

**Bee Pollination of the Endangered Orchid *Calanthe discolor* through a Generalized
Food-Deceptive System**

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Running title: Generalized Food Deception in *Calanthe discolor*

Abstract

Calanthe discolor is a Japanese terrestrial orchid that is cultivated for its beautiful flowers arranged in racemose inflorescences. Although, its propagation for horticultural purposes has been studied extensively, resulting in the successful production of seedlings little is known about the pollinators and breeding system of *C. discolor* in its natural habitat. The current study, which combined field observations and pollination experiments, was conducted to gain further insight into the reproduction of this important orchid species. Three bee species: *Eucera nipponensis*, *Osmia cornifrons* and *Apis cerana japonica*, were found to be effective pollinators, transferring the pollinaria on their heads. However, pollination experiments also revealed that this species was self-compatible, although it was neither autogamous nor apogamous. The fruit set for the open-pollinated flowers was less than 10%, suggesting a high degree of pollinator limitation, possibly as a result of the deceptive nature of this species. These results provide evidence that pollinator specificity is the primary mechanism of reproductive isolation between *C. discolor* and its close relative *C. striata*, because the latter species is known to be exclusively pollinated by carpenter bee.

Keywords: *Apis*, *Calanthe*, *Eucera*, *Osmia*, orchid, pollination biology, pollinator limitation

1 **Introduction**

2
3 The Orchidaceae is the largest family in the plant kingdom, comprising approximately
4 25000 species (Nillson 1992). The tremendous floral diversity and evolutionary
5 radiation of orchid species is often linked to their intimate pollinator relationships
6 (Johnson and Steiner 2000; Tremblay 1992; Cozzolino and Widmer 2005) with 60–70%
7 of orchid species being dependent on discrete pollinator lineages or even single species
8 (Cozzolino and Widmer 2005).

9 The genus *Calanthe* comprises approximately 200 species of primarily
10 terrestrial or lithophilic orchids distributed throughout Africa, Madagascar, China, Japan,
11 tropical Asia, and Australia. While the pollination biology of *Calanthe* species remains
12 largely unknown, seven species have been reported to be autogamous (Catling 1990).
13 Meanwhile, it has also been suggested (Dressler 1993) that the analogous floral features
14 shared with *Epidendrum* sp indicate a role for lepidopteran pollination system. Indeed,
15 one report from the Ryukyu Islands (Japan) has shown that a nymphalid butterfly,
16 *Ideopsis similis similis*, is able to transfer pollinia between flowers of *Calanthe*
17 *triplicata* on its proboscis (Sugiura and Miyanaga 1996). Similarly, the cabbage white
18 butterfly, *Pieris rapae*, has been confirmed as the pollinator of transplanted *C.*
19 *argenteostriata* in southeast China (Zhang et al. 2010). These findings indicate that
20 some *Calanthe* species bearing long spurs can be pollinated by butterflies or moths.
21 However, there is also evidence that other *Calanthe* species might be pollinated by bees.
22 For example, there have been several reports from various locations in Japan that
23 medium-to-large species of bees from the genera *Eucera*, *Xylocopa* and *Apis* can carry
24 the pollinia of *Calanthe* spp. (Ishihara 1957; Ishikawa and Suzuki 1992; Karasawa and
25 Ishida 1998). Indeed, intensive observation of *Calanthe striata* revealed that it was

1 pollinated exclusively by the carpenter bee, *Xylocopa appendiculata circumvolans*
2 (Sugiura 2013), In addition, interval photography showed that *Calanthe reflexa* was
3 pollinated by two bumblebee species: *Bombus diversus diversus* and *B. hypocrita*
4 (Sakata et al. 2013)

5 There are currently twenty-six recorded taxa in the genus *Calanthe* in Japan
6 (Karasawa and Ishida 1998), although over-collection and habitat loss has placed almost
7 all of them in danger of extinction (Environment Agency of Japan 2000). The wide
8 variety of bright colours and pleasant fragrance of *Calanthe* species are highly prized in
9 Japan, and *Calanthe discolor* with its racemose inflorescences is one of the most
10 economically important of the Japanese ornamental orchids (Miyoshi and Mii 1988,
11 1995). However, in its native habitat *C. discolor* is categorized as an endangered species
12 (Environment Agency of Japan 2000) and the probability of its extinction in Japan over
13 the next 100 years is estimated to be almost 100% (Environment Agency of Japan 2000).
14 Despite its popularity, basic information regarding pollinators and other aspects of its
15 reproduction system is lacking, with the exception of a few anecdotal studies suggesting
16 pollination by medium-sized bees (Ishihara 1957; Ishikawa and Suzuki 1992; Karasawa
17 and Ishida 1998).

