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(Citation)

Plants, People, Planet, Early View

(Issue Date)

2026-07-30

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

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(URL)

<https://hdl.handle.net/20.500.14094/0100505431>



Multiple barriers maintain species boundaries in sympatric brood-site deceptive *Gastrodia* orchids

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Funding information

Fusion Oriented REsearch for disruptive Science and Technology, Grant/Award Number: JPMJFR2339; MEXT Promotion of Distinctive Joint Research Center Program, Grant/Award Number: JPMXP0723833155; Japan Science and Technology Agency; Japan Society for the Promotion of Science, Grant/Award Number: JP25H00944

Societal Impact Statement

Gastrodia orchids are inconspicuous forest plants that lack chlorophyll and obtain carbon from fungi. Their dull-colored, ground-level flowers are often hidden among leaf litter, yet they rely on small flies for pollination. We show that two co-occurring *Gastrodia* species remain distinct even though they share similar pollinators. Limited fly movement and reduced reproductive success after cross-species pollination restrict hybrid formation, while fungal requirements may impose additional barriers after seed formation. These findings show how multiple barriers can preserve species differences in cryptic forest orchids and highlight the need to conserve the insects, fungi, and forest-floor microhabitats that support such hidden life histories.

Summary

- Because orchids show remarkable floral diversity and often rely on specific pollinators, pollinator-mediated isolation has traditionally been regarded as the principal reproductive barrier in this family. However, growing evidence indicates that additional mechanisms also contribute to reproductive isolation. *Gastrodia confusa* and *Gastrodia pubilabiata* attract similar drosophilid pollinators through brood-site deception, probably by mimicking fermenting substrates. Despite habitat overlap, morphologically intermediate individuals have not been reported in the wild, suggesting that reproductive barriers other than pollinator specificity restrict natural hybrid formation.
- This study examined the mechanisms and strength of reproductive isolation between these two sympatric *Gastrodia* species by integrating field observations, pollen tube observations, controlled pollination experiments, and molecular analyses.
- Field observations showed that the two species shared largely overlapping drosophilid pollinator assemblages at sympatric sites, but the short-distance movements of individual flies likely limited interspecific pollen transfer under natural spatial arrangements. Interspecific hand-pollination reduced fruit set and the proportion of seeds with well-developed embryos but did not cause complete reproductive

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failure. Molecular analyses detected no F_1 -like genomic profiles and provided only limited evidence of possible natural introgression between *G. confusa* and *G. pubilabiata* at sympatric sites.

- These findings suggest that species boundaries between the two *Gastrodia* species are maintained not by a single complete barrier but by the cumulative effects of multiple partial prepollination and postpollination barriers. Future studies should determine whether seeds produced by interspecific pollination can germinate, whether hybrid seedlings can establish successfully, and whether hybrids retain compatibility with the fungal partners required by either parental species.

KEYWORDS

brood-site deception, *Gastrodia confusa*, *Gastrodia pubilabiata*, hybridization, mycoheterotrophy, postpollination barriers, prepollination barriers, reproductive isolation

1 | INTRODUCTION

Orchidaceae is one of the most species-rich angiosperm lineages and is renowned for its extraordinary diversity of pollination strategies, ranging from reward-based systems to highly specialized forms of floral deception (Cozzolino & Widmer, 2005; Darwin, 1862; Jersáková et al., 2006).

Unlike most flowering plants, which attract pollinators by providing nectar or other rewards, many orchids attract visitors without offering any reward. Although deceptive pollination has evolved independently in several angiosperm clades, it is usually restricted to relatively few species within each family (Jersáková et al., 2006; Renner, 2006). In contrast, nearly half of all orchid species are thought to rely primarily on deception (Ackerman et al., 2023). Food deception is the most common strategy and typically exploits generalized pollinator responses to rewarding flowers without providing nectar or pollen (Ackerman et al., 2023; Jersáková et al., 2006; Johnson & Schiestl, 2016). Other strategies include sexual deception, in which flowers mimic female insect signals to attract males, Batesian floral mimicry, in which rewardless flowers resemble specific rewarding species, and brood-site deception, in which flowers mimic oviposition substrates such as fermenting fruit, carrion, dung, or fungi (Johnson & Schiestl, 2016; Schiestl, 2005; Urru et al., 2011). Brood-site deception is comparatively widespread in orchids, occurring in an estimated 11% of orchid genera (Stökl et al., 2011).

Orchid pollination systems are often highly specialized, involving only one or a few effective pollinator species. Specificity varies among pollination strategies, being the highest in sexually deceptive orchids and somewhat lower in nectar-rewarding and generalized food-deceptive species (Ackerman et al., 2023; Phillips et al., 2009; Schiestl & Schlüter, 2009). Nevertheless, strong pollinator specificity is characteristic of Orchidaceae overall, with a median of one known pollinator species per orchid species across the family (Ackerman et al., 2023; Tremblay, 1992).

Such pollinator specificity likely promotes prepollination reproductive isolation and facilitates coexistence among closely related

species (Pauw, 2013; Phillips et al., 2017; Schiestl & Schlüter, 2009). Phylogenetic analyses across diverse orchid clades also show that speciation is often associated with shifts in pollinator type, suggesting that pollinator shifts can drive orchid diversification (Johnson et al., 1998; Ramírez et al., 2011; van der Niet & Johnson, 2012).

However, accumulating evidence indicates that postpollination barriers are also important for maintaining species integrity in orchids, particularly among food-deceptive species, which often show somewhat lower pollinator specificity (Cozzolino et al., 2004; Pellegrino et al., 2010; Scopece et al., 2007; Waterman & Bidartondo, 2008). Even in sexually deceptive systems, pollinator sharing may occur through chemical convergence (Cortis et al., 2009; Phillips et al., 2013, 2017), and some sexually deceptive species can hybridize in sympatry because of overlapping pollinators and weak mechanical isolation (Cortis et al., 2009). In such cases, postpollination barriers, including reduced hybrid fitness, may restrict hybrid formation and maintain species boundaries.

This study focuses on two fully mycoheterotrophic, brood-site deceptive orchids, *Gastrodia confusa* Honda & Tuyama and *Gastrodia pubilabiata* Y.Sawa. As one of the most species-rich genera of fully mycoheterotrophic plants, *Gastrodia* provides an excellent system for investigating how pollination, fungal dependence, reproductive isolation, and diversification interact in plants that have lost photosynthesis (Martos et al., 2015; Suetsugu, 2022; Suetsugu et al., 2025; Suetsugu & Yamamoto, 2024; Waterman & Bidartondo, 2008). Although brood-site deception is common in orchids (Stökl et al., 2011), pollinator specificity and reproductive isolation among closely related brood-site deceptive orchids have received far less attention than those in food-deceptive and sexually deceptive systems.

A recent study showed that *G. confusa* and *G. pubilabiata* share similar assemblages of drosophilid pollinators attracted to fermenting substrates, at least at allopatric sites (Suetsugu et al., 2026). Although autonomous self-pollination is common in fully mycoheterotrophic plants (Suetsugu, 2015, 2022; Waterman et al., 2013), it has not been

detected in the populations examined for either species (Suetsugu et al., 2026). Furthermore, no putative hybrids with intermediate morphology have been recorded, even under sympatric conditions (Nakajima, 2012; Yukawa, 2015), although hybridization has been reported in other *Gastrodia* species pairs (Suetsugu et al., 2018).

