to annual climatic variation (Masuda *et al.*, 2004; Franks and Weis, 2009; Christie and Strauss, 2019).

Contrary to the two early-acting barriers, pollinator isolation may be more stable in *Arisaema*. In the experimental plots, the flowering phenology of three early-flowering species was clearly delayed by 3–5 weeks compared with that under natural conditions. Moreover, *A. maekawae* and *A. pseudoangustatum* were transplanted outside their altitudinal range. Nevertheless, the species-specific fungus gnats consistently pollinated different *Arisaema* species, indicating the high stability of pollinator specificity. Matsumoto *et al.* (2019) also showed that *Arisaema sikokianum* consistently attracted mushroom-eating insects even when flowering phenology was artificially delayed. Since late-acting barriers can effectively compensate for weakened early-acting barriers (Christie and Strauss, 2019), the strong and stable pollinator isolation mediated by fungus gnats can guarantee the prevention of interspecific hybridization as an alternative reproductive isolation, and contribute to the coexistence of the five *Arisaema* species along the altitudinal gradient of Wakasugi.

The cryptic role of fungus gnats in reproductive isolation could occur much more frequently than expected, because their species-specific pollination was also found in three other plant families (Okuyama *et al.*, 2004; Song *et al.*, 2014, 2015; Thalwitzer *et al.*, 2018). On the other hand, such pollinator isolation may be indirectly affected by some environmental changes because the fungus gnat community is sensitive to clear-cut logging (Økland, 1994), habitat fragmentation (Økland, 1996), forest vegetation (Sueyoshi, 2018) and exotic weed invasion (Toft *et al.*, 2001). In temperate zones, fungus gnat-pollinated plant population is seriously disturbed by increasing deer herbivory (Kato and Okuyama, 2004; Heckel and Kalisz, 2016; Matsumoto *et al.*, 2020). Our results suggest that these habitat disturbances not only may bring about the decline of fungus gnats, but also have unpredictable effects on plant species diversity. Studies on fungus gnat pollination should continue to be conducted to understand the fascinating plant–pollinator interactions.

SUPPLEMENTARY DATA

Supplementary data are available online and consist of the following. Figure S1: flowering phenology of the five *Arisaema* species in the experimental plots. Figure S2: flowering start and end dates of the five examined species in the altitudinal gradient of Wakasugi. Table S1: information on the assemblage of floral visitors and visitation frequency in the altitudinal gradient of Wakasugi. Table S2: information on the assemblage of floral visitors and visitation frequency in ten experimental plots. Table S3: information about additional study sites. Table S4: information on the assemblage of floral visitors and visitation frequency in additional study sites. Table S5: information on the strength of each of the reproductive isolations and their absolute contributions to total reproductive isolation among the five species in the altitudinal gradient of Wakasugi.

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