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**Avian seed dispersal in a mycoheterotrophic orchid *Cyrtosia septentrionalis***

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1   **Abstract:**

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3   **Orchids produce remarkably small seeds lacking endosperm, which are considered**  
4   **to be predominantly wind-dispersed. Here, we report avian seed dispersal in a**  
5   **mycoheterotrophic orchid *Cyrtosia septentrionalis*, which occurs under closed**  
6   **canopies where wind is less dependable. Because some orchids occupy similar**  
7   **habitats and have fleshy fruits, shifts in seed dispersal strategy may be a previously**  
8   **unnoticed mechanism promoting the orchid's evolutionary success.**

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10   Orchidaceae is one of the most diverse groups of flowering plants on Earth. Unlike most  
11   plants, all of the >22,000 species of orchids are heterotrophic in their early life history  
12   stages, obtaining resources from fungi before the production of photosynthetic leaves<sup>1</sup>.  
13   Orchid seeds, therefore, contain minimal energy reserves and are numerous and  
14   dust-like, which maximises the chance of successful encounter with fungi in the  
15   substrate<sup>1</sup>. Despite considerable interest in ways by which orchid flowers are pollinated<sup>2</sup>,  
16   little attention has been paid to how their seeds are dispersed, owing to the dogma that  
17   wind dispersal is their predominant strategy. However, here, we present evidence for  
18   seed dispersal by birds in the orchid *Cyrtosia septentrionalis*.

19       Animal seed dispersal has been implicated in a few orchid genera that produce  
20   fleshy fruits including *Cyrtosia*<sup>3-6</sup>, but a compelling demonstration has hitherto been  
21   lacking. *Cyrtosia septentrionalis* is a non-photosynthetic, fully mycoheterotrophic  
22   orchid found in the understories of temperate forests in Japan and produces red, swollen  
23   fruits that ripen in winter. We monitored the fruits of *Cyrtosia septentrionalis* for a total  
24   of 2427 hours in the field using motion sensor-equipped cameras, which revealed that

1 the their pulp was frequently eaten by four species of birds (Fig. 1 and Table 1). Of  
2 these, *Hypsipetes amaurotis* was the most frequent pulp consumer. We directly  
3 observed defecation by *H. amaurotis* in the field and confirmed through examination of  
4 the faecal pellets that *H. amaurotis* ingested *C. septentrionalis* seeds and thus were not  
5 consuming the pulp alone (seeds per faecal pellet,  $176.1 \pm 24.2$ ;  $N = 20$ ). Of the seeds  
6 sampled from *H. amaurotis* faeces,  $48.8 \pm 6.5$  % ( $N = 10$ ) were viable, a figure that is  
7 comparable to the seeds taken directly from intact *C. septentrionalis* fruits ( $45.2 \pm 5.9$  %,  $N = 10$ ;  $P = 0.34$ ).

9 Compared to other orchids, *C. septentrionalis* possesses several unusual  
10 characteristics that are thought to facilitate ornithochory: red, indehiscent fruit with  
11 sugary pulp, a robust stem that acts as perch, and a lignified testa that protects the  
12 viability of seeds inside avian digestive tracts (Fig. 1). Considering that most orchid  
13 species produce pale-colored, dry capsules that dehisce when ripe to release the  
14 dust-like seeds, the fruit of *C. septentrionalis* is exceptional in Orchidaceae. Red fruits  
15 are usually associated with seed dispersal by diurnal birds<sup>7</sup>. The average sugar content  
16 of the fruit pulp was  $17.1 \pm 0.37$  % ( $N = 20$ ), which is comparable to those of other  
17 bird-dispersed fruits<sup>8</sup>. In addition, the inflorescence stalk of *C. septentrionalis* is  
18 unusually robust; of the 1219 photographs with birds feeding on *C. septentrionalis* fruits,  
19 535 had birds perching directly on the inflorescence stalk (Table 1). Thus, the robust  
20 stalk of *C. septentrionalis* may serve as a perch that facilitates fruit consumption by  
21 birds, similar to the specialised perch of *Babiana ringens* that has been found to  
22 promote bird pollination<sup>9</sup>. Finally, we found that the seeds of *C. septentrionalis* possess  
23 lignified testa, which probably protects the seeds as they pass through the digestive  
24 tracts of birds.

1           Seed dispersal by wind is thought to be successful in open habitats and less  
2   efficient in the understory of densely vegetated forests, where wind is less dependable<sup>10</sup>.  
3   For example, in monocots, 19 of the 21 evolutionary shifts from dry fruits to fleshy  
4   fruits were associated with life in shady habitats, whereas eight of the 11 reversals from  
5   fleshy to dry fruits were associated with life in open sunny habitats<sup>10</sup>. *Cyrtosia*  
6   *septentrionalis* is a fully mycoheterotrophic orchid that has lost its ability to  
7   photosynthesise throughout its life and, instead, depends entirely on fungi for its  
8   nutritive needs. Because mycoheterotrophic plants do not require light, this form of life  
9   has allowed them to succeed in the dark forest understory where there are few  
10   autotrophic competitors<sup>11</sup>. Thus, a shift to bird dispersal may thus have facilitated the  
11   colonisation of such an environment by *C. septentrionalis*.

12           The gross fruit morphology and pigmentation patterns of other members of  
13   *Cyrtosia* parallel those seen in *C. septentrionalis*, suggesting that avian seed dispersal is  
14   widespread among the genus *Cyrtosia*<sup>6</sup>. In addition, fleshy, indehiscent fruit have also  
15   been documented in *Rhizanthella*<sup>4</sup>, a distantly related genus that has also independently  
16   evolved full mycoheterotrophy. Some species of *Vanilla*, although not full  
17   mycoheterotrophs, also have seeds with lignified testa and fleshy fruits<sup>5,12</sup>, which are  
18   also considered to be dispersed by animals. Thus, shifts in seed dispersal mechanisms  
19   may have played a more significant role in promoting the ecological success and  
20   morphological diversity of orchids than previously thought.

