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Pollination strategy of *Gennaria diphylla* (Orchidaceae) on the Canary Islands and on Madeira

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Abstract. *Gennaria diphylla* (Link) Parl. is a terrestrial, inconspicuous green orchid. Its distribution includes the central and western Mediterranean region as well as the Canary Islands and Madeira. Investigations on its pollination biology are scarce. In literature, there are mentions of autogamy and of allogamy. Pollination and pollen import and export were studied on Tenerife and Madeira. The investigations showed autogamy plays a less important role than thought, although a caging experiment showed that the species is well capable of self-pollination. The orchid is pollinated by a variety of small moths, especially of the families Geometridae, Pyralidae and Crambidae. It is flowering in a period when few other plants flower, so there is little competition for pollinators. Complemented by autogamy this seems to be a successful strategy, as the species shows a relatively high average fruit set of 73 %.

Keywords: allogamy, autogamy, *Gennaria diphylla*, Orchidaceae, Madeira, Tenerife.

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Introduction

The Canary Islands are renowned for the high plant diversity, accommodating over 2000 species. About 25 % of all plant species are endemics (Reyes-Betancort *et al.*, 2008; Aedo *et al.*, 2013). Despite the generally high plant diversity, only seven orchid species are found: *Gennaria diphylla* (Link) Parl., *Habenaria tridactylites* Lindl., *Himantoglossum metlesicsianum* (Teschner) Delforge, *Neotinea maculata* (Desf.) Stearn, *Ophrys bombyliflora* Link, *Orchis canariensis* Lindl. and *Serapias parviflora* Parl. (Claessens, 2014, 2015). Interestingly, three out of seven species are Canarian endemics (*Or. canariensis*, *Ha. tridactylites* and *Hi. metlesicsianum*).

Gennaria diphylla is the most common orchid of the Canary Islands. In Spain, it is a much rarer orchid that can be found in southwestern Spain (Cádiz, Granada, Málaga) (Castroviejo *et al.*, 2005). It can be found on Tenerife in the Laurel forests and *Erica arborea*-vegetation, more rarely in the *Pinus canariensis* forests (Pott *et al.*, 2003; Del-Arco *et al.*, 2006), where it grows on acid to neutral soils, preferably in the shade. The plants generally grow in large groups. The flowers emit

a sweet scent, especially at night (Camus & Camus, 1929).

European orchids display a wide variety of pollination mechanisms, ranging from reward pollination to food or sexual deception (Johnson & Nilsson, 1999; Johnson & Edwards, 2000; Tremblay *et al.*, 2004; Schiestl, 2005; Jersáková *et al.*, 2006; Micheneau *et al.*, 2009) or various forms of self-pollination. Autogamy can be advantageous in case of a depauperate insect fauna or for conquering new habitats (Levin, 1972; Nilsson, 1992; Sramkó *et al.*, 2019; Pedersen & Ehlers, 2000). Small changes in flower and column architecture can facilitate autogamous pollination before or during anthesis, or as a final resort if insect pollination by insects fails (Hagerup, 1952; Richards, 1982; Tałaj & Brzosko, 2008; Claessens & Kleynen, 2011; Tałaj & Skierczynski, 2015; Tałaj *et al.*, 2017).

Little research has been done on the pollination strategy of *Gennaria diphylla*. It is said to be predominantly autogamous (Claessens & Kleynen, 2011; Tałaj & Skierczynski, 2015; Marrero *et al.*, 2019; Tałaj *et al.*, 2019). Fernandes *et al.* (2003) reported Noctuidae, Syrphidae and Tipulidae as (potential) pollinators. Being oceanic islands, Madeira and Tenerife

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have a relatively low diversity of Lepidoptera when compared to continental areas of similar size and latitude. Aguiar & Karsholt (2006) listed 317 species of Lepidoptera from Madeira and Báez (2010) listed 499 species from Tenerife. The Lepidoptera fauna of both islands is considered relatively well known, although new species are found almost every year. A number of endemic species occur on both islands.

Aim of this study was to investigate the pollination strategies of *Gennaria diphylla*. We wanted to find out: i.) how important is autogamy in the pollination strategy of the orchid; and ii.) if allogamous pollination takes place, which insects act as pollinators of *G. diphylla*.

Material and methods

We studied the pollination of *Gennaria diphylla*, an orchid with a Western-Mediterranean-Macaronesian distribution, in 2016 and 2018–2020.

