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Bee pollination in the shadows: The role of pseudopollen and agamospermy in the mycoheterotrophic orchid *Gastrodia elata*

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Societal Impact Statement

The family Orchidaceae is renowned for its reliance on specialized pollinators and mycorrhizal symbioses. This study examined how the nonphotosynthetic orchid *Gastrodia elata* thrives on the dim forest floor, focusing on its pollination biology, particularly the chemical properties of pseudopollen and reproductive assurance mechanisms. It was found that *G. elata* mainly relies on *Lasioglossum* bees, attracted by its starch-rich pseudopollen. Additionally, *G. elata* in the studied population can produce fruit through agamospermy (seeds from unfertilized ovules). Overall, the findings reveal that *G. elata* relies on fungi for nutrition and employs reproductive strategies such as mimicking pollen to attract bees and resorting to asexual reproduction when pollinators are scarce.

Summary

- Mycoheterotrophy is an adaptation that allows survival in environments with low irradiance and minimal competition from autotrophic plants. However, such environments can negatively impact bee pollination, as most bees prefer open habitats. Despite this, the mycoheterotrophic orchid *Gastrodia elata* is known to rely on *Lasioglossum* bees for pollination.
- We investigated the reproductive biology of *G. elata* to understand how it overcomes pollinator limitation. Our research focused on the chemical composition and anatomical basis of *G. elata* pseudopollen (a substance that mimics pollen and may facilitate pollination), based on micromorphological and nutritional analyses, as well as observations of pollinator behavior. Additionally, we explored the potential presence of autogamy or agamospermy as mechanisms for reproductive assurance.
- The pseudopollen in *G. elata* originates from the disintegration of the adaxial parenchymatous tissue of the callus and is rich in starch. This contrasts with pseudopollen in most orchids, which typically originates from lip hairs and whose potential attractant is protein. *Lasioglossum* bees pollinating *G. elata* visit multiple flowers and actively collect pseudopollen. Furthermore, agamospermy serves as a reproductive safeguard in shaded habitats where insect-mediated pollination is infrequent, at least in the investigated population.

- Our findings suggest that *Gastrodia elata* pseudopollen plays an important role in enhancing insect-mediated pollination, particularly in relatively open habitats, while agamospermy ensures fruit set in shaded environments of the investigated population. Both pseudopollen production and agamospermy likely help overcome the pollination constraints posed by the mycoheterotrophic lifestyle.

KEYWORDS
agamospermy, *Gastrodia*, *Lasioglossum*, light availability, mycoheterotrophy, Orchidaceae, pollination biology, reproductive assurance

1 | INTRODUCTION

Mycoheterotrophic plants depend on mycorrhizal associations for their carbon demands, marking a pivotal evolutionary departure from the limitations faced by photosynthetic green plants, including the requirement for light energy (Leake, 1994; Merckx, 2013). This shift frees them from the need for complex vegetative structures to intercept light, absorb CO_2, and transport water to replenish losses via stomata, and the synthesis of costly photosynthetic pigments (Ogura-Tsujita et al., 2014). However, the mycoheterotrophic lifestyle introduces a set of challenges, notably an obligatory reliance on mycorrhizal fungi for survival. The specialized reliance could contribute to the rarity, isolation, and speciation of mycoheterotrophic species (Bidartondo, 2005; Taylor et al., 2003, 2004).

The implications of mycoheterotrophy extend into reproductive biology, influencing pollination strategies. Considering their nutritional reliance on fungi, autonomous self-pollination, which reduces the need to invest in pollinator attractants, may emerge as a cost-efficient strategy for mycoheterotrophic plants (Takahashi et al., 1993; Zhang & Saunders, 2000). Yet, some mycoheterotrophs produce vibrant flowers, emit strong fragrances, and offer nectar rewards to attract pollinators (Hentrich et al., 2010; Klooster & Culley, 2009; Zhou et al., 2012), despite the variable efficacy of these visitors. For example, *Epipogium roseum* attracts *Apis cerana cerana* bees but primarily self-pollinates due to its cleistogamous nature (Zhou et al., 2012). Similarly, *Cymbidium macrorhizon* flowers can attract worker bees of *A. cerana cerana* but are predominantly self-pollinated (Suetsugu, 2015). Despite such examples, there are also mycoheterotrophs that lack the capacity for autonomous self-pollination, underscoring the broad spectrum of pollination strategies employed by plants in this group (Hentrich et al., 2010; Klooster & Culley, 2009).

Interestingly, self-pollination in mycoheterotrophs might not solely be a resource-driven adaptation but also a response to the challenges of low-light environments. Mycoheterotrophy is commonly viewed as an adaptation for survival in low-irradiance areas, where competition from autotrophs is minimal (Bidartondo et al., 2004). However, such conditions can hinder plant reproduction, as pollinator activity frequently depends on light availability (Herrera, 1995, 1997). Bees, for example, prefer well-lit areas, potentially reducing pollination success in shaded habitats (Herrera, 1995, 1997). Thus, the development of self-pollination mechanisms in mycoheterotrophs could be an adaptive strategy to thrive in these low-light niches.

In this context, we examine the reproductive biology of *Gastrodia elata*, a mycoheterotrophic orchid widespread in deciduous forests across Asia. The genus *Gastrodia*, with over 100 species—one of the most species-rich genera among mycoheterotrophic plants—spans temperate and tropical regions of Asia, Oceania, Madagascar, and Africa (Ogura-Tsujita et al., 2009; Suetsugu, 2022). It encompasses three major groups of pollination systems: (a) autonomous self-pollination, (b) pollination by drosophilids through brood-site mimicry or mutualism, and (c) pollination by small bees possibly attracted by pseudopollen.

Autonomous self-pollination is a characteristic of many *Gastrodia* species (Kishikawa et al., 2019; Ogaki et al., 2019; Suetsugu, 2022). Many *Gastrodia* species, including *G. clausa*, *G. takeshimensis*, *G. flexistyloides*, *G. kuroshimensis*, and *G. amamiana*, exclusively possess cleistogamous flowers (Hsu et al., 2012; Suetsugu, 2013a, 2014, 2016, 2019). Additionally, species like *G. similis* rely on drosophilids for pollination through chemical mimicry of their oviposition sites (Martos et al., 2015; Suetsugu, 2018). A recent study also demonstrated that *G. foetida* attracts *Drosophila bizonata*, typically mushroom feeders, for pollination by offering decomposing flowers (Suetsugu, 2023). These *Drosophila*-mediated pollination systems, along with self-pollination, may be an adaptation to low-light environments, especially given the prevalence of *Drosophila* in forest understories. Furthermore, certain Australian and Japanese *Gastrodia* species, including *G. elata* and *G. sesamoides*, are pollinated by small bees such as *Lasioglossum* species (Jones, 1985; Kato et al., 2006; Macdonald et al., 2015; Sugiura, 2017), probably attracted by pseudopollen present on the labellum (Jones, 1985; Kato et al., 2006). Notably, *Gastrodia* species pollinated by drosophilids, which rely heavily on olfactory cues, are often tiny (less than 15 cm in plant height), while species pollinated by bees, which also rely on visual cues, tend to be larger (often more than 50 cm in plant height). Considering this, these diverse pollination strategies have probably contributed to the diversification of *Gastrodia* (Suetsugu, 2022). Thus, the genus offers a fascinating model for empirical research to deepen our understanding of reproductive adaptations in mycoheterotrophic plants.