Reproductive character displacement may evolve in sympatry to reduce the costs of hybridization and reproductive interference (Joffard et al., 2022). Potential forms include divergence in flowering phenology, pollinator assemblages, floral morphology, or mating systems. For example, increased selfing may evolve in competitively inferior taxa as a response to reproductive interference from closely related taxa (Katsuhara et al., 2021; Randle et al., 2018).

Here, we examined the mechanisms and strength of prepollination and postpollination reproductive isolation between two sympatric *Gastrodia* species by integrating field observations, controlled pollination experiments, pollen–pistil observations, and molecular analyses. Specifically, we addressed the following questions: (i) Does either species show autonomous self-pollination at sympatric sites? (ii) Is there evidence of divergence in pollinator assemblages between *G. confusa* and *G. pubilabiata* at sympatric sites? (iii) How frequently does interspecific pollen transfer occur via shared drosophilid pollinators? (iv) To what extent does interspecific pollination reduce fruit set and the proportion of seeds with well-developed embryos? (v) Is there molecular evidence of natural introgression between *G. confusa* and *G. pubilabiata*, despite the absence of morphologically intermediate individuals?

2 | MATERIALS AND METHODS

2.1 | Study species

Gastrodia pubilabiata and *G. confusa* are the only *Gastrodia* species on the Japanese mainland known to flower from September to October (Inoue, 2016; Suetsugu et al., 2026). Although *G. confusa* typically flowers 1–2 weeks later than *G. pubilabiata*, variation among individuals is substantial in populations with more than 100 flowering individuals, extending the flowering period at each site to approximately 3–4 weeks. Thus, at sympatric sites, including the three examined here, the flowering periods of the two species usually overlap for approximately 10–20 days, during which individuals occasionally flower simultaneously in close proximity (Figure 1).

The two species were identified in the field by the first author based on diagnostic morphology, following Inoue (2016) and subsequent taxonomic treatments of Japanese *Gastrodia* (Suetsugu, 2019, 2021; Suetsugu et al., 2018; Suetsugu, Aoki, & Kaneko, 2023). *Gastrodia pubilabiata* has dark brown to blackish-brown flowers, a densely pubescent lip, and a short scape, 2–5 cm long, whereas *G. confusa* has lighter greenish-brown flowers, a glabrous lip, and a taller scape, 5–15 cm long. Voucher specimens from one representative flowering individual of each species at each site were deposited in the herbarium of the Department of Biology, Graduate School of Science, Kobe University.

2.2 | Pollinator observations

Fieldwork was conducted from 2012 to 2025 at three sites where *G. confusa* and *G. pubilabiata* co-occur: Higashiomi, Shiga Prefecture, Japan (35.06°N, 136.19°E); Nagoya, Aichi Prefecture, Japan (35.22°N, 136.99°E); and Hachioji, Tokyo, Japan (35.63°N, 139.37°E). At each site, more than 100 flowering individuals of each species were observed over the study period. Pollinator observations focused on cases in which the two species occurred adjacent to one another and flowered simultaneously, allowing potential pollen transfer between the two species to be observed. In the mixed patches used for behavioral observations, the nearest flowering inflorescences of the two species were often less than 20 cm apart. Pollinators were defined operationally as insects that entered *Gastrodia* flowers and exited carrying pollinaria. Observations were conducted during daylight hours (07:00–18:00) in 2- to 8-h sessions, with total observation times of 70 h in Shiga, 60 h in Aichi, and 30 h in Tokyo.

Pollinator behavior was recorded in detail, including approaches to flowers, entry into the trap chamber formed by the labellum and column, acquisition of pollinaria, and the number of flowers pollinated per visit. For visitors moving among inflorescences, we recorded the number of inflorescences visited, whether they were conspecific or heterospecific, and the distances moved. These observations provided the behavioral basis for evaluating whether short-distance fly movements could facilitate or limit interspecific pollen transfer under natural spatial arrangements.

Departing visitors were captured with a sweep net or aspirator for species identification and molecular analysis, allowing pollinator identity, the species identity of attached pollinaria, and potential interspecific pollen transfer to be assessed. In total, 228 drosophilid pollinarian carriers were collected for morphological identification, comprising 125 from *G. confusa* and 103 from *G. pubilabiata* (Table 1). Of these, 227 specimens were identified to species, whereas one female could not be confidently distinguished between *Drosophila auraria* Peng and *Drosophila rufa* Kikkawa & Peng using diagnostic morphology. Drosophilid visitors were identified by the last author using external morphology and, when necessary, male terminalia, following Okada (1956), Toda et al. (1990), Bächli et al. (2004), and Markow and O'Grady (2006). Pollinaria carried by 25 of these drosophilid flies were used for molecular identification of orchid origin by MIG-seq or Sanger sequencing. Voucher specimens were deposited in the Department of Biology, Graduate School of Science, Kobe University.

2.3 | Artificial pollination experiments

At the Shiga site, 20 individuals of each species were enclosed in fine nylon mesh bags before anthesis. Pre-anthesis plants were selected along a 150 m × 50 m transect, with individuals separated by at least 2 m to reduce repeated sampling of the same clone. Although *Gastrodia* species propagate clonally via tubers, vegetative spread is typically less than 1 m (Suetsugu et al., 2014), making it unlikely that plants separated by at least 2 m belonged to the same genet.