Pollinator observations were carried out on two sites on Tenerife (Spain) in January 2016 and February 2019. The first site was located near El Tanque, 28°20'59.8344"N and 16°46'6.1356"W, 820 m a.s.l., on the slopes of an old lava flow (eruption in 1706) of the volcano Montaña Negra. *Gennaria diphylla* grew alongside a path through a relatively moist and shaded gorge. Accompanying species included *Davallia canariensis* (L.) Sm., *Erica arborea* L., *Adenocarpus foliosus* (Aiton) DC., *Bituminaria bituminosa* (L.) C.H.Stirt., *Oxalis pes-caprae* L., *Pinus canariensis* C.Sm., *Ulex europaeus* L., *Centranthus ruber* (L.) DC., and *Habenaria tridactylites*. The second site was near La Montañeta, 28°20'29.4"N and 16°45'16.2"W, 920 m a.s.l., in an open *Pinus canariensis* forest. Accompanying species were *Asphodelus ramosus* L., *Oxalis pes-caprae*, *Erica arborea*, *Neotinea maculata*, *Geranium robertianum* L. and *Bituminaria bituminosa* (L.) C.H.Stirt. Both sites on Tenerife lie in the Thermomediterranean zone and belong to the dry to subhumid pluviaseasonal thermomediterranean bioclimatic zone under the influence of trade-wind clouds (Del-Arco *et al.*, 2006). Climatic data were retrieved from the nearest meteorological station, La Orotava (Worldweatheronline, 2021).

Observations took place during seven days at El Tanque in January 2016 and four more days at El Tanque, and five days at La Montañeta in February 2018, all observations from 19:00 hours until 23:30 hours. Both sites were also inspected for nine hours (El Tanque in 2016 and 2019, La Montañeta in 2019), spread over five days during daylight for the presence of pollinators or visitors.

Additional observations on the pollen import and export were carried out on Madeira in 2018 and on Tenerife in 2020 at the site of La Montañeta. The Madeiran site was located near Faial, 32°47'26.4768"N and 16°51'42.804"W, on the north coast of Madeira at 400 m a.s.l. The orchids grew on a rocky, steep, shaded roadside with a north-eastern exposition. On the moist rocks grew several ferns like *Adiantum capillus-veneris* L., *Asplenium trichomanes* L.,

Cystopteris fragilis (L.) Bernh., *Davallia canariensis*, *Polypodium macaronesicum* Bobrov and *Selaginella kraussiana* (Kunze) A. Braun. Other accompanying plants were *Aeonium glutinosum* Webb & Berthel., *Agapanthus praecox* Willd., *Erica arborea*, *Laurus azorica* (Seub.) Franco and *Pittosporum undulatum* Vent.

Insects carrying, removing and/or depositing pollinaria, visiting several flowers and actively probing the spurs of the orchids were classified as pollinators. Insects sitting on the inflorescence and showing interest in the flower but not probing the flower and carrying no pollinaria were classified as visitors, as proposed by Schatz *et al.* (2005) and Henneresse & Tyteca (2016).

We investigated the import and export of pollinaria of all open flowers, using a powerful flash light and a 12x magnification hand lens. On Tenerife seven plants in two locations and on Madeira nine plants in one location were inspected. We observed if one or both of the pollinaria were absent, if the stigma was pollinated or not and if there were signs of self-pollination. A flower was recorded as pollinated when one or more massulae were observed sticking onto the stigmatic surface. Autogamy was recorded if fragments of pollen or a complete pollinium were observed sticking onto the own stigmatic surface. We inspected 16 plants with a total of 361 flowers.

In order to study autogamy, we caged nine plants that were about to start flowering in February 2020 near La Montañeta (Tenerife), and returned three weeks later to investigate if the plants showed signs of allogamous or autogamous pollination. All open flowers were inspected using a 12x magnification hand lens and a powerful torch. For all flowers we noted if the pollinaria were present, if one or both pollinaria were absent and if a pollinarium or fragments of pollen were visible on the stigma. If pollinaria were missing, this was recorded as allogamy; if fragments of pollen were observed sticking to the stigmatic surface, it was recorded as autogamy.

Lepidoptera were caught using a butterfly net and put in a fumigation jar partly filled with tissue and with some drops of acetone on the bottom. Afterwards the insects were pinned. The Lepidoptera collected for this study were identified either from their wing markings or by careful examination of their genitalia. This was achieved through their extraction and mounting on permanent slide preparations with the following steps: abdomen immersion in 10 % KOH (24 h at room temperature); extraction and cleaning of male/female genitalia on 70 % alcohol; staining with a drop of acid fuchsin; final cleaning in 100 % alcohol; clarification on Eugenol (clover-oil) and mounting on Entellan® (synthetic resin). Slides were then observed on a NIKON Optiphot-2 microscope with an attached digital camera JENOPTIK ProgRes® CT5. All slides and associated specimens are deposited in the Insect Collection of the Madeira Agricultural Laboratory (ICLAM).

Results

Gennaria diphylla is an inconspicuous plant, green to green-yellow and 10–50 cm high. It is characterised by

two cordate, opposite leaves (Figure 1A). The lower leaf is much larger than the upper one. The inflorescence is dense, more or less unilateral and carrying up to 45 flowers (Figure 1B). All perianth segments form a tube at the base, spreading at the tips. The lip base is deepened in a short, rounded spur, containing nectar (Figure 1C).

The column is erect, ovoid and very short (2–3 mm) with widely diverging anther cells (Claessens & Kleynen, 2011, 2016) (Figure 2A-B). The large, club-shaped pollinia have short caudicles, attached to small, ovoid viscid discs. The stigma is three-lobed with two large, globose, convex lateral lobes and a small median lobe.

