1	Bee Pollination of the Endangered Orchid Calanthe discolor through a Generalized
2	Food-Deceptive System
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24	Running title: Generalized Food Deception in Calanthe discolor
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1 Abstract

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3 Calanthe discolor is a Japanese terrestrial orchid that is cultivated for its beautiful 4 flowers arranged in racemose inflorescences. Although, its propagation for horticultural purposes has been studied extensively, resulting in the successful production of $\mathbf{5}$ 6 seedlings little is known about the pollinators and breeding system of C. discolor in its 7 natural habitat. The current study, which combined field observations and pollination 8 experiments, was conducted to gain further insight into the reproduction of this 9 important orchid species. Three bee species: Eucera nipponensis, Osmia cornifrons and 10 Apis cerana japonica, were found to be effective pollinators, transferring the pollinaria 11 on their heads. However, pollination experiments also revealed that this species was 12 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 1314limitation, possibly as a result of the deceptive nature of this species. These results provide evidence that pollinator specificity is the primary mechanism of reproductive 1516isolation between C. discolor and its close relative C. striata, because the latter species 17is known to be exclusively pollinated by carpenter bee.

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

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1 Introduction

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The Orchidaceae is the largest family in the plant kingdom, comprising approximately 25000 species (Nillson 1992). The tremendous floral diversity and evolutionary radiation of orchid species is often linked to their intimate pollinator relationships (Johnson and Steiner 2000; Tremblay 1992; Cozzolino and Widmer 2005) with 60–70% of orchid species being dependent on discrete pollinator lineages or even single species (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 12largely unknown, seven species have been reported to be autogamous (Catling 1990). Meanwhile, it has also been suggested (Dressler 1993) that the analogous floral features 1314 shared with Epidendrum sp indicate a role for lepidopteran pollination system. Indeed, one report from the Ryukyu Islands (Japan) has shown that a nymphalid butterfly, 1516Ideopsis similis similis, is able to transfer pollinia between flowers of Calanthe 17triplicata on its proboscis (Sugiura and Miyanaga 1996). Similarly, the cabbage white 18 butterfly, Pieris rapae, has been confirmed as the pollinator of transplanted C. 19argenteostriata in southeast China (Zhang et al. 2010). These findings indicate that 20some *Calanthe* species bearing long spurs can be pollinated by butterflies or moths. 21However, there is also evidence that other *Calanthe* species might be pollinated by bees. 22For example, there have been several reports from various locations in Japan that medium-to-large species of bees from the genera Eucera, Xylocopa and Apis can carry 2324the pollinia of Calanthe spp. (Ishihara 1957; Ishikawa and Suzuki 1992; Karasawa and Ishida 1998). Indeed, intensive observation of Calanthe striata revealed that it was 25

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

There are currently twenty-six recorded taxa in the genus Calanthe in Japan 5 (Karasawa and Ishida 1998), although over-collection and habitat loss has placed almost 6 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, 1995). However, in its native habitat C. discolor is categorized as an endangered species 11 12(Environment Agency of Japan 2000) and the probability of its extinction in Japan over the next 100 years is estimated to be almost 100% (Environment Agency of Japan 2000). 1314 Despite its popularity, basic information regarding pollinators and other aspects of its reproduction system is lacking, with the exception of a few anecdotal studies suggesting 1516 pollination by medium-sized bees (Ishihara 1957; Ishikawa and Suzuki 1992; Karasawa 17and Ishida 1998).

C. striata is a closely related species that is often classified as an intraspecific 1819variant of C. discolor (Iwatsuki 1995; Kim et al. 2008). The two species share the same 20morphological characteristics during vegetative growth but produce different flowers, 21those of C. striata being larger and emitting a stronger fragrance. The two species also 22differ in their coloration with the flowers of C. striata being uniformly yellow, whereas those of C. discolor are bicolored having brownish sepals and lateral petals with white 2324lips. In addition, C. discolor is often cited as producing nectar (e.g. Kawarasawa 1998), while C. striata is known to be a food deceptive orchid (Sugiura 2013). Since 25

pollinators often have a strong associative learning ability (Biernaskie et al. 2009), 1 $\mathbf{2}$ food-deceptive species are usually expected to be strongly pollinator-limited, and 3 exhibit adaptive traits such as exaggerated floral signals (e.g. elongated spurs; Sletvold 4 and Ågren 2011) and optimized floral phenology (e.g. early flowering; Internicola and $\mathbf{5}$ Harder 2012) to promote pollinator attraction and pollination efficiency. Considering 6 that the diversity of floral characteristics can often be attributed to divergent selection 7 by pollinators, it is important to study whether the floral differences in C. discolor and 8 C. striata are a result of adoptions to different pollinators.