Pseudopollen is a powdery substance resembling pollen, often found on the labella of certain orchids (Davies, 2009; Davies et al., 2013). It is typically produced by the disintegration of multicellular, bead-like trichomes. Pseudopollen is common in the family

Orchidaceae, likely because orchid pollen is often encapsulated in pollinia, rendering it inaccessible to pollen-gathering insects. While it was initially termed for its resemblance to pollen, recent interpretations emphasize its role as "false pollen," indicating a strategy of mimicry and deceit (Davies, 2009). However, emerging evidence suggests that pseudopollen may provide nutritional rewards, such as proteins and starches, potentially meeting the dietary requirements of pollinators (Davies, 2009; Davies & Stpiczyńska, 2008; Zheng et al., 2021). Singer and Koehler (2004) noted that the pollination system of species producing pseudopollen, characterized by pollinators visiting numerous flowers, differs from that of typical deceptive orchids, which tend to receive only a few visits per plant (Tremblay et al., 2005).

Therefore, evidence indicates the existence of two types of pseudopollen: edible pseudopollen, offering real rewards, and deceptive pseudopollen, serving solely as a lure (Davies et al., 2013). Rewarding and deceptive pseudopollen likely evolved under different evolutionary pressures, influenced by distinct pollinator behaviors (Jersáková et al., 2006; Johnson & Schiestl, 2016). Despite pseudopollen being reported in various orchid genera, focused research has largely been limited to a handful of genera, such as *Cypripedium*, *Dendrobium*, *Eria*, *Maxillaria*, and *Polystachya* (Davies, 2009; Zheng et al., 2021), with much of this research concentrating on pseudopollen structure and composition over its ecological role (Davies et al., 2000, 2013; Davies & Turner, 2004a, 2004b). Thus, combining micromorphological and nutritional analyses with observations of pollinator behavior, especially in less-examined taxa, is crucial to fully understand the role of pseudopollen.

So far, the presence of pseudopollen in *Gastrodia* has often been underrepresented in recent reviews on the topic (Davies, 2009; Jiang et al., 2020; Pansarin & Maciel, 2017), and the existence of pseudopollen in *G. elata* remains a subject of debate (Kato et al., 2006; Sugiura, 2017). Kato et al. (2006) documented pseudopollen-like substances on the hind leg scopae of pollinarium-bearing *Lasioglossum* sp., while Sugiura (2017) challenged the production of pseudopollen by the orchid. Sugiura (2017) proposed that it is not pseudopollen but the glossy orange area inside the nectarless perianth chamber of *G. elata* that serves as a deceptive signal for pollinators, simulating nectar secretion. Moreover, our preliminary field observations indicated high fruit set levels in *G. elata*, even in shaded environments that are presumably less favorable for bee pollinators. This suggests the employment of reproductive assurance strategies in the absence of bee pollination. Therefore, our research focused on (i) the chemical composition, anatomical basis, and potential function of *G. elata* pseudopollen, based on micromorphological and nutritional analyses, alongside observations of pollinator behavior, and (ii) the potential presence of autogamy or agamospermy as mechanisms of reproductive assurance.

2 | MATERIALS AND METHODS

2.1 | Study species and site

Gastrodia elata is a fully mycoheterotrophic plant depending on wood-decaying fungi throughout its life cycle (Liu et al., 2024; Suetsugu et al., 2020). It has been recognized as a medicinal herb in Chinese pharmacopoeias since as early as 100 CE (Liu et al., 2021). Commonly referred to as tian ma in Chinese, *G. elata* is employed in traditional Chinese medicine to address a range of neurological symptoms, such as dizziness and neuralgia (Liu et al., 2021).

Gastrodia elata reaches a height of approximately 0.6–1.2 m and is capable of producing around 40–100 flowers on a single stem (Nakajima, 2012). The flowers are characterized by their knobbly and tubular shape, resulting from the fusion of the sepals and petals that only separate at their tips. These flowers feature an urceolate (urn-shaped) perianth chamber, with an opening that is constricted by the column and labellum (Figure 1). Additionally, the presence of a furrow on the ventral side of the column is likely an adaptation to facilitate the entry of bee pollinators, whose thoracic widths match the width of the furrow (Sugiura, 2017). The anther comprises two sectile pollinia consisting of numerous massulae (Nakajima, 2012). The flowers and stems sometimes appear greenish (Figure 1), but this green pigment is not chlorophyll based on chlorophyll fluorescence and liquid chromatography analyses (Kenji Suetsugu, unpublished data). Therefore, the plant does not engage in photosynthesis at all.

The floral ecology of *G. elata* was studied in Koumi-cho, Minamisaku-gun, Nagano Prefecture, in central Japan, from mid to late July during the years 2011 to 2013. During this period, approximately 40 *G. elata* plants were observed in bloom within this population. About 15 individuals growing along mountain paths were found under an open canopy, receiving ample sunlight. In contrast, around 25 plants situated deeper in the forest were located beneath an almost entirely closed canopy. These plants were in shaded environments, where sunlight sporadically penetrated through the tree canopy, creating shifting patches of light on the forest floor that moved with the position of the sun.

2.2 | Micromorphological and nutritional analyses

Morphological and nutritional analyses of the *G. elata* flower, specifically the callus-like structure at the basal part of the labellum potentially representing the pseudopollen (Kato et al., 2006; Figure 2; hereafter referred to as pseudopollen), were conducted using a combination of stereomicroscopy, brightfield microscopy, and scanning electron microscopy (SEM). The initial morphological observations were conducted using either the naked eye or a stereomicroscope. Detailed images of the dissected flowering specimens were captured using an Olympus OM-D E-M1 Mark II digital camera equipped with a M.Zuiko Digital ED 60-mm F2.8 Macro lens and a Raynox MSN-505 close-up lens.