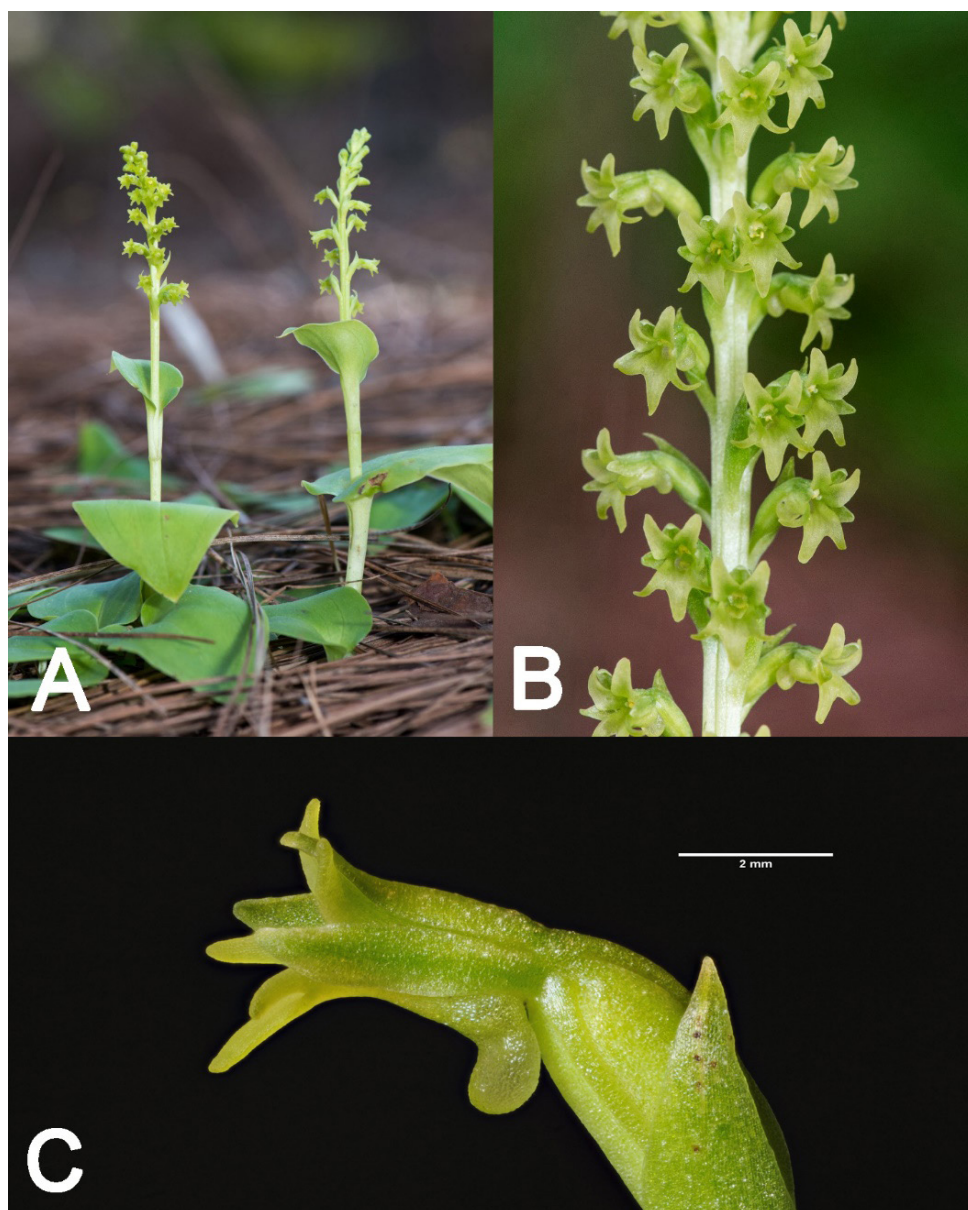


Figure 1. *Gennaria diphylla*. A, Habitus. El Tanque, Tenerife, 19-Jan-2016; B, Close-up of inflorescence. La Montañeta, Tenerife, 24-Jan-2016; C, Side-view of the flower showing the tubular perianth and the short, rounded spur.

In *Gennaria diphylla* autogamy is achieved by certain characteristics of the column that facilitate self-pollination. The main stigmatic lobes, involved in autogamy, are situated on the left and right of the spur entrance, under the anther cells. At anthesis the slits in the anther cells open due to dehydration and the pollinia can easily be dislodged and drop onto the underlying stigma (Claessens & Kleynen, 2011). When dislodged, due to the construction of the pollinarium, they can only pivot sideward and come into contact with the sticky stigmatic surface (Figure 2C). The coherence of the massulae united in the pollinium is rather low; autogamy can also be achieved by massulae falling onto the stigma while

the pollinium is still in the anther. Often the flowers are cleistogamous being already pollinated before anthesis.