18 *C. striata* is a closely related species that is often classified as an intraspecific
19 variant of *C. discolor* (Iwatsuki 1995; Kim et al. 2008). The two species share the same
20 morphological characteristics during vegetative growth but produce different flowers,
21 those of *C. striata* being larger and emitting a stronger fragrance. The two species also
22 differ in their coloration with the flowers of *C. striata* being uniformly yellow, whereas
23 those of *C. discolor* are bicolored having brownish sepals and lateral petals with white
24 lips. In addition, *C. discolor* is often cited as producing nectar (e.g. Kawarasawa 1998),
25 while *C. striata* is known to be a food deceptive orchid (Sugiura 2013). Since

pollinators often have a strong associative learning ability (Biernaskie et al. 2009), food-deceptive species are usually expected to be strongly pollinator-limited, and exhibit adaptive traits such as exaggerated floral signals (e.g. elongated spurs; Sletvold and Ågren 2011) and optimized floral phenology (e.g. early flowering; Internicola and Harder 2012) to promote pollinator attraction and pollination efficiency. Considering that the diversity of floral characteristics can often be attributed to divergent selection by pollinators, it is important to study whether the floral differences in *C. discolor* and *C. striata* are a result of adoptions to different pollinators.

The current study was initiated to investigate four aspects of the reproductive biology of *C. discolor*: (1) to determine the potential for autonomous self-pollination (2) to confirm whether *C. discolor* produces nectar or is food deceptive (3) to identify candidate pollinators and ascertain any pollinator limitation (4) to determine whether *C. discolor* shares the same pollinator assemblage as its close relative *C. striata*.

Materials and Methods

Field observations and pollination experiments of *Calanthe discolor* were conducted in Sanbu City, Chiba Prefecture, from late April to early May of 2012 and 2013. The habitat was a coniferous plantation dominated by *Cryptomeria japonica*. The relative importance of daytime and nighttime visitors was evaluated by walking through the population and checking for the removal of pollinia in the mornings and evenings. This preliminary study revealed that pollinia were only removed during the day, and consequently the detailed observations of floral visitors were scheduled to coincide with the period of highest bee activity (0900 h–1800 h). The floral visitors were carefully observed to assess their pollination behavior, and some were captured for identification

1 immediately after they had visited flowers.

2 The pollination experiments were conducted in early May 2012. Six flowering
3 individuals were selected for the experiment each at the same phenological stage, just
4 prior to anthesis, and bearing more than 15 flower buds. The number of flowers used for
5 the experiment was limited to 15 from each plant, with the surplus flowers being
6 removed before beginning the experiments. Each of the experimental flowers was in
7 fine a mesh net early in the flowering season and assigned to one of three treatments: (1)
8 Pollinator-exclusion, to test for spontaneous self-pollination (6 shoots, 30 flowers), (2)
9 Manual-autogamy to determine self-compatibility by placing pollinia onto the stigmas
10 of the same flowers (6 shoots, 30 flowers), and (3) Manual-allogamy (6 shoots, 30
11 flowers). In addition to these treatments, 69 shoots were randomly tagged to assess the
12 efficiency of pollination under unbagged conditions (69 shoots, 678 flowers in 2012;
13 124 shoots 1340 flowers in 2013). All the allogamous plants were spaced at least 5
14 meters from their nearest neighbor to avoid sampling within genetically identical plant.
15 The experimental plants were intermittently monitored during the subsequent four
16 weeks and scored for fruit set once the capsules had formed. The statistical differences
17 between the pollinator-exclusion, manual-autogamy and manual-allogamy treatments
18 were detected using Fisher's exact test with sequential Bonferroni correction.