For light microscopy, labellar tissues were initially fixed in FAA (a mixture of five parts stock formalin, five parts glacial acetic acid, and ninety parts 50% ethanol), followed by dehydration through an ethanol series. The tissues were then embedded in Technovit 7100 resin (Kulzer, Wertheim, Germany) for sectioning. Sections, 3.5–5 μm thick, were sliced using a rotary microtome and subsequently stained with periodic acid-Schiff's reagent (PAS), Amido Black 10B,

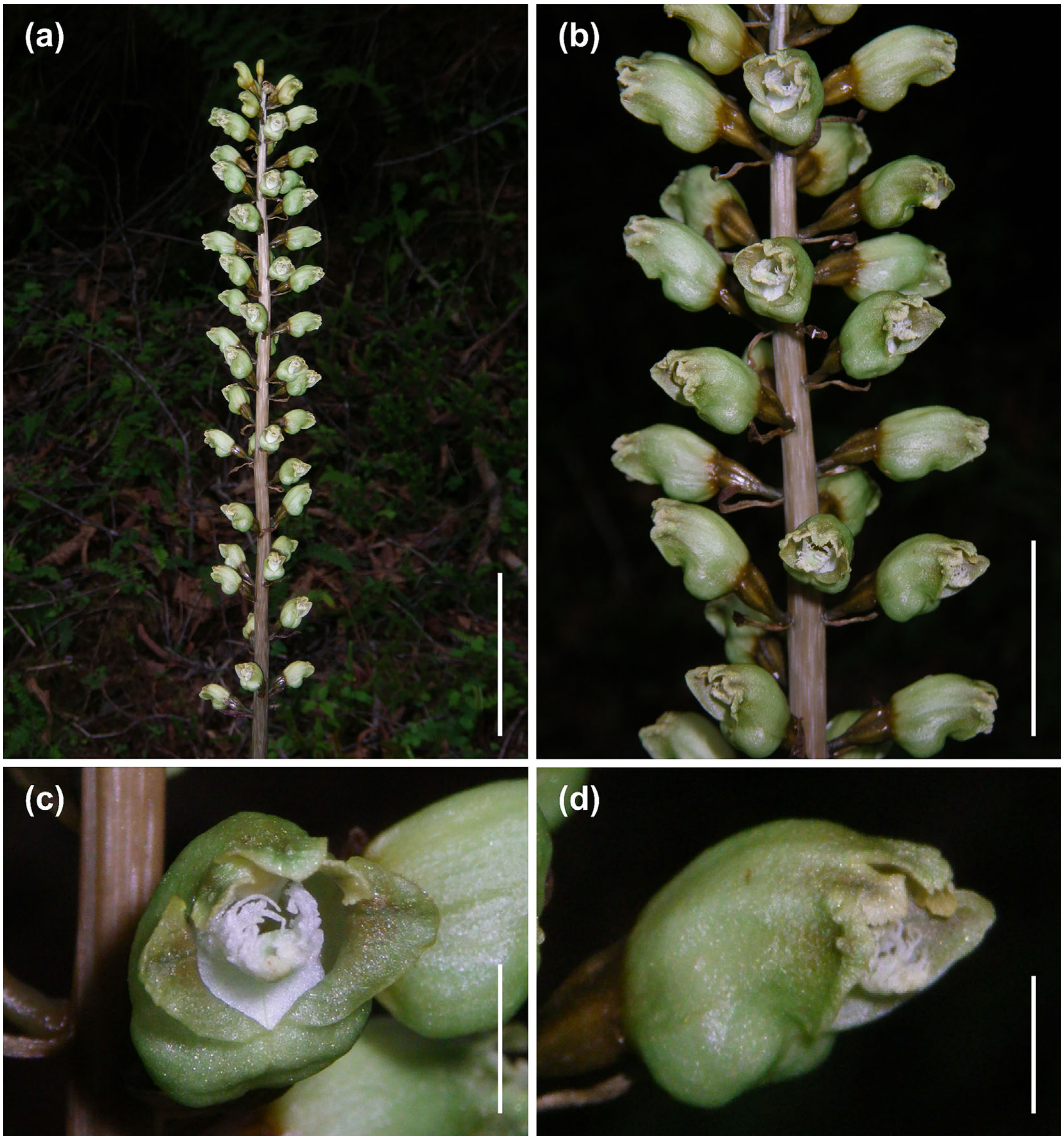

FIGURE 1 *Gastrodia elata*. (a–b) Inflorescence. (c–d) Flower. Scale bars 5 cm (a), 2 cm (b), and 5 mm (c,d). Photographs: Kenji Suetsugu.

and Sudan IV, adhering to protocols from Ruzin (1999) for PAS and Sudan IV and Yeung and Saxena (2005) for Amido Black 10B. These stains were selected for their ability to detect total insoluble polysaccharides (staining reddish-purple), proteins (staining blue), and lipids (staining reddish-orange), respectively. The stained sections were mounted in Entellan New (Merck, Darmstadt, Germany) and examined under an Olympus BX-51 microscope (Olympus, Tokyo, Japan).

For SEM observation, the preparation of labellar tissues followed the procedure for brightfield microscopy, including fixation and dehydration steps. Tissues were then subjected to critical-point drying with CO_2, essential for maintaining their microstructure. The dried samples were mounted on aluminum stubs using double-sided sticky tape and sputter-coated with platinum to improve electron conductivity for SEM imaging. Observations were made using a Hitachi Miniscope TM-1000 (Hitachi, Tokyo, Japan).

2.3 | Flower visitors

Over 3 years, from 2011 to 2013, we conducted direct observations of flower visitors during daylight hours from 8:00 to 18:00, totaling approximately 40 h (ca. 10 h in 2011, ca. 10 h in 2012, and ca. 20 h in 2013). The individuals growing under an open canopy and the plants situated deeper in the forest were observed for nearly equal amounts of time each year (i.e., approximately 20 h each). To record the behavior of insect visitors, we either walked around the study area or sat near flowering *G. elata* plants. We noted the frequency, duration, and patterns of visits for each floral visitor.

We observed and documented the behaviors of insects visiting the flowers, including their approach to the flowers, landing on a floral organ, entering the labellum, and crawling into the flower. Key aspects of pollinator behavior, such as the removal or deposition of a pollinium during their visit, as well as the harvesting of pseudopollen, were