According to Worldweatheronline (2021), the average temperature for La Orotava is 16–18 °C in January and 15–18 °C in February; the average rainfall is 24.7 mm in January and 28.8 mm in February. In 2016, 2019 and 2020 the average monthly temperature was normal (18–19 °C), but the precipitation was very low (1.9–4.5 mm). These meteorological conditions provided ideal circumstances for observing and catching pollinators. However, in 2018 the average temperature was lower (16 °C) with almost twice as much precipitation as usual (51.6 mm), and only very little potential pollinators were observed.

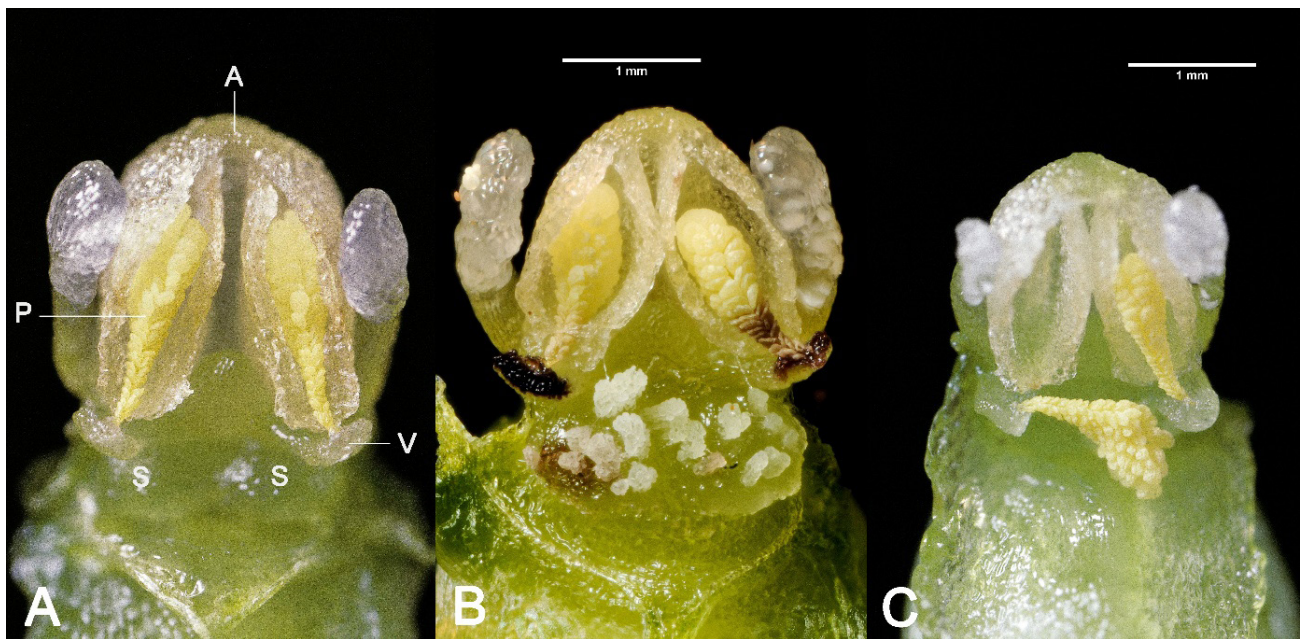


Figure 2. *Gennaria diphylla*, column. A, Front view of the column; B, The stigmatic lobes are covered with massulae whereas the pollinaria are still inside the anther; C, All perianth segments are removed. This is an example of autogamous pollination: one pollinarium has fallen sideward onto the stigmatic surface.

A= Anther, P=Pollinarium, V=Viscidium, S=Stigmatic lobe.

In total 31 insects belonging to 16 different species were captured on *Gennaria diphylla* (Table 1). 14 species out of them were carrying one or more pollinaria of *G. diphylla* and were seen visiting several flowers. They were classified as pollinator (Schatz *et al.*, 2005; Henneresse & Tyteca, 2016). During daylight we never observed any insects visiting or pollinating *Gennaria diphylla*. Moths appeared at dusk and were most active in complete darkness. However, at temperatures below 16 °C the moths showed hardly any activity, at dusk it got too cold for them. Foraging behaviour of the Crambidae

differed from that of the Geometridae and Noctuidae. Crambidae (Figure 3) stayed for five to 30 minutes on an inflorescence, staying immobile for some time and then moving or starting to feed on the nectar present in the spur again. They moved very little between flowers, often alternated periods of immobility with periods of feeding and often went from flower to flower on the same inflorescence. The Geometridae (Figure 4) and Noctuidae flew from flower to flower, probing many flowers during a foraging bout, which lasted from ten seconds up to two minutes.

Table 1. Pollinators and visitors of *Gennaria diphylla* on Tenerife identified in this study.