9 The current study was initiated to investigate four aspects of the reproductive 10 biology of *C. discolor*: (1) to determine the potential for autonomous self-pollination (2) 11 to confirm whether *C. discolor* produces nectar or is food deceptive (3) to identify 12 candidate pollinators and ascertain any pollinator limitation (4) to determine whether *C. discolor* shares the same pollinator assemblage as its close relative *C. striata*.

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15 Materials and Methods

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17Field 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 18 19 habitat was a coniferous plantation dominated by *Cryptomeria japonica*. The relative 20importance of daytime and nighttime visitors was evaluated by walking through the 21population and checking for the removal of pollinia in the mornings and evenings. This 22preliminary study revealed that pollinia were only removed during the day, and consequently the detailed observations of floral visitors were scheduled to coincide with 2324the 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 25

1 immediately after they had visited flowers.

 $\mathbf{2}$ 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 $\mathbf{5}$ the experiment was limited to 15 from each plant, with the surplus flowers being removed before beginning the experiments. Each of the experimental flowers was in 6 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; 124 shoots 1340 flowers in 2013). All the allogamous plants were spaced at least 5 1314 meters from their nearest neighbor to avoid sampling within genetically identical plant. The experimental plants were intermittently monitored during the subsequent four 1516 weeks and scored for fruit set once the capsules had formed. The statistical differences 17between the pollinator-exclusion, manual-autogamy and manual-allogamy treatments were detected using Fisher's exact test with sequential Bonferroni correction. 18

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

microscope to assess the ratio of seeds with an embryo compared to those without. The 1 $\mathbf{2}$ 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.

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

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Results 8

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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. Suetsugu 2013; Sugiura 2013), were detected. Members of the Diptera and Coleoptera 13were also observed visiting the flowers of C. discolor, although they did not exhibit 14typical pollinator behaviour, with most individuals merely alighting or resting on the 1516 flowers. However, food-seeking behavior was occasionally observed for the hoverfly 17Episyrphus balteatus and false blister beetle Oedemeronia lucidicollis, although they were unable to carry pollinia as a result of their small body size. The agromyzid fly, 1819Japanagromyza tokunagai, known to infest various species of Japanese orchids (e.g. 20Suetsugu 2013; Sugiura 2013) was also observed visiting the flowers of C. discolor to 21lay its eggs in the young ovaries.

The most frequent visitors were bee species including Eucera nipponensis 22(28% of total floral visits), Osmia cornifrons (23%) and Apis cerana japonica (10%). 2324All three species exhibited nectar seeking and pollinating behaviour; shortly after 25alighting on the labellum, they held the labellum with their fore and middle legs and

inserted their proboscises deeply into the spur (Fig. 2D). Whilst probing, their heads 1 $\mathbf{2}$ 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 $\mathbf{5}$ inflorescence before leaving. However, occasionally individuals of E. nipponensis and O. cornifrons (two and three individuals, respectively) visited multiple flowers within 6 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 12bore fruit corresponding to a total fruit set of 6.5% and 1.2%, respectively. The large discrepancy between the open-pollinated samples and the artificially fertilized flowers 13(both autogamous and allogamous) suggests that C. discolor experiences a high degree 14pollinator limitation in its native habitat. Although the manual autogamous treatment 1516produced a lower fruit set than the allogamous treatment, this difference was not significant (Fisher's exact test, P = 0.85), indicating that C. discolor is fully 17self-compatible. Furthermore, the seed mass and proportion of seeds with an embryo 18 did not significantly differ between the two treatments (Student's *t*-test, P = 0.06, P =19200.42, respectively, Table. 2).

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The results of the spur dissection experiment suggested that C. discolor does not produce nectar since no nectar secretions were detected in any of the 20 flowers 2223examined.

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25Discussion

A large discrepancy was observed between the fruit set of the open-pollinated subjects 1 $\mathbf{2}$ (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 pollinator-limitation, but is also consistent with the trend for relatively low fruit set 5 found in most orchid species (Neiland and Wilcock 1998; Tremblay et al. 2005). 6

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 to C. discolor, indicates that like other Calanthe species (Sakata et al. 2013; Sugiura 122013), C. discolor adopts a generalized strategy of food deception, whilst these results 1314 should not be considered conclusive as it possible that other populations do produce nectar, or that C. discolor is adapted to mimic a rewarding plant (or a set of plants) that 1516 did not occur in this particular location. Generalized food deceptive species often rely 17on pollination by naive insects and/or insects whose food resources have become depleted and thus early flowering is flavoured (Internicola et al. 2008, Internicola and 1819Harder 2012). The flowering time of C. discolor (late April to early May) is early to 20middle stage of the main pollinator Eucera activity (April to June; Enju 2013). Thus, C. 21discolor ultilize not so much naive pollinator as pollinator whose food resources have become depleted. Because there are no sympatric co-blooming plants which share 2223pollinators in our study site, the habitat separation from other nectariferous plants may 24be important for successful exploitation.