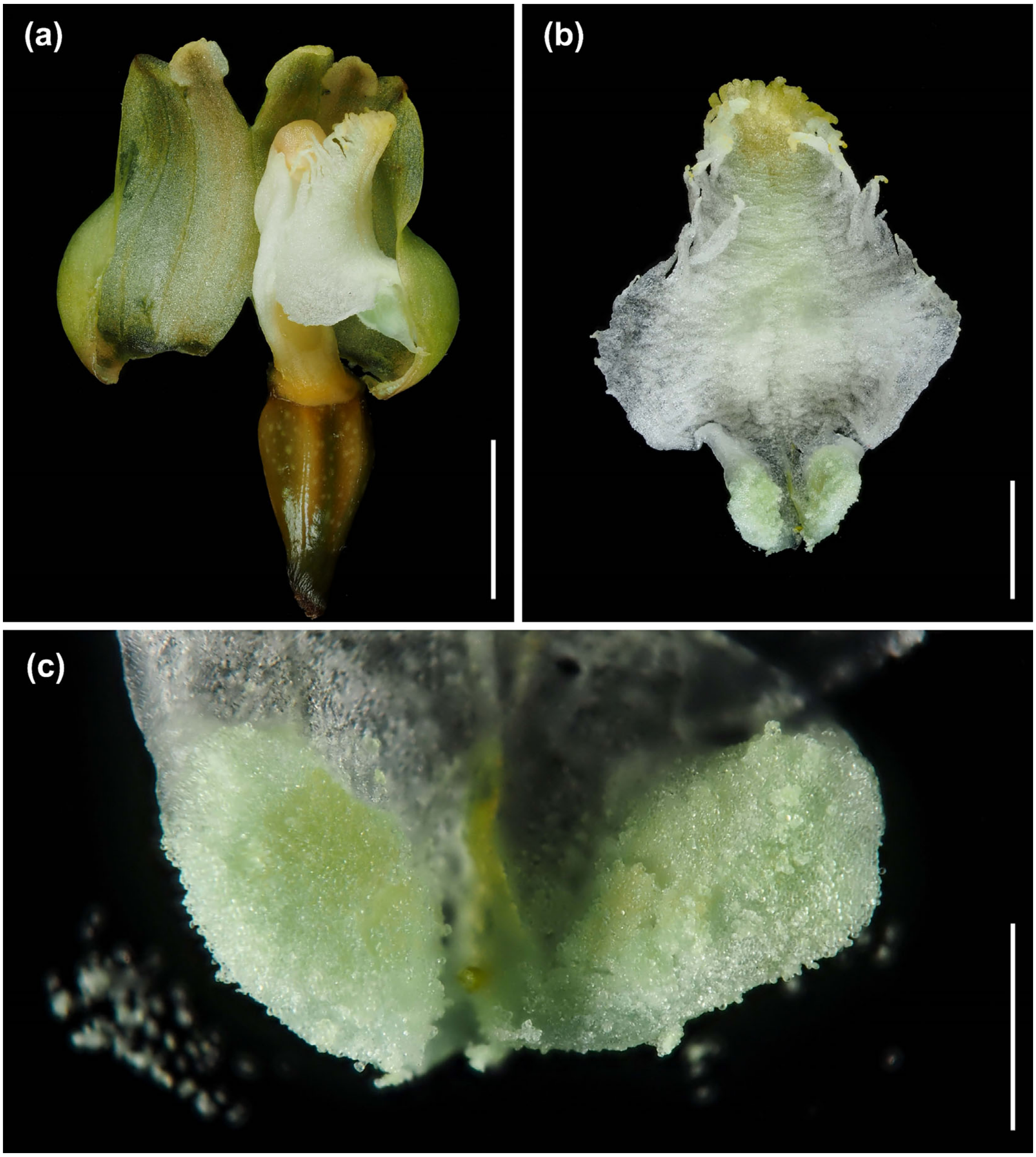

FIGURE 2 *Gastrodia elata* flower. (a) Longitudinal section. (b) Labellum. (c) Close-up of the basal part of the labellum with pseudopollen-producing calli. Scale bars 5 mm (a), 2 mm (b), and 1 mm (c). Photographs: Takuto Shitara.

carefully recorded. Additionally, a subset of the observed floral visitors was captured using a sweep net or aspirator. To minimize disturbance to the behavior of the flower visitors, we limited the capturing process to only those individuals that were necessary for identification purposes.

Due to the challenges of directly observing interactions with pseudopollen within the tubular structure of the flower, we also examined the frequency of pseudopollen carriage among pollinarium-laden halictid bees ($n = 25$). Furthermore, we collected labella from both freshly opened flowers and those visited by pollinators (10 samples from each category). These labella were dissected, and the clawed base of the labellum was examined under a microscope to assess whether pseudopollen was harvested during pollinator visits.

2.4 | Artificial pollination experiments

Inflorescences of *G. elata* were initially covered with fine mesh (0.25 mm; Wataya, Kyoto, Japan) during the bud stage to exclude flower visitors in mid-July 2011. Upon flower opening, each inflorescence was assigned to one of four treatments: (i) autonomous self-pollination treatment, where inflorescences remained bagged to exclude pollinators (50 flowers, five individuals); (ii) artificial self-pollinated treatment, involving stigma saturation with a pollinium from the same plant (50 flowers, five individuals); (iii) artificial cross-pollinated treatment, using a pollinium from a different plant at least five meters away (50 flowers, five individuals); and (iv) agamospermy treatment, entailing removal of pollinaria and leaving the flower unpollinated (50 flowers, five individuals). Post-treatment, plants were re-covered with mesh bags to exclude insects for the remainder of the experiment. Additionally, several inflorescences were tagged to allow fruit development under natural conditions (289 flowers from five individuals).

Over the subsequent 4 weeks, the experimental plants were intermittently monitored. Fruit set was then compared across the different treatments using Fisher's exact test. Seed viability was assessed by evaluating the proportion of seeds with a well-developed embryo in 100 randomly selected seeds from each capsule. For seeds developed under natural conditions, this assessment was conducted on a random sample of 50 fruits. To assess the impact of the pollination treatment on fruit set and seed set, we constructed a generalized linear mixed model with a binomial error (logit link). Due to overdispersion indicated by the residuals, the quasibinomial family was used in the analysis of seed set. Both models included "treatments" as an explanatory variable and "plant ID" as a random term. Multiple comparisons among treatments were performed using Tukey multiple comparisons.

2.5 | Epifluorescence microscopy

Following the indication of agamospermy in *G. elata* from our pollination experiments, we examined the occurrence of sexual fertilization under controlled conditions in mid-July 2012. This involved bagged, artificial self-pollinated, and artificial cross-pollinated treatments (10 flowers from five individuals in each category). The manual pollinations, both self-pollinated and cross-pollinated ones, were performed according to the methodologies detailed in the pollination experiments.

Approximately 96 h post-pollination, columns were excised from the flowers. These columns were first fixed in FAA and then softened using 1 N KOH for 1 h at 60°C on a block heater. This was followed by thorough washing in distilled water. For staining, the columns were treated with 0.1% aniline blue in K_3PO_4 buffer (pH 8.5) for 2 h at room temperature. After a brief rinse in distilled water, the samples were mounted in 50% glycerol. They were then gently squashed to spread the cellular material and subsequently examined under UV microscopy under an Olympus BX-51 microscope.