Family	Species	Sex	Number of individuals	Pollinator	Location
Crambidae	<i>Cornifrons ulceratalis</i> Lederer	male and female	3	yes	El Tanque, Tenerife
Crambidae	<i>Euchromius ocellus</i> (Haworth)	female	1	no	El Tanque, Tenerife
Crambidae	<i>Eudonia angustea</i> (Curtis)	male and female	4	yes	El Tanque and La Montañeta, Tenerife
Crambidae	<i>Eudonia lineola</i> (Curtis)	male	5	yes	El Tanque and La Montañeta, Tenerife
Erebidae	<i>Schrankia costaeistrigalis</i> (Stephens)	male	1	yes	El Tanque, Tenerife
Geometridae	<i>Ascotis fortunata</i> (Blachier)	?	1	yes	El Tanque, Tenerife
Geometridae	<i>Costaconvexa centrostrigaria</i> (Wollaston)	?	1	yes	El Tanque, Tenerife
Geometridae	<i>Cyclophora maderensis</i> (Bethune-Baker)	male and female	5	yes	El Tanque and La Montañeta, Tenerife
Geometridae	<i>Episauris kiliani</i> Rebel	?	1	yes	La Montañeta, Tenerife
Geometridae	<i>Eupithecia</i> cf. <i>rosai</i> Pinker	?	1	no	El Tanque, Tenerife
Geometridae	<i>Eupithecia tenerifensis</i> (Rebel)	?	2	yes	El Tanque, Tenerife
Geometridae	<i>Gymnoscelis insulariata</i> (Stainton)	?	1	yes	El Tanque, Tenerife
Geometridae	<i>Gymnoscelis ruffasciata</i> (Haworth)	female	2	yes	El Tanque, Tenerife
Noctuidae	<i>Agrotis segetum</i> (Denis & Schifferrmüller)	?	1	yes	El Tanque, Tenerife
Noctuidae	<i>Ctenoplusia limbirena</i> (Guenée)	?	1	yes	La Montañeta, Tenerife
Tortricidae	<i>Acroclita sonchana</i> Walsingham	female	1	yes	El Tanque, Tenerife