19 All the mature indehiscent fruits from 6 experimental plants, as well as 20
20 fruits from 20 open-pollinated plants were collected in late September 2012, and silica
21 dried for two weeks. Two from the manual-autogamy and one from the
22 manual-allogamy treatments were excluded from the analysis as the mature fruits could
23 not be detected in September. It is possibly that these fruits had aborted subsequent to
24 the initial survey in late May. The dry seeds within each capsule were weighed to the
25 nearest 0.1 mg before 500 seeds were selected for dissection under a stereoscopic

1 microscope to assess the ratio of seeds with an embryo compared to those without. The
2 effect of the pollination treatments on seed weight and the proportion of seeds having an
3 embryo were tested using the Student's *t*-test.

4 In addition, twenty flowers were selected at random from 5 individual *C.*
5 *discolor* plants in late April of 2012, and their spurs dissected under a stereoscopic
6 microscope to investigate whether the flowers produced nectar.

7 **Results**

8
9
10 The field observations revealed that the most abundant insect visitors were
11 hymenopterans, with no adult lepidopteran visitors being observed. However, the larva
12 of the lepidopteran *Lemyra imparilis*, known to be florivore of other orchids (e.g.
13 Suetsugu 2013; Sugiura 2013), were detected. Members of the Diptera and Coleoptera
14 were also observed visiting the flowers of *C. discolor*, although they did not exhibit
15 typical pollinator behaviour, with most individuals merely alighting or resting on the
16 flowers. However, food-seeking behavior was occasionally observed for the hoverfly
17 *Episyrphus balteatus* and false blister beetle *Oedemeronia lucidicollis*, although they
18 were unable to carry pollinia as a result of their small body size. The agromyzid fly,
19 *Japanagromyza tokunagai*, known to infest various species of Japanese orchids (e.g.
20 Suetsugu 2013; Sugiura 2013) was also observed visiting the flowers of *C. discolor* to
21 lay its eggs in the young ovaries.

22 The most frequent visitors were bee species including *Eucera nipponensis*
23 (28% of total floral visits), *Osmia cornifrons* (23%) and *Apis cerana japonica* (10%).
24 All three species exhibited nectar seeking and pollinating behaviour; shortly after
25 alighting on the labellum, they held the labellum with their fore and middle legs and

1 inserted their proboscises deeply into the spur (Fig. 2D). Whilst probing, their heads
2 were pushed against the tip of the column, thereby receiving pollinaria, or if already
3 laden transferring it to the stigmatic surface. The total time spent per flower was
4 typically less than 10 seconds, with the bees usually visiting one flower per
5 inflorescence before leaving. However, occasionally individuals of *E. nipponensis* and
6 *O. cornifrons* (two and three individuals, respectively) visited multiple flowers within
7 an inflorescence in succession.

8 The pollination experiments revealed that the three treatments:
9 pollinator-exclusion, manual autogamy and manual allogamy resulted in quite different
10 levels of fruit set, with the proportion for each treatment being 0%, 86.7%, and 93.3%,
11 respectively. In contrast, of the 678 and 1340 open-pollinated samples, only 44 and 16
12 bore fruit corresponding to a total fruit set of 6.5% and 1.2%, respectively. The large
13 discrepancy between the open-pollinated samples and the artificially fertilized flowers
14 (both autogamous and allogamous) suggests that *C. discolor* experiences a high degree
15 pollinator limitation in its native habitat. Although the manual autogamous treatment
16 produced a lower fruit set than the allogamous treatment, this difference was not
17 significant (Fisher's exact test, $P = 0.85$), indicating that *C. discolor* is fully
18 self-compatible. Furthermore, the seed mass and proportion of seeds with an embryo
19 did not significantly differ between the two treatments (Student's t -test, $P = 0.06$, $P =$
20 0.42 , respectively, Table. 2).

21 The results of the spur dissection experiment suggested that *C. discolor* does
22 not produce nectar since no nectar secretions were detected in any of the 20 flowers
23 examined.

24 25 Discussion

1 A large discrepancy was observed between the fruit set of the open-pollinated subjects
2 (6.5% in 2012 and 1.2% in 2013) and the samples from both the manual autogamous
3 and allogamous pollination treatments (86.7%, and 93.3%, respectively). These results
4 suggest that the population investigated experiences a high degree of
5 pollinator-limitation, but is also consistent with the trend for relatively low fruit set
6 found in most orchid species (Neiland and Wilcock 1998; Tremblay et al. 2005).