2.6 | Pollinaria removal and massulae deposition

Given the potential for agamospermy in *G. elata*, which may obscure the role of insect-mediated pollination success, we concentrated on counting pollinaria removal from the anther and massulae deposition on the stigma in mid to late July 2012. As with most orchids, *G. elata* pollen grains are aggregated into pollinia, making their removal and deposition easily observable and quantifiable in the field. This methodology serves as an indicator of insect-mediated male and female reproductive success. We randomly tagged eight flowering individuals—four in shaded environments and four in open environments—and left them under natural pollination conditions. Throughout the flowering season, we regularly inspected the experimental plants at least every 2 days, progressively documenting pollinaria removal and massulae deposition to avoid losing track of massulae that may dissolve into the stigmatic secretion. However, due to their knobbly and tubular floral shape, identifying massulae deposition on the stigma was challenging in some cases without destructive examination. Therefore, at the end of the flowering season, we dissected the floral tubes to reassess the presence of massulae deposition on the stigma. In cases where previously confirmed massulae deposition marks were no longer visible, we assumed the massulae had dissolved into the stigmatic secretion and kept them in our count. Additionally, newly discovered instances of massulae deposition on the stigma were added to the count. Subsequently, we compared the proportion of pollinaria removal and deposition between shaded and open environments using a generalized linear model with a binomial error (logit link).

3 | RESULTS

3.1 | Micromorphological and nutritional analyses

The pseudopollen of *G. elata* was identified as a pale blue translucent material, predominantly located on the rear part of the callus. It was harvestable, similar to regular pollen grains (Figure 2a–c). The adaxial surface of the callus, densely coated with pseudopollen, consists of homogenous cells ranging from rounded and ellipsoidal to widely fusiform shapes (Figure 3a–c).

Transverse sections of the callus revealed that these cells originate from the fragmentation of adaxial parenchymatous tissue, likely facilitated by schizogenous development of intercellular spaces (i.e., the cellular separation of contiguous primary walls through the middle lamella) (Figure 4a–d). The cells contain large, centrally placed vacuoles with translucent contents, surrounded by a parietal cytoplasm with amyloplasts accumulating starch grains (Figure 4d). The starch accumulation is not uniform with some cells containing large, conspicuous starch grains, while others have smaller, less conspicuous ones. Amido Black 10B staining positively marked only the cytoplasm and nucleus, indicating the absence of solid protein bodies or aleurone grains (Figure 4a–d). The absence of Sudan IV staining indicates the lack of lipids in the tissue.

3.2 | Observation of insect visitors

During our study, a limited number of insect visitations to *G. elata* flowers were documented. The agromyzid fly, *Japanagromyza tokunagai*, known for its larvae that feed on orchid seeds, was occasionally observed visiting the plants to lay eggs on the young ovaries and/or stems ($n = 19$). However, *J. tokunagai* did not enter the inner part of the flower or contact the column. Similarly, the psilid fly *Chyliza vittata*, another species whose larvae feed on orchids, visited the plants for egg laying on the young ovaries or inside the perianth chamber

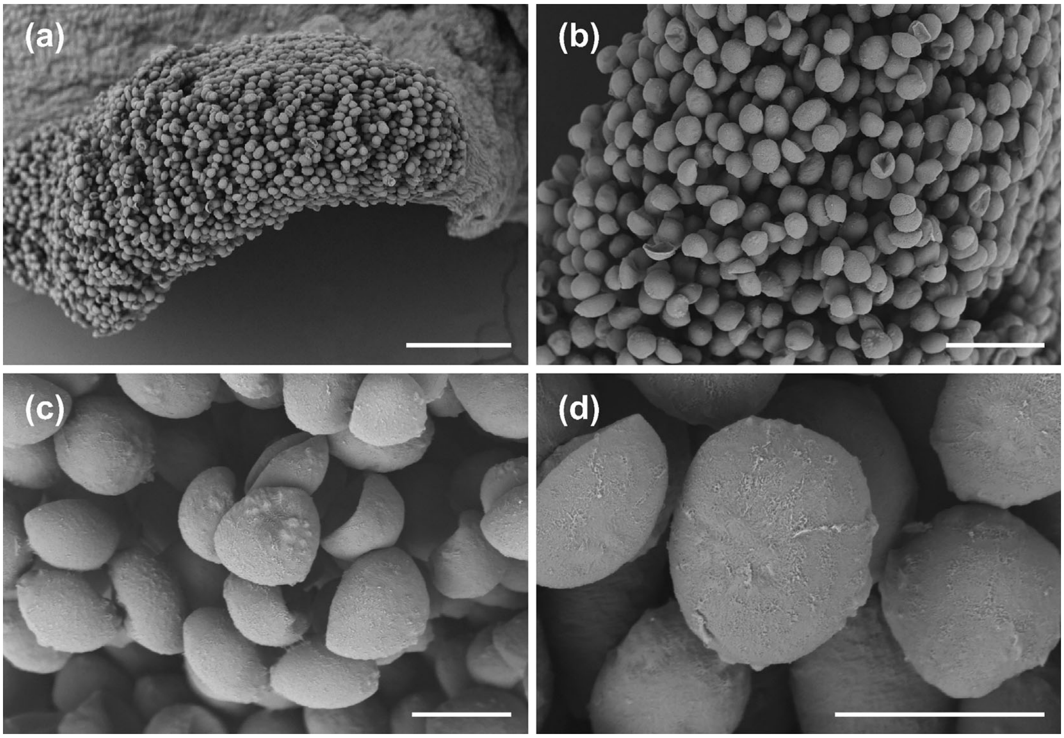

FIGURE 3 Scanning electron micrographs of *Gastrodia elata* labellum. (a) Basal part of the labellum with pseudopollen-producing callus. (b,c) Pseudopollen-producing callus. (d) Close-up of pseudopollen cells. Scale bars 300 μm (a), 100 μm (b), and 30 μm (c–d). Photographs: Takenori Yamamoto.