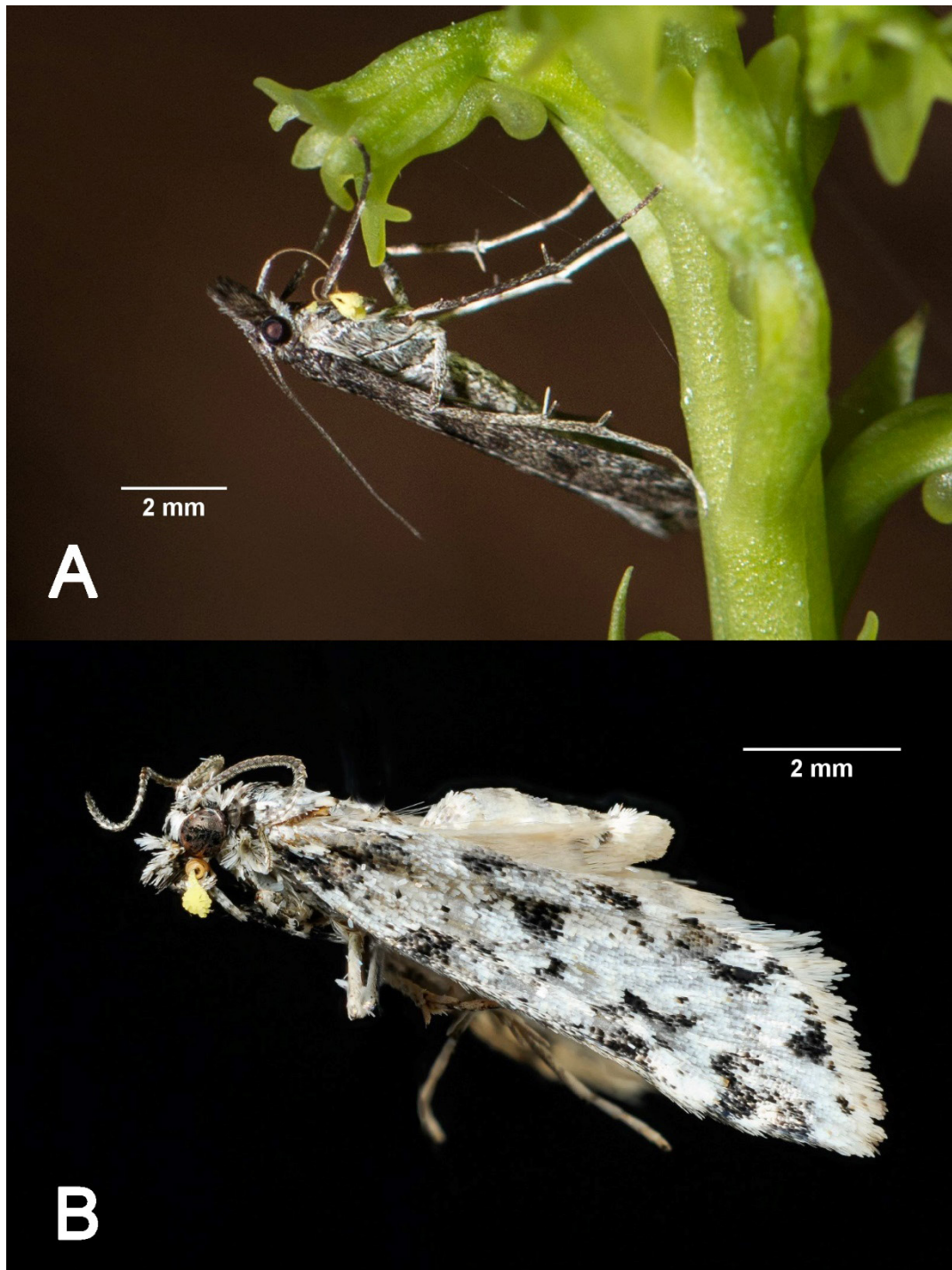


Figure 3. A, *Eudonia angustea* (Crambidae) pollinating *Gennaria diphylla*; B, *Eudonia lineola* (Crambidae) with pollinarium of *G. diphylla* attached to the proboscis.

Pollinaria import and export was studied in unbagged, open flowers of 16 plants with 361 open flowers (Table 2). In 17.2 % of all flowers the pollinaria were still present and the flower was not pollinated. In total 59.8 % of all flowers were pollinated; 14.1 % of all pollinated flowers showed autogamous pollination. A total of 5 % of all flowers were pollinated, whereas the own pollinaria were still present in the anther.

In the bagged plants, 126 flowers out of a total of 149 open flowers were self-pollinated; 23 open flowers showed no sign of autogamy; both pollinaria were still present in the anther cells (Figure 5). The proportion of self-pollinating plants (84.6 %) is overwhelmingly significantly more than 50 %; a binomial test gives a one-tailed p-value that is indistinguishable from zero.