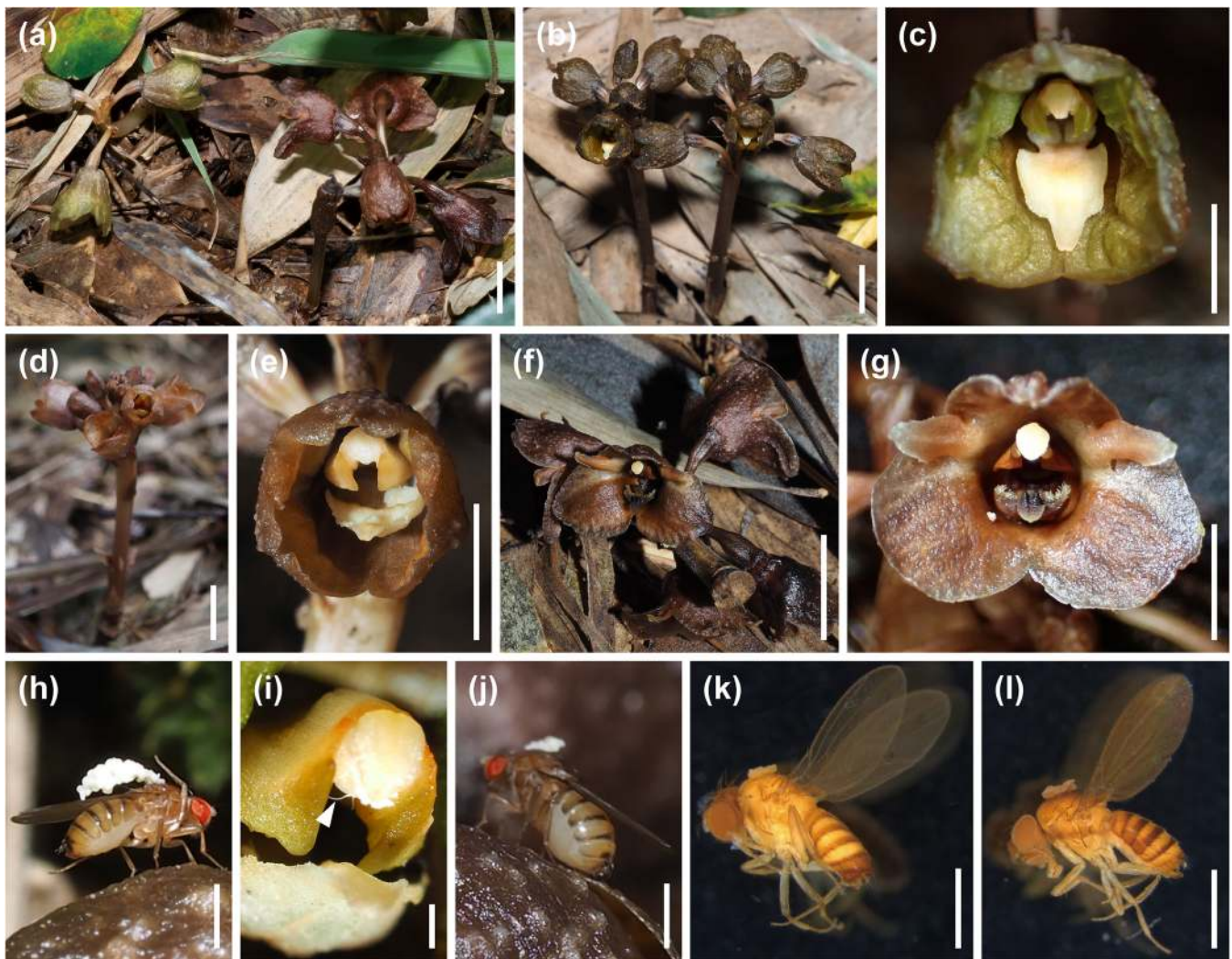


FIGURE 1 Pollination ecology of *Gastrodia confusa* and *G. pubilabiata*. (a) *Gastrodia confusa* (left) and *G. pubilabiata* (right) growing sympatrically. (b, c) Flowering plants and a flower of typically colored *G. confusa*. (d, e) Flowering plant and flower of *G. confusa* with a brown floral tube. (f, g) Flowering plant and flower of *G. pubilabiata*. (h) *Drosophilid* pollinator visiting *G. confusa*. (i) Flower of *G. confusa* containing a *drosophilid* egg; the arrowhead indicates the dorsal appendages of the egg. (j) *Drosophilid* pollinator visiting *G. pubilabiata*. (k) *Drosophila rufa* carrying pollinaria of *G. confusa*. (l) *D. rufa* carrying pollinaria of *G. pubilabiata*. Scale bars: 1 cm (a, b, d, f), 5 mm (c, e, g), and 1 mm (h–l). Photographs by Kenji Suetsugu.

Four pollination treatments were applied to assess breeding system, pollinator limitation, and hybridization potential: (1) autonomous self-pollination, in which flowers were bagged from pre-anthesis until the end of anthesis to exclude pollinators; (2) hand self-pollination, in which pollinaria were removed and manually applied to the stigma of the same flower, followed by bagging; (3) hand cross-pollination, conducted as in Treatment 2 but using pollinaria from a conspecific individual at least 2 m away; and (4) interspecific hand-pollination, conducted as in Treatment 2 but using pollinaria from a heterospecific individual. Interspecific crosses were performed reciprocally, with each species serving as the maternal parent. In *G. confusa*, inflorescences bearing at least four flowers were preferentially selected, allowing all four treatments to be applied to the same individual and resulting in 20 flowers, one from each of 20 individuals, for each treatment. Because many *G. pubilabiata* individuals bore only three flowers, each individual received three of the

four treatments; the omitted treatment was rotated among individuals so that all treatments were represented, resulting in 15 flowers, one from each of 15 individuals, for each treatment. After pollination, inflorescences of both species were rebagged to prevent insect access. To estimate fruit set under open pollination, we marked an additional 15 unmanipulated *G. confusa* individuals bearing 70 flowers and 19 *G. pubilabiata* individuals bearing 65 flowers before anthesis and left them accessible to pollinators.

Plants were monitored for 4 weeks, and fruit set, defined as the proportion of flowers that developed into fruits, was compared among treatments. In addition, the proportion of seeds with well-developed embryos was assessed just before full maturation because seed traits cannot be reliably evaluated after capsule dehiscence. For each capsule, 100 seeds were randomly selected and examined under a microscope for the presence or absence of a well-developed embryo.

TABLE 1 Species composition of drosophilid flies carrying *Gastrodia* pollinaria at sympatric sites, with data presented separately for each site.

Visited orchid species (site)	Drosophilid species	Males	Females
<i>Gastrodia confusa</i> (Aichi)	<i>Drosophila bizonata</i>	4	1
	<i>Drosophila lutescens</i>	0	17
	<i>Drosophila rufa</i>	2	9
	<i>Drosophila sternopleuralis</i>	0	12
	<i>Drosophila suzukii</i>	2	0
	<i>Liodrosophila aerea</i>	0	1
<i>Gastrodia confusa</i> (Shiga)	<i>Drosophila lutescens</i>	1	20
	<i>Drosophila rufa</i>	2	19
	<i>Drosophila sternopleuralis</i>	0	3
	<i>Drosophila simulans</i>	1	0
	<i>Drosophila auraria</i> or <i>D. rufa</i>	0	1
<i>Gastrodia confusa</i> (Tokyo)	<i>Drosophila bizonata</i>	2	6
	<i>Drosophila lutescens</i>	2	5
	<i>Drosophila rufa</i>	2	4
	<i>Drosophila sternopleuralis</i>	1	8
<i>Gastrodia pubilabiata</i> (Aichi)	<i>Drosophila bizonata</i>	1	0
	<i>Drosophila lutescens</i>	2	5
	<i>Drosophila rufa</i>	3	4
	<i>Drosophila sternopleuralis</i>	1	8
	<i>Drosophila suzukii</i>	2	0
<i>Gastrodia pubilabiata</i> (Shiga)	<i>Drosophila lutescens</i>	0	1
	<i>Drosophila rufa</i>	3	24
	<i>Drosophila sternopleuralis</i>	0	17
	<i>Liodrosophila aerea</i>	0	2
<i>Gastrodia pubilabiata</i> (Tokyo)	<i>Drosophila bizonata</i>	0	8
	<i>Drosophila lutescens</i>	1	4
	<i>Drosophila rufa</i>	2	4
	<i>Drosophila sternopleuralis</i>	6	3
	<i>Liodrosophila aerea</i>	0	2

Fruit set and the proportion of seeds with well-developed embryos were analyzed using generalized linear mixed models (GLMMs) with binomial error distributions and logit link functions. Treatment was included as a fixed effect, and plant identity was included as a random effect to account for non-independence among flowers from the same individual. Pairwise comparisons were performed using Tukey-adjusted post hoc tests.

2.4 | Pollen–pistil interactions

To examine the postpollination barrier suggested by reduced fruit set and the lower proportion of seeds with well-developed embryos after interspecific hand-pollination, we assessed pollen germination and pollen tube growth after intraspecific and interspecific pollination

using an aniline blue staining method adapted from Pellegrino et al. (2010), with minor modifications.