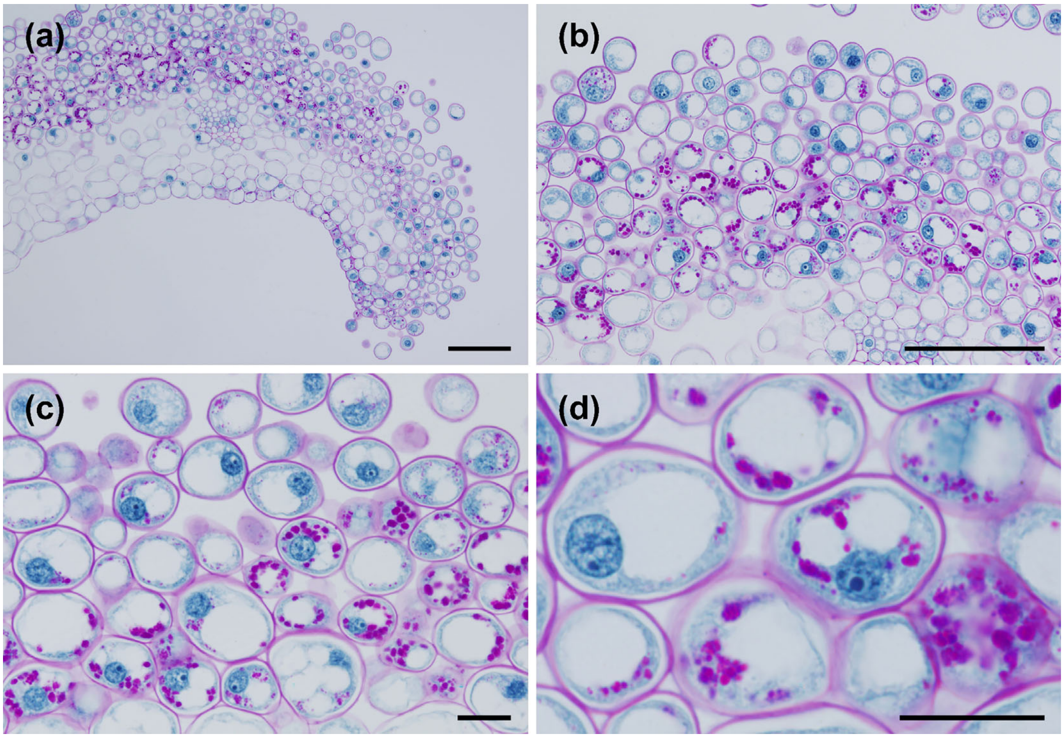

FIGURE 4 Transverse sections of the basal part of the *Gastrodia elata* labellum, showing pseudopollen adaxially. Sections were treated with Amido black 10B and PAS, indicating the presence/absence of proteins (stain blue) and insoluble polysaccharides (stain reddish purple), respectively, in pseudopollen cells. Scale bars 100 μm (a,b) and 20 μm (c,d). Photographs: Takenori Yamamoto.

($n = 11$). Some *C. vittata* were also seen using the flower as a mating site. Nonetheless, these interactions did not contribute to pollination. The only pollinating insects observed entering the flower were the sweat bee *Lasioglossum apristum* (Figure 5).

In our observations, 37 individuals (28 in open environments and 9 in shaded environments) of *L. apristum* landed on either the outer surface of the flower or the mid-lobe of the labellum. Out of these, 27 individuals crawled into the floral tube. Unfortunately, the narrow entrance of the perianth chamber hindered full observation of the bee behavior inside the chamber. However, they were probably actively collecting pseudopollen from the basal part of the labellum, given their vigorous leg movements. This was further supported by at least 16 instances where pollinarium-bearing bees were seen with *G. elata* pseudopollen on their hind leg scopae (Figure 5). The comparison between the labella of untouched flowers and those visited by pollinators underscores the significant impact of bee activity on pseudopollen collection. All fresh flowers had abundant pseudopollen ($n = 10$), while little remained on the labella of flowers after visits by *Lasioglossum* bees ($n = 10$). Furthermore, all flowers visited by *Lasioglossum* bees showed evidence of pollinaria removal or deposition.

The behavior of the sweat bee during its visit, including its exit strategy and leg movements, indicated that after collecting pseudopollen from the labellum, the bees turned around inside the base of the flower and emerged headfirst but upright. This motion allowed them to receive the stigmatic secretion that adheres the pollinarium to their thorax. If the bees were already carrying pollinaria, some massulae were deposited on the stigmatic surface. On average, bees spent 40.7 ± 21.8 s inside each flower ($n = 9$; mean ± SD), visiting approximately 5.1 ± 3.0 flowers in a single flight or visiting bout, with the maximum number of flowers visited being 11. Notably, *Lasioglossum* bees were observed facilitating pollinaria transfer between *G. elata* individuals located within 1 m of each other ($n = 2$), and it was

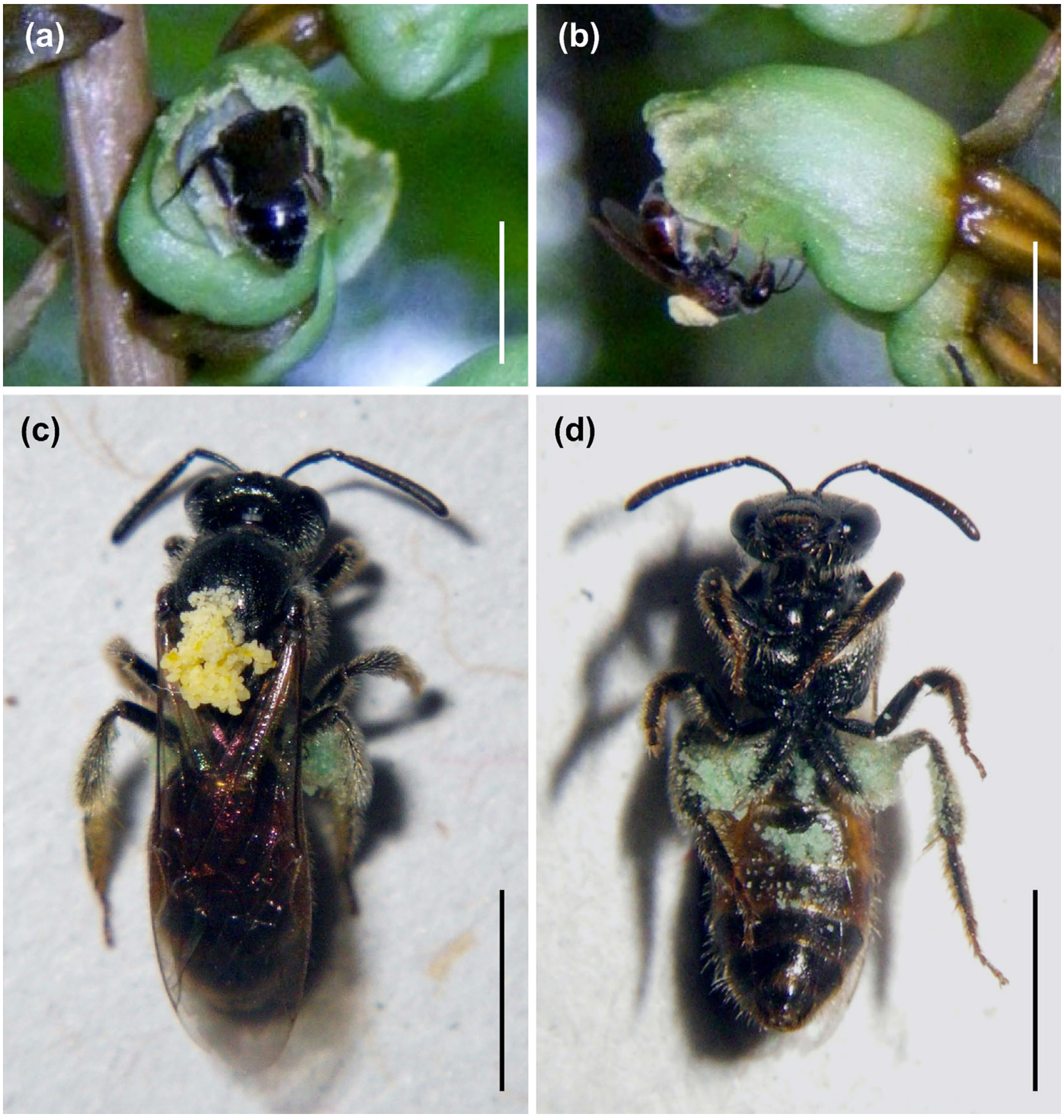

FIGURE 5 Pollinator of *Gastrodia elata*. (a) *Lasioglossum apristum* entering the tubular flower. (b) *L. apristum* with pollinia on its thorax. (c,d) *L. apristum* with pollinia on its thorax and pseudopollens on the underside of the abdomen and tibial scopae. Scale bars 5 mm (a,b) and 3 mm (c,d). Photographs: Kenji Suetsugu.

frequently noted that bees bore pollinaria upon their first visit to an inflorescence ($n = 10$). These observations suggest the bee contributes not only to geitonogamous but also to cross-pollination.