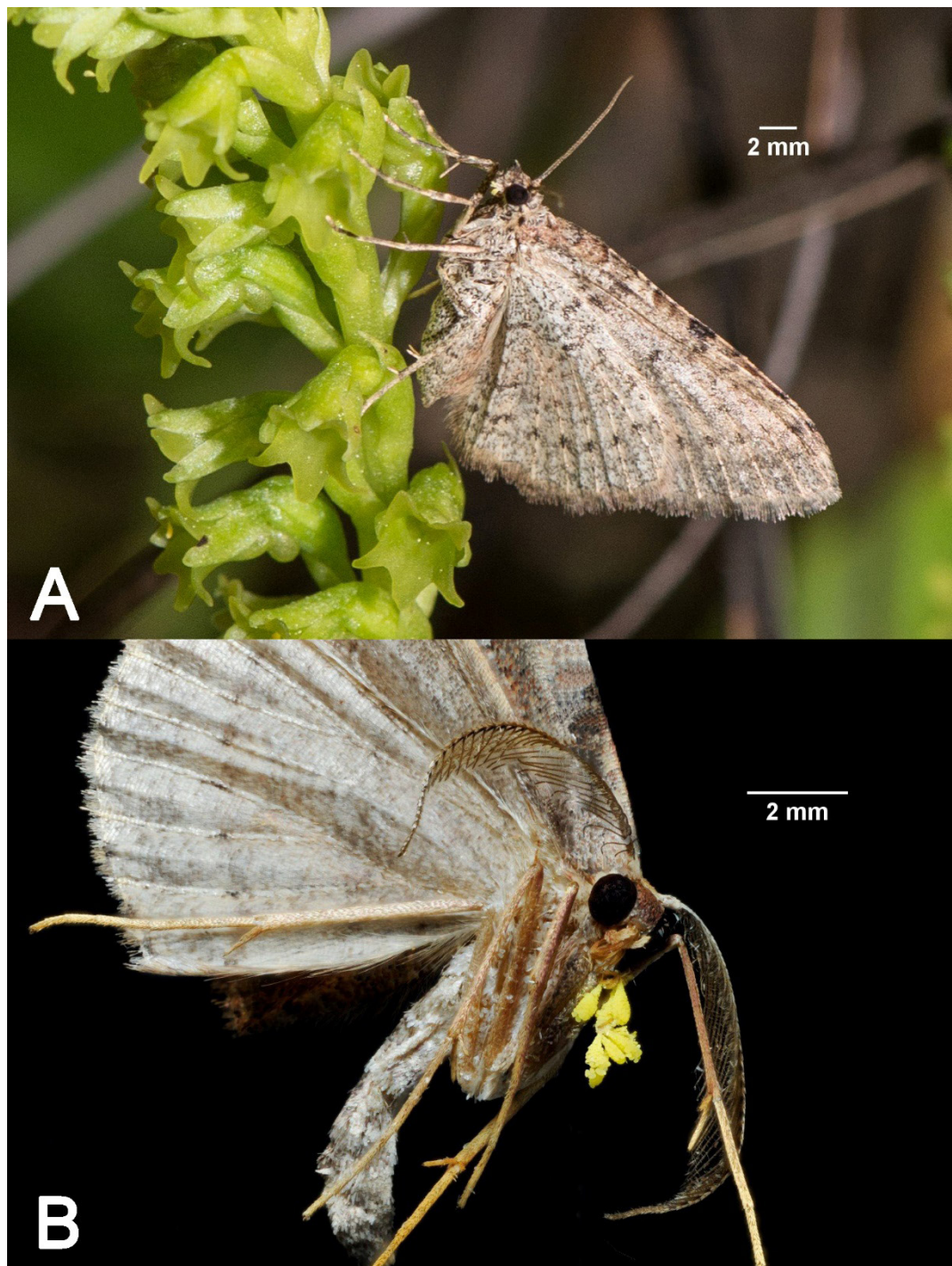


Figure 4. A, A female of *Costaconvexa centrostrigaria* (Geometridae) pollinating *Gennaria diphylla*, various pollinaria are attached to the proboscis; B, A male of *Cyclophora maderensis* (Geometridae) with a bunch of pollinaria attached to the proboscis.

Discussion

Gennaria diphylla flowers in winter, a period in which there are few insects (Gangwere, 1973; Shapiro, 1978). Moreover, during its flowering peak there are only few other accompanying flowering plant species. Flowers in harsh conditions (high elevation, extreme climatic conditions) or on remote islands show a higher degree of autogamy (Hagerup, 1951; Lloyd, 1992; Barrett, 1996; Taylor *et al.*, 2019), and in such conditions autogamy can be beneficial (Jain, 1976), as it enables the production of seeds in absence of pollination agents for the reproductive assurance (Darwin, 1877; Kores, 1980;

Cheptou, 2011). Autogamy can also be disadvantageous for plants (Darwin, 1877; Charlesworth & Charlesworth, 1987; Claessens & Kleynen, 2018), because the viability of its offspring is reduced due to inbreeding depression (Charlesworth & Charlesworth, 1999; Ayasse *et al.*, 2010; Herlihy & Eckert, 2002). Baker's Rule dictates that self-compatible species are more successful colonisers of islands (Baker, 1955). In *G. diphylla* the autogamy could allow the plant to survive in pollinator-depauperate conditions. The strategy of both allogamous and autogamous pollination resembles that of *Pseudorchis albida*, an orchid that also has a tubular

flower, a short spur containing nectar and wide-open thecae (Claessens & Kleynen, 2012). This species shows both allogamous and autogamous pollination, assuring a high fruit set (Claessens & Kleynen, 2011).

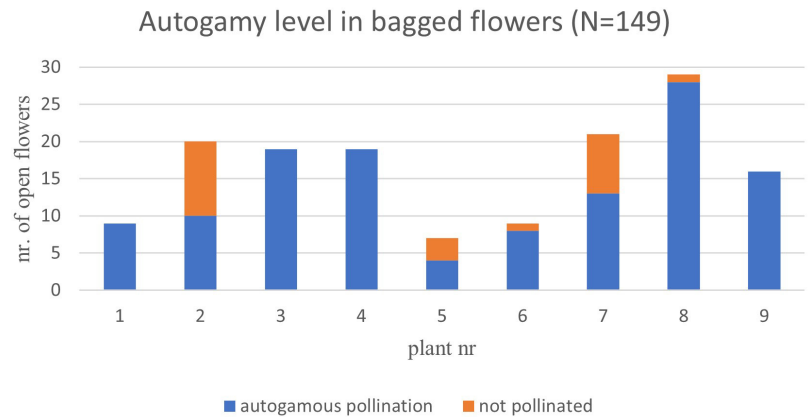


Figure 5. Autogamy level of *Gennaria diphylla* in bagged flowers, La Montañeta (Tenerife), February 2020.

Table 2. Pollination of 16 plants of *Gennaria diphylla* in three locations. Plants nr. 1–9: Faial (Madeira) 19-Feb-2018; 10–13: El Tanque (Tenerife) 18-Feb-2019; 14–16: La Montañeta (Tenerife) 17-Feb-2019.

Plant nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	total
Number of flowers with																	
pollinaria present, not pollinated		6	6	1	4	6	8	10	1	1		1	1	7	6	4	62
pollinaria present, pollinated			3		1	1	1		1	1	2	1	4		3		18
pollinaria absent, not pollinated	5	2	3	2	5	1		5	3	1		1		1	1	1	31
one pollinarium absent, not pollinated	1		3	4	2	4	1	3	4		1		2	1	4	4	34
one pollinarium absent, pollinated			1		3	6	2	2	3	4	4	11	6	3	3	5	53
pollinaria absent, pollinated	2	5	7	2	12	1	2	3	10	24	16	12	9	2	2	3	112
autogamous pollination	1	3	3	4	4		1		1	8	8	6	8	2	1	1	51
																	361

Gennaria diphylla displays allogamous features like a slight scent and nectar production. The major compounds of its volatiles are terpenes and lilac alcohols (Nilsson, 1978), which are attractants for various night-flying moths (Pleyps *et al.*, 2002; Jürgens *et al.*, 2003; Sexton & McQueen, 2004). It grows in habitats with few other nectar producing species, showing few pollinator visits per hour (Fernandes *et al.*, 2003). The green colour and the exclusive attractiveness for night-flying moths indicate that this orchid relies on its floral fragrance for long distance attraction, and the floral scent may have evolved specifically for long distance attraction (Fernandes *et al.*, 2007). The high variation in floral fragrance as found on Madeira is thought to be a strategy for establishing pollinator fidelity (Fernandes *et al.*, 2007).