For each species, 10 flowers from 10 individuals received interspecific pollination, and another 10 flowers from 10 individuals received intraspecific cross-pollination. Because this procedure was destructive, it was conducted independently of the hand-pollination experiments.

At 72 h after pollination, columns and ovaries were excised, softened in 1 M KOH at 60°C for 30 min, rinsed in distilled water, and stained with 0.1% aniline blue in K_3PO_4 buffer (pH 8.5) for 2 h at room temperature. Samples were then rinsed, mounted in 50% glycerol, gently squashed, and examined using a fluorescence microscope (BX60; Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a U-MNIBA filter cube for interference-blue excitation (excitation filter BP 470–490 nm, dichroic mirror DM 505 nm, and barrier filter BA 515–550 nm) to visualize pollen tubes.

2.5 | Molecular analysis

Because morphology alone cannot reliably detect introgression, we used MIG-seq to obtain genome-wide SNP markers suitable for assessing genomic separation and potential admixture, following Suyama et al. (2022). In total, 88 plants identified in the field as *G. confusa* and 38 identified as *G. pubilabiata* were sampled. Among the *G. confusa* samples, two individuals from the Tokyo site had brown floral tubes that partly resembled those of *G. pubilabiata* in coloration but otherwise showed no clear deviation from typical *G. confusa*; these individuals were therefore included for detailed assessment of internal floral morphology and genotyping.

DNA libraries were also prepared from pollinaria collected from 25 drosophilid flies visiting *G. confusa* and *G. pubilabiata* (Dataset S1), because pollinarium morphology did not allow species-level identification and molecular identification was required when complete visitation sequences were unavailable. Sequencing was performed on two platforms. A total of 141 samples were sequenced on a MiSeq system (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit v3 (150 cycles) with 80-bp paired-end reads. The remaining 10 samples were sequenced on an AVITI24 platform (Element Biosciences, San Diego, CA, USA) using an AVITI Cloudbreak Freestyle 2 × 75 Sequencing Kit, generating 58-bp paired-end reads. After quality control, 144 samples retained sufficient reads for downstream analyses. Raw reads were deposited in the DDBJ under BioProject accession number PRJDB42663. For both the MiSeq and AVITI24 sequencing platforms, the first 17 sequencing cycles, corresponding to the SSR and anchor regions, were skipped for both Read 1 and Read 2 using the DarkCycle option.

Primer sequences and low-quality reads were removed using Trimmomatic 0.39 (Bolger et al., 2014). To standardize read length across sequencing platforms, MiSeq reads were trimmed to 58 bp. After quality control, 25,791,349 reads (mean ± SD = 179,107 ± 15,404 per sample) were retained from 33,016,848 raw reads (mean ± SD = 229,284 ± 17,330 per sample). Quality-controlled reads were aligned to the genome of *Gastrodia menghaiensis* Z.H.Tsi & S.C.Chen (Jiang et al., 2022) using BWA 0.7.17 (Li, 2013) with default settings. SNPs were called using the Stacks 2.68 pipeline (Rochette et al., 2019). In the populations program, SNPs with high heterozygosity ($H_o \geq 0.6$) or a minor allele count < 3 were excluded. Only the first SNP per locus was retained to avoid linkage bias. To minimize platform-specific bias between the MiSeq and AVITI24 datasets, only SNPs detected in at least 50% of the samples sequenced on each platform were retained for downstream analyses. After filtering, 262 SNPs were used for population genetic analyses.

A maximum-likelihood tree was inferred with RAxML v8.2.10 (Stamatakis, 2014) using a GTR model with Lewis's ascertainment-bias correction (Lewis, 2001) and 1000 bootstrap replicates. A Neighbor-Net network was generated in SplitsTree App 6.4.13 (Huson & Bryant, 2024) based on an uncorrected *p*-distance matrix after excluding ambiguous sites. Population structure was analyzed in STRUCTURE 2.3.4 (Pritchard et al., 2000), with 30 replicates per *K*, 100,000 burn-in iterations, and 100,000 MCMC steps. The most likely *K* was identified using the ΔK method (Evanno et al., 2005) in Structure

Harvester (Earl & vonHoldt, 2012), and results were summarized in CLUMPAK (Kopelman et al., 2015). Because experimentally produced *F*₁ reference genotypes were unavailable, individuals were regarded as putative *F*₁-like hybrids when they showed genomic profiles with a mixture of SNP patterns characteristic of both parental species, particularly near-equal STRUCTURE assignment to the two parental clusters and intermediate placement in phylogenetic or network analyses.

Because prolonged ethanol storage likely reduced SNP recovery from some pollinaria attached to drosophilid individuals in MIG-seq, pollinaria that could not be assigned by MIG-seq, together with selected adult voucher samples and selected pollinaria for confirmation, were identified by Sanger sequencing of the nuclear internal transcribed spacer (ITS) region. The ITS region was amplified using the universal primers IT1 (5'-TCGTAACAAGGTTTCCGTAGGT-3') and IT2 (5'-GTAAGTTTCTTCTCCTCCGCT-3') (Tsai et al., 2004). PCR products were purified with Exo-SAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced bidirectionally using the amplification primers, a BigDye Terminator v3.1 Cycle Sequencing Kit, and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The resulting sequences were deposited in GenBank under accession numbers LC885095–LC885114.

3 | RESULTS

3.1 | Concordant drosophilid visitation patterns

Gastrodia confusa and *G. pubilabiata* were pollinated by similar assemblages of drosophilid flies that exhibited comparable behaviors at sympatric sites (Figures 1 and 2). In all three populations, the pollinator assemblages of both species were dominated by fruit-feeding drosophilids, particularly *D. rufa* Kikkawa & Peng, 1938, *Drosophila sternopleuralis* Okada & Kurokawa, 1957, and *Drosophila lutescens* Okada, 1975, although both orchids were also occasionally pollinated by mycophagous species, including *Drosophila bizonata* Kikkawa & Peng, 1938, at the Aichi and Tokyo sites (Table 1). Minor differences in pollinator composition among sites likely reflected local variation in drosophilid communities because both orchids were pollinated by largely the same drosophilid taxa. However, *D. lutescens* contributed more frequently to pollination in *G. confusa* than in *G. pubilabiata* in all populations, suggesting modest differences in pollinator composition.

Drosophilid behavior was consistent with previous observations of these two *Gastrodia* species (Suetsugu et al., 2026). Flies typically landed on the floral surface or labellum tip and frequently entered the trap chamber formed by the labellum and column. Female drosophilids were occasionally observed ovipositing on the labellum, perianth tube, or column. Once inside the chamber, the labellum lifted, guiding flies toward the reproductive column and confining them within a narrow floral tunnel. The only exit was located between two toothlike structures near the anther, where flies acquired pollinaria on the dorsal thorax. Pollinarium-bearing flies often remained on the flowers or perched on nearby branches, repeatedly attempting to remove the pollinaria with their legs before eventually ceasing and flying away