7 Although *C. discolor* has previously been categorized as a nectariferous orchid,
8 based on the presence of a flower spur (Karasawa and Ishida 1998), the dissection
9 experiments produced no evidence of nectar secretion. The absence of floral rewards of
10 any kind, combined with the nectar-seeking behaviour of the observed pollinators, as
11 well as the absence of nectariferous flowers with a similar morphology and phenology
12 to *C. discolor*, indicates that like other *Calanthe* species (Sakata et al. 2013; Sugiura
13 2013), *C. discolor* adopts a generalized strategy of food deception, whilst these results
14 should not be considered conclusive as it possible that other populations do produce
15 nectar, or that *C. discolor* is adapted to mimic a rewarding plant (or a set of plants) that
16 did not occur in this particular location. Generalized food deceptive species often rely
17 on pollination by naive insects and/or insects whose food resources have become
18 depleted and thus early flowering is flavoured (Internicola et al. 2008, Internicola and
19 Harder 2012). The flowering time of *C. discolor* (late April to early May) is early to
20 middle stage of the main pollinator *Eucera* activity (April to June; Enju 2013). Thus, *C.*
21 *discolor* utilize not so much naive pollinator as pollinator whose food resources have
22 become depleted. Because there are no sympatric co-blooming plants which share
23 pollinators in our study site, the habitat separation from other nectariferous plants may
24 be important for successful exploitation.

1 Since pollinators often have a strong associative learning ability (Biernaskie et
2 al. 2009), it might be expected that generalized food-deceptive species would be
3 avoided (Li et al. 2011), with only a few non-rewarding flowers being visited before the
4 pollinators switch to alternative species (Dafni and Ivri 1981). This low pollinator
5 visitation, could in turn reduce the reproductive success of deceptive orchids (Neiland
6 and Wilcock 1998; Tremblay et al. 2005). The data from the current study provided
7 further support for this hypothesis with most pollinators only visiting one flower per
8 inflorescence resulting in a low fruit set (6.5% in 2012 and 1.2% in 2013). The value is
9 much lower than the average for both nectariferous and nectarless temperate orchids
10 ($37.1\% \pm 3.2\%$, $n = 84$ and $20.7\% \pm 1.7\%$, $n = 130$, respectively; Tremblay et al. 2005),
11 even considering the fact that annual fruiting success in orchids varies considerably
12 (Curtis 1954; Sugiura et al. 2001; Sugiura et al. 2002; this study). Low pollination has
13 also been linked to forest fragmentation (Tomimatsu and Ohara 2002; Huang et al.
14 2009). However, this seems unlikely since the study site was dominated by *Cryptomeria*,
15 which has a dense canopy, and unmanaged *Cryptomeria* plantations are known to
16 support very few understory plants (Ishii et al. 2008). Under such circumstance forest
17 fragmentation might actually improve the richness and abundance of nectariferous
18 plants, and indirectly favor insect visitation rates to orchids.

19 The data from the pollination experiments also confirmed that *Calanthe*
20 *discolor* is self-compatible, as no significant differences in fruit set were detected
21 between the manual-autogamy and manual-allogamy treatments. Many investigations of
22 orchid breeding systems have demonstrated that self-pollination results in significantly
23 lower rates of embryo formation relative to cross-pollination (e.g. Tremblay et al. 2005;
24 Smithson 2006; Vale et al. 2011, 2013 but also see Gale 2007; Suetsugu and Fukushima
25 2013), even when there are no significant differences between the fruit set resulting

1 from manual-autogamy and manual-allogamy treatments . This tendency appears to be
2 particularly evident in pollinator-dependent species, suggesting that inbreeding can
3 affect these species more than autogamous species (Tremblay et al. 2005). However,
4 there appeared to be no evidence of such inbreeding depression in the
5 pollinator-dependent *C. discolor*, with no significant difference being detected between
6 the proportion of seeds with a developed embryo from the manual-autogamy and
7 manual-allogamy treatments,. In addition, the seed mass and the proportion of seeds
8 with a developed embryo in unbagged plants were similar to those of manual-autogamy
9 and manual-allogamy treatments. Whilst it is possible that resource limitation
10 negatively affected seed mass and the proportion of seeds with embryo, similar
11 tendency on unbagged and controlled treatments suggested that pollinators would be as
12 efficient as artificial pollination, in terms of quality of fruit.