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## 22   **Methods**

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## 24   **Field study**

1 Field studies were carried out between December 2008 and January 2009, and between  
2 November 2010 and February 2011 in Gochi Town, Higashi-Ohmi City, Shiga  
3 Prefecture, Japan. Consumers of *C. septentrionalis* fruits were observed in the field  
4 throughout the duration of the field study by remote cameras, which had built-in  
5 infrared motion sensors (Bushnell Trophy Cam Field Scan Night Vision, Bushnell  
6 Corporation, USA or Sensor Camera Fieldnote, Marif Co. Ltd., Yamaguchi, Japan).  
7 Each camera was set up 1 meter away from six to nine *C. septentrionalis* plants to  
8 determine the species composition of the animals feeding on the fruits. In 2011, we  
9 collected 20 fruits that had been observed being consumed by *H. amauroti* and  
10 measured the sugar concentrations of fruit pulp using a refractometer (Bellingham &  
11 Stanley, Kent, UK). We deposited a voucher specimen of *Cyrtosia* with *H. amauroti*  
12 feeding marks in KYO.

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#### 14 **Seed viability**

15 Orchid seeds are believed to be particularly vulnerable to stress because their testa  
16 consists of a thin layer of non-lignified cells and are unlikely to withstand the digestive  
17 fluids of birds<sup>13</sup>. Therefore, we tested the viability of *Cyrtosia* seeds defecated by birds.

18 We observed bird visits by hiding in the vegetation and collected faecal pellets  
19 whenever we observed defecation by *H. amauroti*. We used 10 such faecal pellets for  
20 the below seed viability test. The faecal pellets were washed in distilled water, and 50  
21 seeds per faecal pellet were randomly chosen for a total of 500 seeds. Viability was  
22 tested using the 2,3,5-triphenyl tetrazolium chloride staining method as previously  
23 described<sup>14</sup>. In brief, seeds were pretreated in 5% Ca(ClO)<sub>2</sub> (W/V) + 1% Tween-80  
24 (V/V) for 6 hours. After the treatment, the seed testa was removed with tweezers, and

the embryos were placed on filter paper discs immersed in a solution of 1% TTC (Tokyo Chemical Industry, Japan) in pH 7 water adjusted with 1M NaOH. The filter paper discs were placed in sealed Petri dishes wrapped in aluminum foil, and incubated at 30°C for seven days. The viability of defecated seeds was compared with that of the same number of seeds collected directly from the fruits of 10 plants. Viability differences between seeds from fruits and faeces were compared using Student's *t* test.

### **Seed coat anatomy**

The test of seed viability revealed that seeds extracted from the faeces of *H. amaurotis* remained intact. This led us to investigate the anatomy of the seed testa to explore possible characteristics that allowed the seeds to withstand the digestive fluids of animals. Seeds were fixed in FAA and then dehydrated in graduated *t*-butyl alcohol, after which they were saturated with paraffin. Sections (7 µm) were cut with a microtome and attached to Haupt's adhesive-treated microscope slides. After the removal of the paraffin, slides were stained with Safranin O (1% w : v in 50% v : v ethanol, 5 min). With this staining technique, lignified tissues and secondary cell walls were stained red<sup>15</sup>.

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## 19 **Acknowledgements**

20 We thank Drs. B. Anderson and S. Nakamura for providing comments that improved  
21 this manuscript and S. Mori for field assistance. This work was partially supported by  
22 the Japan Society for the Promotion of Science (No. 12J00602, K.S., and No. 22247003,  
23 M.K.).

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1    **Author contributions**

2    K.S. designed the study, conducted the experiment, and composed the manuscript. A.K.  
3    and M.K. contributed to the study design and the manuscript composition.

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1   **Figure legend**

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3   **Figure 1 | *Cyrtosia septentrionalis* and its seed dispersal agents.** (a–b) Reproductive  
4   stem, (c) *Cettia diphone* perching on the stem, (d) *Hypsipetes amaurotis* feeding on the  
5   fruit pulp, (e) fruits with feeding marks, (f) horizontal section of the fruit, (g) seeds  
6   defecated from *H. amaurotis*, and (h) cross section of a seed. Bar = 100 µm.



**Table 1 | List of bird species captured by motion-sensor camera.** Camera recordings were conducted for 219 hours between December 2008 and January 2009, and for 2208 hours between November 2010 and February 2011. Numbers given are the total numbers of (a) frames that captured each bird species, and (b) individuals that fed on the fruits. Birds were regarded as the same individual when captured in consecutive frames. Numbers in parentheses are the frames with individual birds perching on the inflorescence stalk.

|                             | 2008–2009  |                 | 2010–2011  |                 |
|-----------------------------|------------|-----------------|------------|-----------------|
|                             | (a) Frames | (b) Individuals | (a) Frames | (b) Individuals |
| <i>Cettia diphone</i>       | 103 (10)   | 41 (6)          | 1 (1)      | 1 (1)           |
| <i>Hypsipetes amaurotis</i> | 49 (5)     | 21 (3)          | 988 (501)  | 146 (59)        |
| <i>Turdus pallidus</i>      | 4 (1)      | 4 (1)           | 68 (11)    | 20 (7)          |
| <i>Garrulus glandarius</i>  | 0          | –               | 1 (1)      | 1 (1)           |