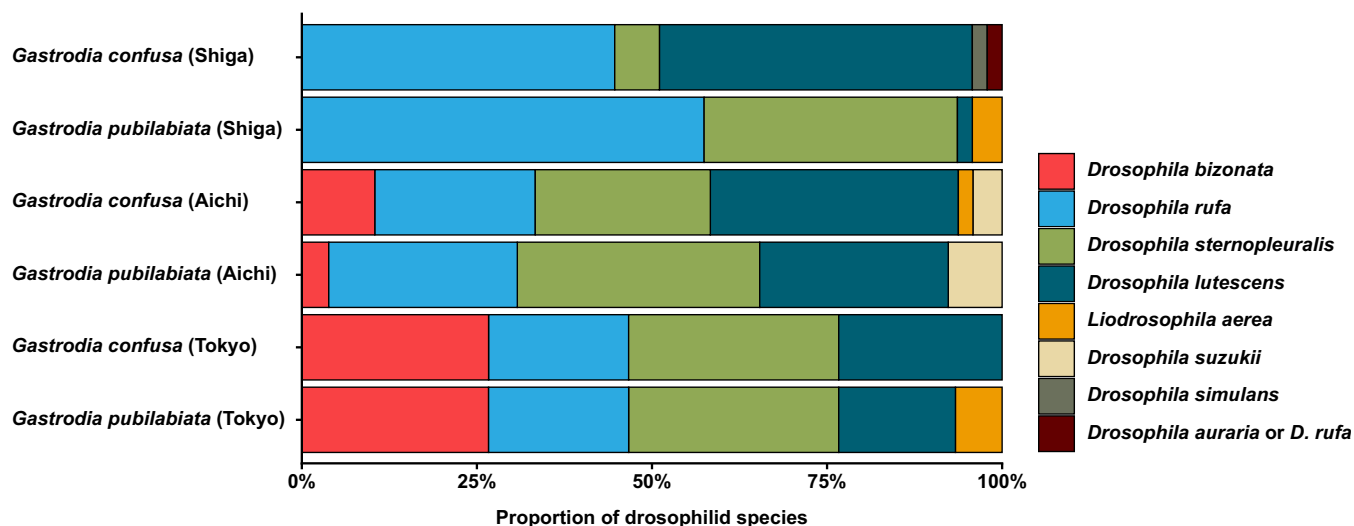


FIGURE 2 Proportional species composition of pollinarium-carrying drosophilid flies associated with *Gastrodia confusa* and *G. pubilabiata* at sympatric sites. Bars show the proportional composition of drosophilid taxa recorded from each orchid species at the Shiga, Aichi, and Tokyo sites. Colors indicate drosophilid taxa.

(Figure 1). Although some flies ultimately moved beyond the observation area, many remained on or returned to the same inflorescence, visiting the same or other flowers within it.

When complete visitation sequences were observed, flies visited multiple flowers within a single inflorescence in 40.0%–66.7% of cases for *G. confusa* and 50.0%–66.7% of cases for *G. pubilabiata*. When additional conspecific or heterospecific plants were present nearby, flies occasionally moved among individuals, creating opportunities for both intraspecific and interspecific pollen transfer. The observed between-flower movement distances of pollinarium-bearing flies (mean $\pm$ SE) were 39.9 ± 19.6 cm ($n = 11$) between *G. confusa* individuals, 20.8 ± 8.3 cm ($n = 4$) from *G. confusa* to *G. pubilabiata*, 23.4 ± 14.4 cm ($n = 5$) from *G. pubilabiata* to *G. confusa*, and 53.0 ± 24.3 cm ($n = 12$) between *G. pubilabiata* individuals.

3.2 | Pollinator limitation and reduced interspecific reproductive success

All *G. confusa* and *G. pubilabiata* flowers bagged before anthesis failed to set fruit (Table 2), confirming the absence of autonomous self-pollination. By contrast, hand self-pollination produced high fruit set in both species (86.7% in *G. pubilabiata* and 85.0% in *G. confusa*), and hand cross-pollination yielded similarly high fruit set (86.7% in *G. pubilabiata* and 90.0% in *G. confusa*). In both species, hand self- and cross-pollination produced significantly higher fruit set than open pollination (30.8% in *G. pubilabiata* and 34.3% in *G. confusa*; $P < 0.01$ for all relevant comparisons), indicating pollinator limitation.

Reciprocal interspecific hand-pollination between *G. confusa* and *G. pubilabiata* produced fruit set of 50.0% when *G. confusa* was the maternal parent and 26.7% when *G. pubilabiata* was the maternal parent. In *G. pubilabiata*, interspecific pollination resulted in significantly

lower fruit set than either self- or cross-pollination ($P < 0.05$). In *G. confusa*, fruit set after interspecific pollination was numerically lower than after cross-pollination ($P = 0.052$) and self-pollination ($P = 0.103$), but these differences were not statistically significant.

After both intraspecific and interspecific pollen deposition on stigmas, pollen tubes emerged from massulae, entered the stigma, and reached the ovary within 72 h (Figure 3). The proportion of seeds with well-developed embryos did not differ among hand self-pollination, hand cross-pollination, and open pollination in either species ($P > 0.15$ in all cases). In contrast, interspecific pollination in both directions produced significantly lower proportions of embryo-bearing seeds than any other treatment in either species ($P < 0.001$ in all comparisons).

3.3 | Limited interspecific pollen transfer and introgression

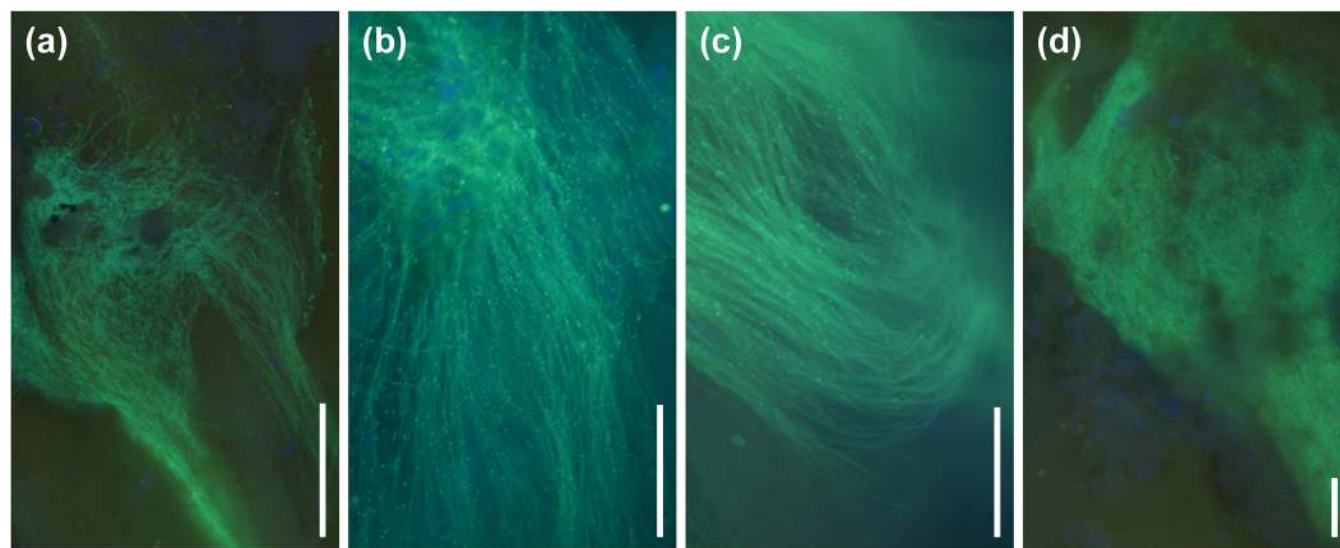
MIG-seq SNP genotyping revealed clear genetic separation between the two species at sympatric sites. Neither the maximum-likelihood tree nor the Neighbor-Net analysis identified individuals with clearly intermediate, F_1 -like genomic profiles, although a few individuals were positioned slightly closer to the other species. STRUCTURE supported these patterns, with the highest ΔK at $K = 2$, indicating two genetic clusters corresponding to *G. confusa* and *G. pubilabiata* (Figure 4).