13 The floral diversity of orchids has often been linked to the intimate and
14 complicated interactions they have with their pollinators (Gill 1989). However, most
15 estimates of a high incidence of single-pollinator species were based on observations of
16 unique pollination systems, such as the sexually deceptive ones (Mant et al. 2002;
17 Soliva and Widmer 2003). In contrast, subsequent studies have shown that generalized
18 food deception orchids often attract a guild of locally available insect visitors
19 (Cozzolino et al. 2004, 2005). The field observations in the current study supported this
20 opinion, with at least three bee species (*Eucera nipponensis*, *Osmia cornifrons* and *Apis*
21 *cerana japonica*), belonging to two different families, being observed pollinating the
22 flowers of *C. discolor*.

23 No evidence was found to support the hypothesis of Dressler (1993) that
24 *Calanthe* species are likely to be pollinated by lepidopterans on the basis of their floral
25 features being analogous to those of the lepidopteran-pollinated genus *Epidendrum*.

1 Instead, the current study confirmed that *C. discolor*, similar to its close relative *C.*
2 *striata* (Sugiura 2013), successfully attracts bees for pollination, whilst this species has
3 relatively long spurs (ca. 7-10 mm) and these is longer than the tongue lengths of the
4 pollinators (ca. 5-7 mm). Although a long spur is often thought to be associated with the
5 Lepidopteran pollination syndrome, rather than bee pollination (Dressler 1993), there is
6 growing evidence that the actual fauna pollinating a particular species is determined by
7 complex factors that include not only floral syndromes but also the local availability of
8 pollinators and historical adaptations to a habitat (Xie et al. 2013). Furthermore, it is
9 possible that the evolution of nectarless flowers may have released from selective
10 constraint associated with mechanical fit of pollinator proboscis with access to nectar
11 (Huang and Fenster 2007). Indeed, it has also been demonstrated that long spurs is
12 favored by other bee-pollinating deceptive orchids (Sletvold and Ågren 2011). Further
13 investigation is needed to reveal whether the phenotypic selection to longer spur exists
14 in *C. discolor*.

15 Curiously, both *C. discolor* and *C. striata* have been considered conspecific by
16 some authors (Kim et al. 2008), despite the differences in their flower size, coloration
17 and fragrance (Park et al. 2010). The current study provides further evidence for
18 reproductive isolation between these two species (i.e. difference of pollinator
19 preference: *C. discolor* being pollinated by *E. nipponensis*, *O. cornifrons* and *A. cerana*
20 *japonica*, while *Calanthe striata* seems to be pollinated exclusively by large carpenter
21 bees; Sugiura 2013). Furthermore, *C. discolor* lacks the strong and/or very sweet scents
22 which is associated with *Xylocopa*-pollinated orchids (e.g. Braga 1977; Díaz and Vale
23 2001; Sugiura 2013). Although it should be noted that differences of floral visitors can
24 be partially explained by local availability of pollinators in our study and Sugiura's
25 study site, the floral characteristics, such as perianth size and the height of the

1 rostellum/stigma and fragrance are also likely to reflect adaptations to their own
2 pollinator assemblages. However, since *C. discolor* is known to hybridize with *C.*
3 *striata* in wild populations (Kim et al. 2008) further investigation is required to
4 ascertain whether these species share pollinator assemblages in sympatric populations.

