TITLE:

Slippery flowers as a mechanism of defence against nectar-thieving ants

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2   **Title: Slippery flowers as a mechanism of defence against nectar-thieving ants**

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11   Running title: Slippery flowers deter nectar-thieves

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1   **Abstract**

2   -Background and Aims

3   The great diversity of floral characters among animal-pollinated plants is commonly understood

4   as the result of coevolutionary interactions between plants and pollinators. Floral antagonists,

5   such as nectar thieves, also have the potential to exert selection on floral characters, but

6   adaptation against floral antagonists has attracted comparatively little attention. We found that the

7   corollas of hornet-pollinated *Codonopsis lanceolata* (Campanulaceae) and the tepals of

8   bee-pollinated *Fritillaria koidzumiana* (Liliaceae) are slippery to nectar-thieving ants living in the

9   plant's habitat; because the flowers of both species have exposed nectaries, slippery perianths

10   may function as a defence against nectar-thieving ants.

11   -Methods

12   We conducted a behavioural experiment and observed perianth surface microstructure by

13   scanning electron microscopy to investigate the mechanism of slipperiness. Field experiments

14   were conducted to test whether slippery perianths prevent floral entry by ants, and whether ant

15   presence inside flowers affects pollination.

16   -Key Results

17   Scanning electron microscopy observations indicated that the slippery surfaces were coated with

18   epicuticular wax crystals. The perianths lost their slipperiness when wiped with hexane. Artificial

bridging of the slippery surfaces using non-slippery materials allowed ants to enter flowers more

frequently. Experimental introduction of live ants to the *Codonopsis* flowers evicted hornet

pollinators and shortened the duration of pollinator visits. However, no statistical differences

were found in the fruit or seed sets of flowers with and without ants.

-Conclusions

Slippery perianths, most likely based on epicuticular wax crystals, prevent floral entry by ants

that negatively affect pollinator behaviour. Experimental evidence of floral defence based on

slippery surfaces is rare, but such a mode of defence may be widespread amongst flowering

plants.

Key words:

Codonopsis lanceolata; *Fritillaria koidzumiana*; nectar-theft; nectar-thieving ant; floral

antagonist; floral defence; floral larceny; epicuticular wax; wax crystals; plant–insect

interactions; ant–plant interactions; pollination

1   **INTRODUCTION**

2   The remarkable diversity of floral characters among animal-pollinated plants is commonly

3   understood to be the result of coevolutionary interactions between plants and pollinators. This is

4   exemplified by numerous studies of how the colour, odour and shape of flowers mediate effective

5   attraction of pollinators and their physical contact with anthers and stigmas (e.g. Fenster *et al.*

6   2004; Papadopulos *et al.* 2013; Johnson and Wester 2017; De Jager and Peakall 2019; Kemp *et al.*

7   2019). However, flowers are visited by both legitimate pollinators and floral antagonists, the

8   latter including florivores, seed predators, predators of pollinators, and nectar thieves. Such floral

9   antagonists may also exert selective force on floral evolution (Irwin *et al.* 2004; McCall and

10   Irwin 2006; Willmer 2011), but adaptation to such floral antagonists has attracted comparatively

11   little research interest.

12   Nectar-foraging ants generally belong to the floral antagonists (Willmer *et al.* 2009). They

13   are globally ubiquitous and have a major impact on terrestrial ecosystems (Hölldobler and

14   Wilson 1990). A considerable number of plants have independently established mutualisms with

15   ants by offering them rewards and utilising their ecological impact for protection and dispersal;

16   plants in more than 100 families are engaged in defensive mutualisms with ants by producing

17   extrafloral nectar (Weber *et al.* 2015), and plants in more than 80 families exhibit seed dispersal

mutualisms with ants (Giladi 2006). However, examples of pollination mutualism involving ants

are rare (Dutton and Frederickson 2012). This is thought to be because of their inability to fly,

small and hairless body surface (although several ant species are covered with setae (Beattie *et al.*

1984)), frequent grooming behaviour, and chemical compounds on their body that inhibit pollen

viability (Beattie *et al.* 1984; Hull and Beattie 1988; Dutton and Frederickson 2012).