Although two field-identified *G. confusa* individuals were positioned slightly nearer to *G. pubilabiata* in the Neighbor-Net network and showed weak STRUCTURE admixture signals, and one putative *G. pubilabiata* individual showed a similar pattern, their floral color, lip pubescence, and scape height matched the diagnostic morphology of their assigned species. Their placement within species-specific lineages in the maximum-likelihood tree further suggests that these weak admixture signals do not provide strong evidence of recent

TABLE 2 Effects of pollination treatments on fruit set and the proportion of seeds with well-developed embryos in *Gastrodia confusa* and *Gastrodia pubilabiata* at the Shiga site.

Plant species	Treatment	Bagged	Hand self-pollination	Hand cross-pollination	Interspecific pollination	Open
<i>Gastrodia confusa</i>	Fruit set (%)	0 (20)	85.0 ^a (20)	90.0 ^a (20)	50.0 ^{ab} (20)	34.3 ^b (70)
	Seeds with well-developed embryos (%)	—	87.6 ± 4.8 ^b	86.8 ± 4.4 ^b	51.3 ± 12.7 ^a	87.9 ± 4.3 ^b
<i>Gastrodia pubilabiata</i>	Fruit set (%)	0 (15)	86.7 ^b (15)	86.7 ^b (15)	26.7 ^a (15)	30.8 ^a (65)
	Seeds with well-developed embryos (%)	—	87.0 ± 4.7 ^b	89.1 ± 3.9 ^b	18.8 ± 17.0 ^a	87.4 ± 4.2 ^b

Note: Fruit set is expressed as the percentage of flowers that developed into fruits, with sample sizes shown in parentheses. The proportion of seeds with well-developed embryos is presented as mean ± SD. Different superscript letters within each row indicate significant differences among treatments ($P < 0.05$). Bagged treatments were not included in post hoc comparisons because no fruits were produced.

**FIGURE 3** Fluorescence micrographs showing pollen tube growth after intraspecific and interspecific pollination. (a) Pollen tube growth after intraspecific pollination of *Gastrodia confusa*. (b) Pollen tube growth on the stigma of *G. confusa* after pollination with *G. pubilabiata* massulae. (c) Pollen tube growth on the stigma of *G. pubilabiata* after pollination with *G. confusa* massulae. (d) Pollen tube growth after intraspecific pollination of *G. pubilabiata*. Scale bars: 300 μm. Photographs by Kenji Suetsugu.

interspecific gene flow. Two individuals with a brown floral tube had genetic profiles fully consistent with *G. confusa*, with no evidence of introgression from *G. pubilabiata* in either STRUCTURE or Neighbor-Net analyses. Their internal floral morphology was also consistent with *G. confusa*, and, except for floral color, they could not be distinguished from other conspecific individuals (Figure 5).

Molecular identification of pollinaria confirmed that drosophilid pollinators sometimes carried heterospecific pollinaria (Figure 4), consistent with field observations of interspecific visits. For flies visiting *G. confusa*, MIG-seq assigned 14 pollinaria, of which 11 were identified as *G. confusa* and three as *G. pubilabiata*. Sanger sequencing identified three additional pollinaria from flies visiting *G. confusa* that could not be assigned by MIG-seq, including two *G. confusa* and one *G. pubilabiata*. Thus, four of 17 pollinaria (23.5%) carried by flies visiting *G. confusa* were heterospecific. For flies visiting *G. pubilabiata*, MIG-seq assigned four pollinaria, two to each orchid species. Sanger sequencing identified four additional pollinaria from flies visiting

G. pubilabiata that could not be assigned by MIG-seq, including one *G. confusa* and three *G. pubilabiata* (Dataset S1). Thus, three of eight pollinaria (37.5%) carried by flies visiting *G. pubilabiata* were heterospecific. Overall, heterospecific pollinarium carriage occurred in both directions, although most pollinaria matched the orchid species being visited.

4 | DISCUSSION

This study evaluated the mechanisms and strength of reproductive isolation between the sympatric, fully mycoheterotrophic, brood-site deceptive orchids *G. confusa* and *G. pubilabiata*. Although none of the reproductive barriers examined here was complete on its own, genomic analyses provided little evidence of recent gene flow. A few individuals showed weak admixture-like genomic signals that could reflect limited introgression, but no individual showed the strongly admixed