3.3 | Breeding system

Relatively high fruit set rates (69.5–86.0%) were observed in all treatments, including bagged and emasculated conditions, indicating that *G. elata* possesses a reproductive assurance mechanism through agamospermy and can bear fruit without pollinators (Dataset S1). Notably, there was no significant difference in fruit set among bagged, emasculated, artificially selfed, and artificially crossed treatments (Tables 1 and S1). However, the open treatment, which permits natural pollination, resulted in the lowest fruit formation. This reduced rate in open conditions may be partly due to seed-parasitic insects like *J. tokunagai*. In addition, the proportion of seeds with an embryo in bagged and emasculated conditions was significantly lower than those in artificial cross-pollinated conditions, suggesting some negative impacts of agamospermy and selfing (Tables 1 and S1).

The presence of a prominent rostellum in the flower structure impeded direct contact between the pollinia and stigma, indicating that autogamy is unlikely to occur in flowers under bagged conditions. This is corroborated by our findings under UV excitation, where no pollen tube formation or pollen deposition was observed in these conditions. Conversely, in both artificial self-pollinated and cross-pollinated treatments, most pollen grains had germinated, creating dense clusters of several thousand tubes extending to the base of the column (Figure S1), suggesting successful fertilization.

There were significant differences in pollinaria removal and massulae deposition on stigmas between shaded and open environments. Pollinaria removal in open environments was 37.1% (92/248), significantly higher than the 10.7% (26/244, $P < .001$) in shaded environments. Massulae deposition on stigmas was also higher in open environments at 42.3% (105/248) compared with 12.7% (31/244, $P < .001$; Table S1) in shaded environments.

4 | DISCUSSION

4.1 | Pseudopollen and its interaction with a pollinator

Gastrodia elata within the studied population is exclusively pollinated by *Lasioglossum* bees, aligning with recent findings that highlight *Lasioglossum* pollination in *G. elata* (Kato et al., 2006; Sugiura, 2017), suggesting a general predilection of this species for sweat bee pollinators. Intriguingly, some *Gastrodia* species in Australia are also pollinated by *Lasioglossum* spp. (Macdonald et al., 2015), indicating an affinity for *Lasioglossum* bees in many *Gastrodia* species, possibly due to floral morphometric filtering that restricts the range of pollinator taxa (Martos et al., 2015; Sugiura, 2017).

Lasioglossum bees were observed collecting pseudopollen from the labellum of *G. elata* in the present study. The function of pseudopollen in orchids—whether primarily as a deceptive lure or offering genuine rewards—remains a topic of debate. This debate is fueled by the focus of existing research on the structural, cellular, and developmental aspects of pseudopollen, with in-depth studies on pollinator interactions with flowers producing pseudopollen being relatively scarce (Pansarin & Maciel, 2017; Roubik, 2000; Zheng et al., 2021). The foraging behavior of *Lasioglossum* bees on *G. elata* sheds light on the potential functionality of its pseudopollen. Notably, these bees frequently visit an average of 5.1 ± 3.0 flowers ($n = 11$; mean ± SD) per foraging trip. This pattern stands in contrast to the visitation patterns seen in other nectarless orchids, which typically attract visits to only a single or a few flowers per pollinator visit (Internicola & Harder, 2012; Johnson & Schiestl, 2016; Tremblay et al., 2005). The frequent visits to multiple flowers by *L. apristum* suggest that these bees either derive enough nutrition from the pseudopollen of *G. elata* or are unable to discern its lack of rewards.

Moreover, deceptive pollination strategies are typically associated with very low pollination success rates (Neiland & Wilcock, 1998; Tremblay et al., 2005). Yet, *G. elata* shows considerably higher pollination rates in well-lit areas compared with other orchids relying on deception, suggesting that its pseudopollen alleviates pollen limitation by potentially satisfying the nutritional requirements of pollinators. Conversely, *G. elata* in dimly lit areas still shows low pollination rates despite the presence of pseudopollen, which may have driven the evolutionary pressure towards agamospermy.

Histological analyses have revealed the presence of numerous amyloplasts stained with PAS in *G. elata* pseudopollen, indicative of a rich starch content, which could serve as a nutritional reward. Similar starch contents have been documented in the pseudopollen of other orchids (Davies et al., 2000, 2002; Davies & Turner, 2004a, 2004b; Jones, 1985). The presence of starch, coupled with active pseudopollen collection by pollinators and their behavior of visiting multiple flowers, suggests that *G. elata* pseudopollen might serve as an edible reward for visiting insects (Davies et al., 2013). However, it should be noted that even if pseudopollen primarily attracts insects through deceit, these pollinators might have limited opportunities to learn

TABLE 1 Effects of pollination treatment on fruit set and proportion of the seeds with an embryo in *Gastrodia elata*.

Treatment	Bagged	Artificial self-pollinated	Artificial cross-pollinated	Agamospermy	Open
Fruit set (%)	80.0^{a}	84.0^{a}	86.0^{a}	80.0^{a}	69.5^{b}
Seeds with embryo	88.0 ± 5.3^{a}	89.7 ± 5.1^{ab}	91.7 ± 4.5^{b}	87.8 ± 5.3^{a}	90.5 ± 4.5^{ab}

Note: Different superscript letters indicate significant differences ($P < .05$) among treatment groups. The proportion of seeds with an embryo is expressed as the mean ± SD.

about the deception, as the resources are probably consumed by their offspring rather than by the pollinators themselves. In this respect, it is noteworthy that pseudopollen cell contents exhibit variable levels of starch accumulation. Consequently, additional research is needed to investigate how pollinators process pseudopollen once it is brought back to their nests and its effect on their fitness.