Since pollinators often have a strong associative learning ability (Biernaskie et 1 $\mathbf{2}$ 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 $\mathbf{5}$ visitation, could in turn reduce the reproductive success of deceptive orchids (Neiland and Wilcock 1998; Tremblay et al. 2005). The data from the current study provided 6 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 $(37.1\% \pm 3.2\%, n = 84 \text{ and } 20.7\% \pm 1.7\%, n = 130$, 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 13also been linked to forest fragmentation (Tomimatsu and Ohara 2002; Huang et al. 142009). However, this seems unlikely since the study site was dominated by Cryptomeria, which has a dense canopy, and unmanaged Cryptomeria plantations are known to 1516support very few understory plants (Ishii et al. 2008). Under such circumstance forest 17fragmentation might actually improve the richness and abundance of nectariferous plants, and indirectly favor insect visitation rates to orchids. 18

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

from manual-autogamy and manual-allogamy treatments. This tendency appears to be 1 $\mathbf{2}$ 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 the proportion of seeds with a developed embryo from the manual-autogamy and 6 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.

13The floral diversity of orchids has often been linked to the intimate and complicated interactions they have with their pollinators (Gill 1989). However, most 14estimates of a high incidence of single-pollinator species were based on observations of 1516 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 food deception orchids often attract a guild of locally available insect visitors 1819(Cozzolino et al. 2004, 2005). The field observations in the current study supported this 20opinion, with at least three bee species (Eucera nipponensis, Osmia cornifrons and Apis 21cerana japonica), belonging to two different families, being observed pollinating the 22flowers of C. discolor.

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

Instead, the current study confirmed that C. discolor, similar to its close relative C. 1 $\mathbf{2}$ 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 growing evidence that the actual fauna pollinating a particular species is determined by 6 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 possible that the evolution of nectarless flowers may have released from selective 9 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 investigation is needed to reveal whether the phenotypic selection to longer spur exists 1314 in C. discolor.

Curiously, both C. discolor and C. striata have been considered conspecific by 1516 some authors (Kim et al. 2008), despite the differences in their flower size, coloration 17and fragrance (Park et al. 2010). The current study provides further evidence for reproductive isolation between these two species (i.e. difference of pollinator 18 19preference: C. discolor being pollinated by E. nipponensis, O. cornifrons and A. cerana 20japonica, while Calanthe striata seems to be pollinated exclusively by large carpenter 21bees; Sugiura 2013). Furthermore, C. discolor lacks the strong and/or very sweet scents 22which is associated with Xylocopa-pollinated orchids (e.g. Braga 1977; Díaz and Vale 2001; Sugiura 2013). Although it should be noted that differences of floral visitors can 2324be partially explained by local availability of pollinators in our study and Sugiura's study site, the floral characteristics, such as perianth size and the height of the 25

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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6	Acknowledgements
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8	We thank Makoto Kato and Yuta Nakase for identifying the bees and hoverflies. This
9	study was partly supported by the Japan Society for the Promotion of Science Research
10	Fellowships for Young Scientists Grant to KS.
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1 Figure legends



Figure 1. Flowers of *Calanthe discolor* and its visitors. (a) Flowering individuals, (b)
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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Insect appairs	Incost order	No. of	Pollinia	Pollinia
	Insect order	visitors	removal	deposition
Eucera nipponensis	Hymenoptera	11	8	3
Osmia cornifrons	Hymenoptera	9	5	2
Apis cerana japonica	Hymenoptera	4	2	0
Episyrphus balteatus	Diptera	5	0	0
Eupeodes corollae	Diptera	2	0	0
Japanagromyza tokunagai	Diptera	1	0	0
<i>Sphaerophoria</i> sp.	Diptera	1	0	0
Oedemeronia lucidicollis	Coleoptera	2	0	0
Macrolagria rufobrunnea	Coleoptera	2	0	0
<i>Lemyra imparilis</i> (larva)	Lepidoptera	3	0	0

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

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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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