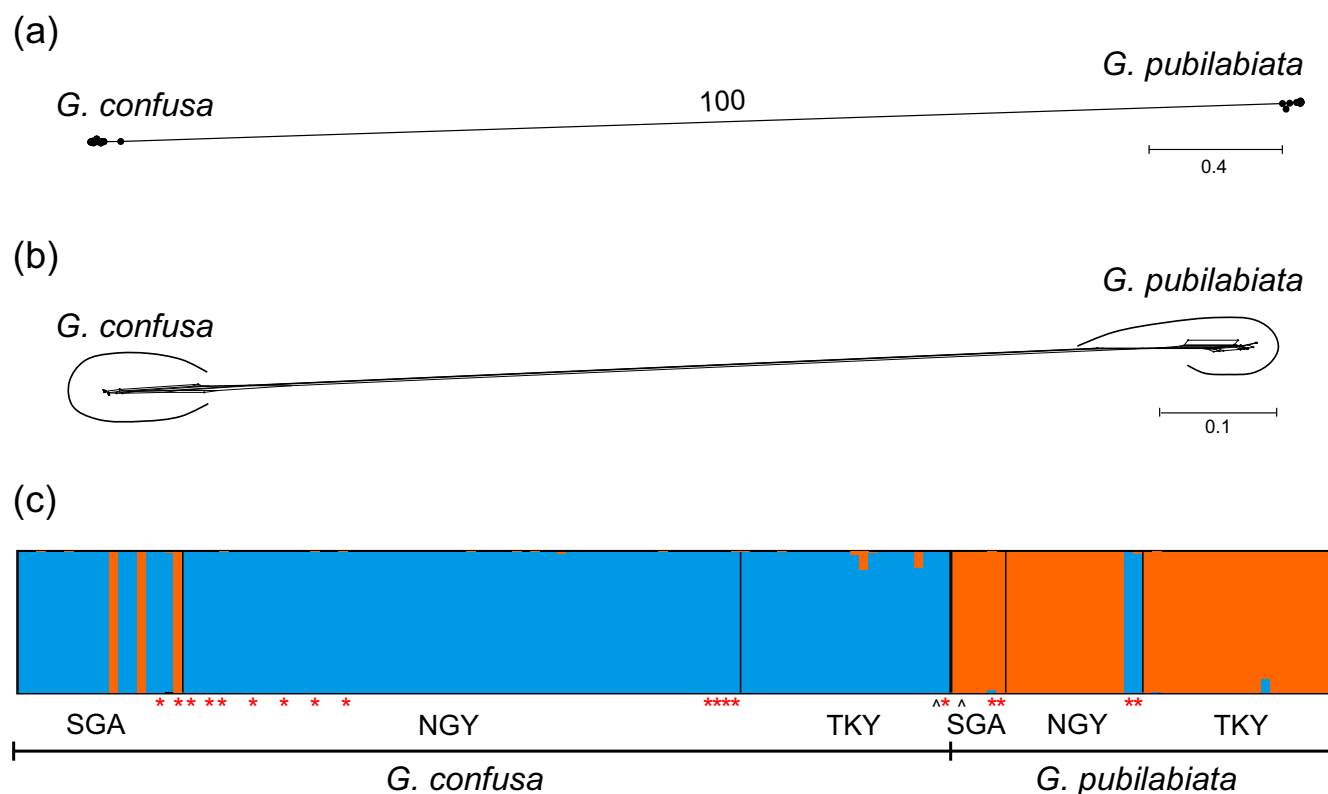


FIGURE 4 Genome-wide SNP analyses of *Gastrodia confusa* and *G. pubilabiata* at sympatric sites. (a) Maximum-likelihood phylogeny; only interspecific bootstrap support values are shown. Black dots indicate terminal tips. (b) Neighbor-Net network based on uncorrected p-distances. (c) STRUCTURE bar plot at $K = 2$, with species boundaries indicated by thick black vertical lines and site boundaries by thin black vertical lines. Pollinarium-derived samples are marked with red asterisks, and carets indicate *G. confusa* individuals with a brown floral tube. SGA, Shiga; NGY, Nagoya/Aichi; TKY, Tokyo. Sample IDs are provided in Dataset S1.

SNP profile expected for an F_1 hybrid. These results suggest that natural introgression between *G. confusa* and *G. pubilabiata* is minimal and that species boundaries are maintained by the combined action of multiple partial barriers.

Reproductive isolation, including both prepollination and postpollination barriers, is fundamental to the maintenance of species boundaries (Coyne & Orr, 2004). In plants, prepollination barriers include geographic isolation, phenological divergence, floral morphological differences, and divergent pollinator assemblages, which can collectively reduce interspecific pollen transfer among sympatric taxa (Lowry, Modliszewski, et al., 2008; Lowry, Rockwood, & Willis, 2008). Post-pollination barriers include pollen–pistil incompatibility, hybrid embryo inviability, and F_1 sterility (Lowry, Modliszewski, et al., 2008; Lowry, Rockwood, & Willis, 2008). Although orchids are often regarded as classic examples of specialized plant–pollinator interactions, recent studies have increasingly emphasized reproductive barriers beyond pollinator specificity (Cozzolino et al., 2004, 2005; Pellegrino et al., 2010; Scopece et al., 2007; Waterman & Bidartondo, 2008; Zhang & Gao, 2017).

At sympatric sites, *G. confusa* and *G. pubilabiata* were visited by highly overlapping assemblages of drosophilid flies. Both species appear to use brood-site deception by mimicking fermenting

substrates and exploiting sensory biases that attract fruit-feeding drosophilids to decaying fruit (Suetsugu et al., 2026). This strategy likely attracts a broad range of local drosophilid species and promotes extensive pollinator sharing. For fully mycoheterotrophic *Gastrodia*, such a relatively generalized association may be advantageous because dependence on a single specialized pollinator could impose additional constraints on already vulnerable life histories (Waterman et al., 2013). The moist, shaded forest-floor habitats occupied by many *Gastrodia* species may be less favorable for many bees and other common pollinators than for drosophilid flies, which are often abundant in these environments. The abundance of drosophilids may, therefore, have favored their recruitment as *Gastrodia* pollinators, whereas the scarcity of alternative pollinators may have constrained shifts to other partners (Suetsugu et al., 2026).

We found little evidence that mechanical isolation substantially reduced interspecific pollen transfer. Although differential pollen placement on shared pollinators can act as a prepollination barrier (Sun et al., 2011; Waterman et al., 2011), pollinaria of both species were consistently attached to the upper thorax. The pollination mechanism in *Gastrodia*, in which small dipterans are guided through a restricted floral passage and acquire pollinaria on a limited body region, is comparable to the masdevalliform mechanism described for