Previous reviews have suggested that pseudopollen has evolved independently on at least six occasions within the family Orchidaceae (Jiang et al., 2020; Pansarin & Maciel, 2017; Zheng et al., 2021). These evolutions have been identified in Catasetinae (Pansarin & Maciel, 2017), Dendrobiinae (Davies & Turner, 2004a), Eriinae (Davies & Turner, 2004b), Maxillariinae (Davies et al., 2000), Polystachyinae (Davies et al., 2002), and Cypripedioideae (Jiang et al., 2020; Zheng et al., 2021). *Gastrodia* has independently acquired pseudopollen compared with other pseudopollen-producing groups. Morphologically, *G. elata* pseudopollen shares similarities with those found in members of the *Polystachya* sect. *Polystachya* and various *Maxillaria* species, characterized by rounded, ellipsoidal, or lemon-shaped individual component cells (Davies et al., 2000, 2002). Nonetheless, while these species produce moniliform multicellular hairs that detach or fragment into individual cells or short cell chains (Davies, 2009), the pseudopollen of *G. elata* originates from the fragmentation of the adaxial parenchymatous tissue of the callus, enriching our comprehension of pseudopollen diversity.

4.2 | Agamospermy as a reproductive assurance

Gastrodia elata at the investigated site exhibits the capability for agamospermous seed production. This study has also highlighted a decrease in insect-mediated reproductive success, such as pollinaria removal and massulae deposition, in shaded habitats. Mycoheterotrophy enables *G. elata* to thrive in these shaded environments with minimal competition from autotrophic plants (Bidartondo et al., 2004). However, these low-light conditions pose some reproductive challenges, as bees, including species of *Lasioglossum*, which prefer open areas, are less abundant in shaded environments (Sakagami et al., 1974). This diminished reproductive success in shaded areas underscores the challenges mycoheterotrophic orchids face in such ecological niches.

Conversely, our pollination experiments indicated similar fruit set ratios in emasculated, bagged, artificial self-pollination, and artificial cross-pollination treatments, indicating that agamospermy serves as a reliable form of reproductive assurance for *G. elata*. Given that *G. elata* is a threatened plant with depauperate populations (Kato et al., 2006), agamospermy might help mitigate the extinction risk due to pollination failure. Nonetheless, Sugiura (2017) reported lower fruit-set ratios under shaded conditions for *G. elata* on Rebun Island, northern Japan, indicating that agamospermy may not always be an effective reproductive strategy. Additionally, in China, where the plant is cultivated for medicinal purposes, hand pollination is practiced to produce seeds, indicating the absence or minimal contribution of agamospermy (Zhou et al., 2005). Therefore, the occurrence and effectiveness of agamospermy might vary among different populations, reflecting ecological factors such as the availability and abundance of suitable pollinators (Suetsugu, 2013b; Whitehead et al., 2018).

Gastrodia elata at our study site likely employs both agamospermy and bee pollination for reproduction. Although the contribution of outcrossing should ideally be genetically analyzed, the combined contributions of agamospermy and outcrossing are plausible for *G. elata* in the investigated population. This is because (i) pollinarium-laden *Lasioglossum* bees actively visit *G. elata* flowers with pseudopollen; (ii) *G. elata* pollen tubes on the stigma develop vigorously, unlike many obligate apomictic plants without viable pollen; and (iii) most agamospermous species are facultative (Tucker & Koltunow, 2009). It is known that obligate selfing and obligate agamospermy carry significant risks, such as the accumulation of deleterious mutations and increased extinction risk (Hollister et al., 2015; Muller, 1964; Shimizu & Tsuchimatsu, 2015). The opportunity for outcrossing may be particularly crucial, especially for mycoheterotrophic plants heavily reliant on their mycorrhizal partners (Suetsugu, 2020), given that the Red Queen hypothesis posits that outcrossing is maintained through antagonistic host–parasite interactions (Ladle, 1992).

Mycoheterotrophic plants lacking reproductive assurance often face significant pollinator limitations (Hentrich et al., 2010; Klooster & Culley, 2009; Suetsugu, 2013c, 2015). Therefore, the importance of mixed mating (a blend of outcrossing and selfing) in mycoheterotrophic species from the Ericaceae, Gentianaceae, and Orchidaceae families has been highlighted (Hentrich et al., 2010; Klooster & Culley, 2009). *Gastrodia sesamoides*, for instance, resorts to self-pollination as a backup in the absence of bee pollination (Jones, 2006; Macdonald et al., 2015). Selfing that occurs after opportunities for outcrossing (delayed selfing) is often interpreted as a mating system that combines the advantages of selfing and outcrossing. Similarly, delayed agamospermy likely combines the advantages of outcrossing when feasible and selfing when necessary. We speculate that agamospermy is not confined to individuals in shaded habitats but occurs at a late developmental stage in all unpollinated flowers in *G. elata*, as both open-site and shaded-site individuals can produce fruit even when bagged and emasculated.

While agamospermy is less common than autonomous self-pollination (Xiao et al., 2021), and mixed mating typically refers to a mix of selfed and outcrossed progeny (Whitehead et al., 2018), a mixed mating system combining agamospermy and outcrossing could represent a convergent strategy under similar selective pressures. Intriguingly, the bumblebee-pollinated mycoheterotrophic orchid *Epipogium aphyllum* is known to reproduce through both sexual and asexual means, though apomixis might play a minimal role in this species (Krawczyk et al., 2016). Moreover, agamospermy has been suspected in *Gastrodia cunninghamii*, which likely also has selfing ability (Lehnebach et al., 2005). In summary, mixed mating strategies, encompassing not only selfing and outcrossing but also agamospermy and outcrossing, might be more widespread among mycoheterotrophic plants than previously acknowledged.

5 | CONCLUSIONS

Our comprehensive study indicates that the pseudopollen of *Gastrodia elata* plays an important role in attracting *Lasioglossum* pollinators, while *G. elata* also exhibits the capability for fruit production through agamospermy. Consequently, the dual strategy of employing pseudopollen and agamospermy appears to be an adaptive mechanism to ensure reproductive success in this nonphotosynthetic orchid, which typically faces pollination challenges due to its mycoheterotrophic lifestyle. Davies and colleagues have suggested that the characteristics of pseudopollen may provide valuable insights into its taxonomy and ecology (Davies, 2009; Davies et al., 2013). Intriguingly, Sugiura (2017) questioned the presence of pseudopollen in *G. elata* on Rebun Island, suggesting possible intraspecific variation in its morphology or even in its presence among different populations of *G. elata*. Future research focusing on the morphology and nutritional content of pseudopollen in intraspecific variations of *G. elata* and other *Gastrodia* species will enhance our understanding of the complex interactions between orchids and their pollinators. Additionally, examining the prevalence of agamospermy across the distribution range of *G. elata* and its relationship to the presence of effective pollinators will offer further insights into the reproductive strategies employed by this unique orchid species.

AUTHOR CONTRIBUTIONS

K. S. conceived and designed the study. K. S. collected the materials and investigated the reproductive biology. T. Y. and K. S. conducted the morphological and nutritional analyses. K. S. wrote the original draft with input from T. Y. All authors revised the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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

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