Nevertheless, ants often visit flowers and consume floral nectar, and are thus mainly regarded as

nectar thieves. In addition to consuming nectar, they sometimes attack and deter legitimate

pollinators, thereby decreasing pollination success (Galen and Cuba 2001; Tsuji *et al.* 2004; Ness

2006; Lach 2008; Hansen and Müller 2009; Cembrowski *et al.* 2014). Such negative effects of

ants are likely to drive the evolution of ant-deterring mechanisms. For example, the floral odours

of a wide range of tropical flowers are repellent to ants (Junker and Blüthgen 2008), and several

compounds commonly found in floral odour, such as linalool and 2-phenyl ethanol, exhibit such

ant repellence (Junker *et al.* 2011). Besides chemical deterrence, extrafloral nectar has been

suggested to function as a decoy (Villamil *et al.* 2019), and various floral features have been

proposed to act as physical barriers to ants (Willmer *et al.* 2009), but experimental studies to test

such functions are limited (e.g. Galen and Cuba 2001; Tagawa 2018; Villamil *et al.* 2019).

1   *Codonopsis lanceolata* (Campanulaceae) is a perennial vine that produces pendent and

2   campanulate flowers that are almost exclusively pollinated by hornets (Fig. 1A–C). There are five

3   short nipple-shaped spurs on the corners of the corolla base, and the nectar in each spur is often

4   visible as droplets (Fig. 1B). The exposed nature of the nectar suggests that the flower is

5   susceptible to nectar thieving by ants. During preliminary observation, we found that ants visited

6   the flowers and often slipped off the corolla while attempting to walk on it. Further inspections

7   indicated that the abaxial surface and the distal half of the adaxial surface of the corolla of *C.*

8   *lanceolata* are slippery to ants, whereas the basal half of the adaxial surface, where the pollinators

9   cling to the flower with their forelegs during visitation (Figure 1C), is not slippery (see Figure 2;

10   Supplemental Movie S1, S2). We also discovered slippery perianths in *Fritillaria koidzumiana*

11   (Liliaceae), which similarly bears campanulate flowers and has exposed nectaries. In the present

12   study, we hypothesise that the slippery perianths in *C. lanceolata* and *F. koidzumiana* are a

13   physical defence against nectar-thieving ants and test whether 1) their perianths possess special

14   surface structures that make them slippery to ants (Federle *et al.* 1997; Gaume *et al.* 2002), 2) the

15   slipperiness prevents ants from entering the flowers under natural conditions, and 3) the presence

16   of nectar-thieving ants has a negative effect on pollination.

17

1   **MATERIALS AND METHODS**

2   *Materials*

3   *Codonopsis lanceolata* is a perennial herbaceous vine that occurs along forest edges of evergreen

4   and deciduous forests in temperate areas of East Asia. Each individual produces 1–30 flowers

5   from September to October. The corolla is creamy white with variable degrees of purple patches

6   and lines and a diameter of 23.7 ± 0.7 mm (mean ± SE, n = 6) (Fig. 1A–C). Each flower may

7   hold as much as 94.0 ± 25.1 μL (mean ± SE, n = 3) of nectar during the day (Fig. 1B). As is

8   common to most Campanulaceae, the flower is protandrous. As the bud opens, the pollen on the

9   stamen is deposited and presented on the style column, at which stage the stigma remains

10   unexposed (male stage; Fig. 1B). On the second day, the stigma becomes exposed (female stage;

11   Fig. 1C). The flower withers on the third day. The flowers are almost exclusively visited by

12   hornets (genus *Vespa*; Hymenoptera, Vespidae) that carry abundant pollen on their thoraces (Fig.

13   1C) and are thus likely effective pollinators (Inoue *et al.* 1990; Funamoto 2019) (K. Takeda,

14   personal observations). We used plant individuals growing at the Ashiu Forest Research Station

15   of Kyoto University (35°18'N, 135°43'E, Kyoto Prefecture, Japan), Sakauchi (35°37'N,

16   136°22'E, Gifu Prefecture, Japan), and Shiramine (36°10'N, 136°37'E, Ishikawa Prefecture,

17   Japan) for the experiments and observations described below.

1   *Fritillaria koidzumiana* is a spring ephemeral endemic to central Japan that grows on the

2 forest floors of cool-temperate deciduous forests. Each mature individual produces a single

3 flower at the shoot tip between late March and April. The flower is a broad campanulate, white to

4 cream coloured with green and purple dots, and has a diameter of approximately 12 mm (Fig.