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7
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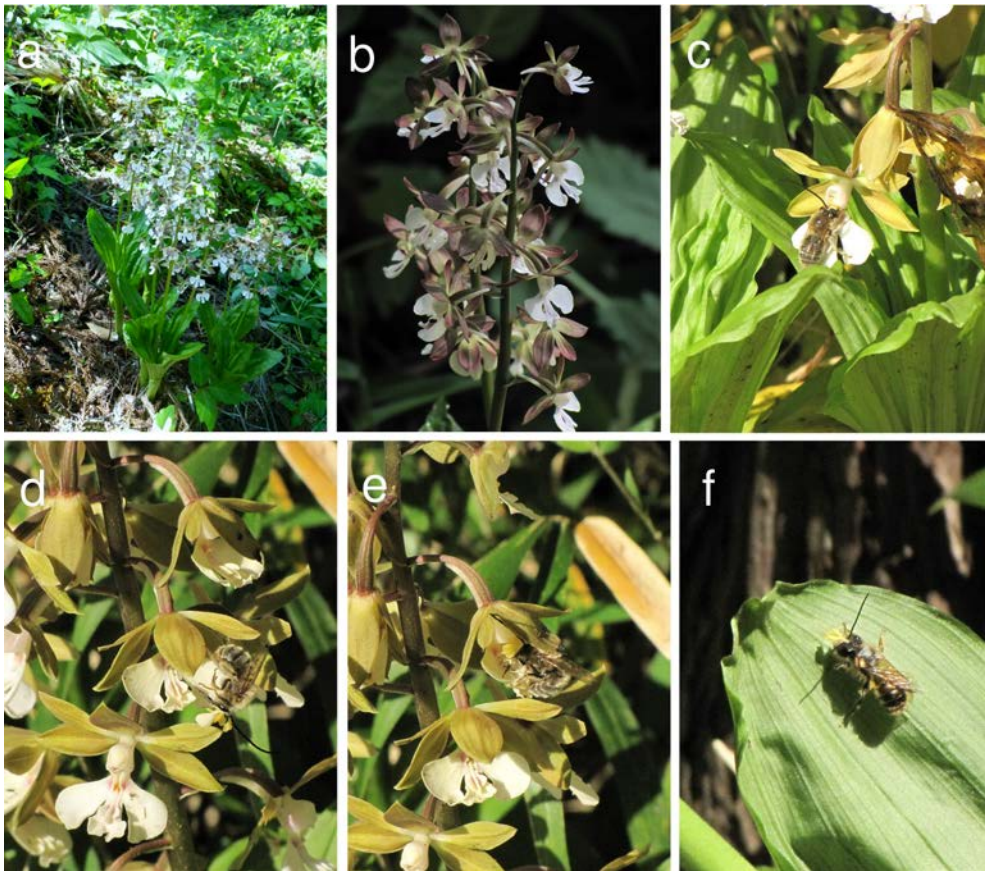
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1 Figure legends



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3 Figure 1. Flowers of *Calanthe discolor* and its visitors. (a) Flowering individuals, (b)
4 inflorescence, (c-e) *Eucera nipponensis* visiting a flower and carrying pollinaria on its
5 head, (f) *Osmia cornifrons* carrying pollinaria on its head.

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Table 1. The identity of insect taxa and total number of insect visitors

Insect species	Insect order	No. of visitors	Pollinia removal	Pollinia deposition
<i>Eucera nipponensis</i>	Hymenoptera	11	8	3
<i>Osmia cornifrons</i>	Hymenoptera	9	5	2
<i>Apis cerana japonica</i>	Hymenoptera	4	2	0
<i>Episyrphus balteatus</i>	Diptera	5	0	0
<i>Eupeodes corollae</i>	Diptera	2	0	0
<i>Japanagromyza tokunagai</i>	Diptera	1	0	0
<i>Sphaerophoria</i> sp.	Diptera	1	0	0
<i>Oedemeronia lucidicollis</i>	Coleoptera	2	0	0
<i>Macrolagria rufobrunnea</i>	Coleoptera	2	0	0
<i>Lemyra imparilis</i> (larva)	Lepidoptera	3	0	0

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Table 2. The effect of pollination treatment on fruit set, seed mass and proportion of seeds with embryo.

Treatment	Pollinator exclusion	Manual autogamy	Manual allogamy	Unbagged
Fruit set	0/30	26/30	28/30	44/678
Seed mass (mg)	-	20.8 ± 6.2	23.6 ± 6.5	29.1 ± 6.9
Seed with embryo (%)	-	86.9 ± 9.5	87.5 ± 7.8	87.9 ± 7.5

N/n, number of developed fruits/number of examined flowers; Seed mass and seeds with embryo are indicated mean ± SD.

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