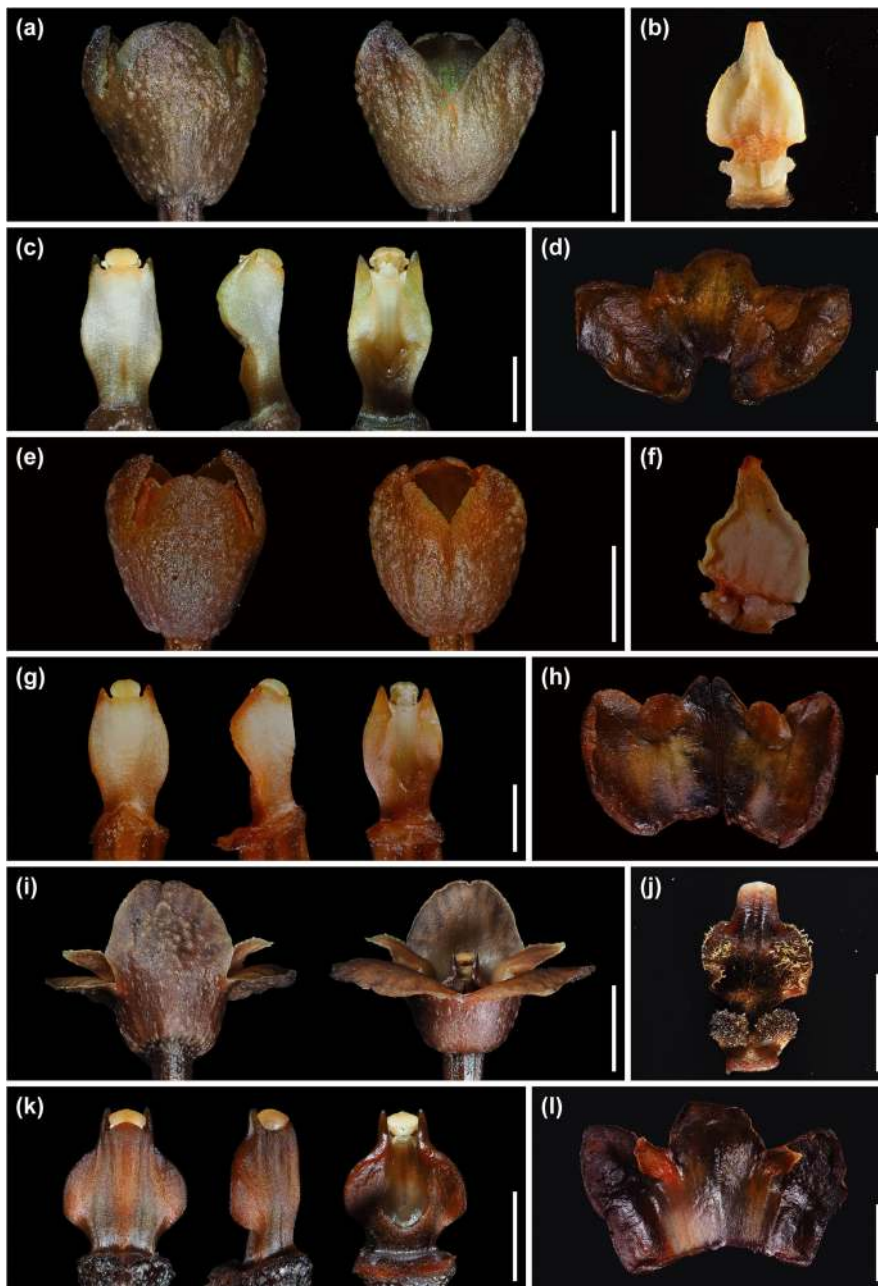


FIGURE 5 Diagnostic floral morphology of typically colored *Gastrodia confusa* (Kenji Suetsugu STG05155; a–d), *G. confusa* with a brown floral tube (Kenji Suetsugu STG05175; e–h), and *G. pubilabiata* (Kenji Suetsugu STG05185; i–l). For typically colored *G. confusa*: (a) flower, top view at left and bottom view at right; (b) lip, adaxial surface; (c) column, different views from left to right; and (d) dissected and flattened perianth tube. For *G. confusa* with a brown floral tube: (e) flower, top view at left and bottom view at right; (f) lip, adaxial surface; (g) column, different views from left to right; and (h) dissected and flattened perianth tube. For *G. pubilabiata*: (i) flower, top view at left and bottom view at right; (j) lip, adaxial surface; (k) column, different views from left to right; and (l) dissected and flattened perianth tube. Scale bars: 5 mm (a, d, e, h, i, l) and 3 mm (b, c, f, g, j, k). Photographs by Yoshiaki Kitada.

Pleurothallidinae (Gil-Amaya et al., 2025; Karremans & Díaz-Morales, 2019). This similarity between phylogenetically unrelated orchid lineages suggests convergent evolution toward an efficient mechanism for exploiting dipteran behavior, while also implying limited scope for major changes in pollinarium placement. Thus, pollen placement itself is unlikely to have substantially reduced interspecific pollen transfer. Within the traits examined here, we found no clear indication of reproductive character displacement in sympatry, such as divergence in pollinator assemblages or the evolution of autonomous self-pollination.

Despite extensive pollinator overlap, opportunities for interspecific pollen transfer appeared relatively limited under natural conditions. Although heterospecific pollinarium carriage was detected in both

directions, the observed proportions were based on flowers monitored under conditions of close spatial proximity and flowering overlap. Thus, under the broader spatial and phenological structure of natural populations, these proportions likely overestimate the frequency of heterospecific pollinarium carriage and, consequently, the opportunities for successful interspecific pollen transfer. Modest phenological separation likely reduces opportunities for natural hybrid formation, as *G. pubilabiata* typically begins flowering 7–14 days earlier than *G. confusa*, despite partial temporal overlap (Osborne et al., 2020; Sianta & Kay, 2021). In addition, even at co-occurring sites, the two species are not fully intermixed spatially. Because drosophilid pollinators appear to move only short distances, this partial spatial segregation likely promotes conspecific pollen transfer under natural conditions.

Postpollination barriers likely further strengthen reproductive isolation between *G. confusa* and *G. pubilabiata*. Although interspecific pollen tubes reached the ovary, interspecific crosses produced markedly lower proportions of seeds with well-developed embryos. This pattern suggests late-acting postpollination barriers, potentially involving reduced fertilization success or early embryo failure. Additional late-acting barriers may also contribute to the scarcity of hybrids in natural populations. One plausible, although currently untested, mechanism is reduced mycorrhizal compatibility in hybrid individuals, because *G. confusa* and *G. pubilabiata* differ somewhat in their mycorrhizal associations (Kinoshita et al., 2016). Because fully mycoheterotrophic plants depend entirely on fungal partners, hybrid seeds may germinate poorly, and the resulting seedlings may establish poorly, if compatibility with the fungal communities associated with either parental species is reduced or lost (Jacquemyn et al., 2016, 2018).

Nevertheless, even limited gene flow between closely related species may have broader evolutionary implications for *Gastrodia*, one of the most species-rich genera of mycoheterotrophic plants (Suetsugu, 2022). Notably, *Gastrodia* × *nippo-uraiensis* Suetsugu & T.C.Hsu has been described as a natural hybrid between *G. foetida* Koidz. (treated as *Gastrodia nipponica* (Honda) Tuyama in that study) and *G. uraiensis* T.C.Hsu & C.M.Kuo (Suetsugu et al., 2018). Because this hybrid was found at a site where one parental species was absent, it may persist beyond the immediate area of hybrid formation. Moreover, such hybrids may colonize novel microhabitats if they acquire trait combinations and fungal associations that permit establishment outside the ecological ranges of either parent. This hypothesis warrants explicit testing. Future studies combining high-resolution molecular phylogenetics with ecological data from broad sampling of *Gastrodia* species should assess how fungal dependence, pollinator specificity, hybridization, selfing ability, and geographic isolation have jointly shaped diversification within this genus (Kinoshita et al., 2016; Martos et al., 2015; Suetsugu, Aoki, & Kaneko, 2023; Suetsugu, Fukushima, et al., 2023; Suetsugu et al., 2025).

In summary, *G. confusa* and *G. pubilabiata* shared largely overlapping drosophilid pollinator assemblages at sympatric sites, but short-distance fly movements, modest phenological separation, and partial spatial segregation likely reduced interspecific pollen transfer under natural conditions. Reduced fruit set and impaired embryo development after interspecific pollination further limited hybrid formation, although neither barrier was complete. Hybrid seedlings may also experience reduced compatibility with the fungal communities associated with either parental species, although this possibility remains to be tested. Collectively, our results indicate that no single barrier examined here fully explains reproductive isolation; rather, multiple partial prepollination and postpollination barriers together maintain species boundaries between these two *Gastrodia* species. This study provides a detailed assessment of reproductive isolation in brood-site deceptive orchids and, together with recent work on nectar-producing, food-deceptive, and sexually deceptive orchids, highlights the importance of considering multiple reproductive barriers beyond pollinator specificity in Orchidaceae.

AUTHOR CONTRIBUTIONS

Kenji Suetsugu conceived and designed the study and conducted the fieldwork and pollination experiments. Akihiro Nishimura, Shun K. Hirota, and Kenji Suetsugu performed the molecular experiments. Shun K. Hirota and Kenji Suetsugu conducted the molecular analyses. Masahito T. Kimura identified drosophilid pollinators. Kenji Suetsugu drafted the manuscript. All authors reviewed and approved the final version.

ACKNOWLEDGMENTS

We thank Sayoko Mori, Kaito Inoue, and Yoshiaki Kitada for invaluable support in the field and Kazuma Takizawa, Hidehito Okada, and Michiko Ishida for assistance with laboratory procedures. This study was supported by the FOREST Program (JPMJFR2339, KS) of the Japan Science and Technology Agency, JSPS KAKENHI (JP25H00944, KS) from the Japan Society for the Promotion of Science, and the MEXT Promotion of Distinctive Joint Research Center Program (JPMXP0723833155, KS).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The MIG-seq data have been deposited in the DDBJ Sequence Read Archive under accession number PRJDB42663, and Sanger-derived ITS sequences have been deposited in GenBank under accession numbers LC885095–LC885114. All other primary data supporting the findings are included in the article and supplementary data files.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Suetsugu, K., Nishimura, A., Hirota, S. K., & Kimura, M. T. (2026). Multiple barriers maintain species boundaries in sympatric brood-site deceptive *Gastrodia* orchids. *Plants, People, Planet*, 1–14. <https://doi.org/10.1002/ppp3.70252>