This study demonstrates that *Gennaria diphylla* shows a high degree of allogamous pollination and is visited by various insect families including, Crambidae, Geometridae, Erebidae, Noctuidae and Tortricidae. The number and diversity of pollinators on both Tenerife and

Madeira indicate that allogamous pollination is far more important than previously assumed. The orchid has a generalised pollinator spectrum, a phenomenon that can be seen in plants on remote islands (Taylor *et al.*, 2019). A similar phenomenon of very different pollinator classes was observed in another Canarian orchid, *Habenaria tridactylites* (Claessens *et al.*, 2019). Crambidae and Geometridae seem to be the main pollinators of *G. diphylla* on Tenerife. On Madeira, Noctuidae were the main visitors (Fernandes *et al.*, 2003), however their status as pollinator has yet to be confirmed. Due to the mild climate the moths do not completely disappear in winter but remain present, though in reduced numbers.

The study of pollen export and import in flowers of *Gennaria diphylla* on Tenerife and Madeira revealed, that the flowers in which the pollinaria were present in absence of pollination were mostly top flowers that had just opened and probably were not yet visited. The high number of cross-pollinated flowers (45.7 %) compared to the self-pollinated flowers (14.1 %) in unbagged plants, shows that pollen transport by pollen vectors

plays a major role in the pollination of *G. diphylla*. In 5 % of all flowers, the flowers were pollinated while the pollinaria were still in the anther, which could be a sign of some inefficiency in the attachment of the pollinaria to the potential pollinator. Other studies (Nilsson, 1978; Claessens & Kleynen, 2011, 2016) also show a low percentage of imprecise pollinaria placement. This is not surprising, since *G. diphylla* is pollinated by quite different pollinator families, both by short-tongued and long-tongued pollinators. In such a system pollinaria placement is less precise than in a highly specialised flower-pollinator relationship, like in the genus *Ophrys* where there is often one or a very limited number of potential pollinators which are similar sized and shaped (Ayasse *et al.*, 2010; Xu *et al.*, 2012).

Almost 85 % of all caged plants were self-pollinated, showing that in unfavourable conditions the species is capable of producing fruits through autogamous pollination. Crambidae crawl or fly from flower to flower on a single plant and therefore cause a high degree of geitonogamous pollination. Fruit set varies from 61.5 % to 89.5 %, the mean is 73.2 % (122 plants with 2837 flowers) (Claessens & Kleynen, 2016). Even in times of shortage of insect visitors, the orchid is well pollinated. The relatively high fruit set, comparable to other rewarding, moth-pollinated species (e.g., *Gymnadenia conopsea* (L.) R.Br.) showing an average fruit set of 74 %, seems to be the result of adaptation to both allogamous and autogamous pollination.

Interestingly, on Tenerife we observed an overlap in pollinators between the early-flowering *Habenaria tridactylites* and the later flowering *Gennaria diphylla*: the main pollinators of the first species were also pollinators of the second one. At the end of anthesis of *Ha. tridactylites* the first flowers of *G. diphylla* opened. Especially *Cyclophora maderensis* (Figure 4B) seems to play an important role in the pollination of both orchid species (Claessens *et al.*, 2019). It is a common moth on Tenerife and Madeira (Wagner, 2020) which flies all year round in many generations, and thus can act as a reliable pollinator. Oceanic islands show a relatively low number of insects compared to the mainland (Olesen, 2001), so it is not surprising to find a large overlap in pollinator spectrum, even though the flower morphology, especially the accessibility of the nectar, is quite different between both orchid species (long spur *versus* short spur). The subsequent flowering of both species offers the moths food sources in times in which food supply is scarce, enabling *G. diphylla* to tap into the same pollinator resources as *Ha. tridactylites*. Whether the majority of the pollinaria are derived from the same flower and/or plant or not remains to be investigated.

Conclusions

Gennaria diphylla is a common orchid both on Tenerife and Madeira. The orchid is inconspicuous, entirely green and flowers in winter, when accompanying flowers are scarce. Despite those unfavourable conditions the species shows a high fruit set. This is achieved by adapting to

allogamous as autogamous pollination. In this study, we show that *G. diphylla* is regularly pollinated by small moths, mostly Crambidae and Geometridae. A caging experiment showed the species is capable of self-pollinating; a high percentage of the caged plants showed high fruit set. This adaptation to two different pollination modes is found in some other European orchids, e.g. *Pseudorchis albida*, and allows a relatively high fruit set, even in harsh periods when few pollinators are to be found. Flowering in winter seems a risky strategy, but an advantage is that there is little competition for pollinators, which enhances the chance that the few pollinators available are likely to visit the plants.

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