5 1D–F). Its nectary is band-shaped and located along the centre of each of the six tepals (Fig. 1E).

6 The nectaries are exposed and easily distinguishable by their green colour. The flowers are

7 pollinated by mining bees *Andrena* (Hymenoptera, Andrenidae) (Fig. 1F), mainly *A. benefica*

8 (Naruhashi *et al.* 2006). We used plant individuals from Tsurugi (36°26'N, 136°38'E, Ishikawa

9 Prefecture, Japan) for the analyses presented below.

10

11 *Behavioural assay*

12 Previous studies of slippery plant surfaces, such as those in the pitcher plant *Nepenthes*

13 (Nepenthaceae) and the stems of *Macaranga* myrmecophytes (Euphorbiaceae) (e.g. Federle *et al.*

14 1997; Gaume *et al.* 2002), have shown that the slipperiness is caused by epicuticular wax crystals.

15 Thus, we examined whether epicuticular wax is also responsible for the slipperiness of *C.*

16 *lanceolata* and *F. koidzumiana* perianths. The behavioural assays were conducted in the field at

17 the Ashiu population for *C. lanceolata*, and at the Tsurugi population for *F. koidzumiana*.

1   Because most *C. lanceolata* flowers occurred high up on the vine, we used flowers detached from

2   the plants, whereas in *F. koidzumiana*, we used flowers as they occurred on the plants. We first

3   investigated ant behaviour on flowers whose wax had been removed. Because plant epiculaticular

4   waxes are soluble in non-polar solvents, we gently wiped the perianths with glass wool (thickness,

5   2–6 µm, AS ONE Corporation, Osaka, Japan) soaked with pure hexane (Wako Pure Chemical,

6   Osaka, Japan). We compared the proportions of ants that slipped off the wax-removed flowers to

7   those on control flowers whose perianths were left untreated. Four ant species (Hymenoptera,

8   Formicidae), which have been observed foraging for floral nectar in the natural habitats of the

9   studied plant species, were used for this experiment; *Nylanderia flavipes*, *Pristomyrmex*

10   *punctatus*, and *Formica japonica* for *C. lanceolata*, and *N. flavipes* and *Lasius japonicus* for *F.*

11   *koidzumiana*. We carried out experiments on both the adaxial (hereafter, inner or inside) and

12   abaxial (hereafter, outer or outside) surfaces of the corolla for *C. lanceolata*, but only on the outer

13   surface of the tepals for *F. koidzumiana*. This is because *F. koidzumiana* possesses hairy

14   processes on the edges of the outer-whorl tepal and along the boundary of the nectary on the

15   inner tepal surface (Fig. 2C), and ants could walk by gripping them. In the case of *C. lanceolata*,

16   two flowers from each of ten individuals were used for experiments; one was used for the

17   hexane-wipe treatment and the other as a non-treated control. Both male- and female-stage

1   flowers were used, but floral age was randomized between treatments. In the case of *F.*

2   *koidzumiana*, one flower from each of 40 individuals was used for experiment; 20 were treated

3   with a hexane-wipe and 20 were used as non-treated controls. We used freshly opened flowers

4   that retained abundant pollen on anthers. Difference in the sample sizes between the two species

5   simply reflects availability of flowers. We conducted one trial for one individual of each ant

6   species on both sides of every flower. The detailed procedures of the experiment are described in

7   the Supplementary Material.

8

9   *Microscopy*

10   We investigated whether epicuticular wax crystals are present on the slippery portions of the

11   perianths by using scanning electron microscopy (SEM). The flowers (*C. lanceolata* from Ashiu,

12   n = 3; *F. koidzumiana* from Tsurugi, n = 4) were sampled from separate individuals in the field,

13   kept in Ziploc® bags (S. C. Johnson & Son, Inc., US) for less than 6 h before being brought back

14   to the laboratory, and frozen at –25°C until examination. Subsequently, the flower samples were

15   freeze-dried for 12h using a vacuum freeze dryer (FDU-1200, Tokyo Rikakikai Co. Ltd., Tokyo,

16   Japan), cut into ca. 5 x 5 mm fragments and fixed onto specimen holders with double-sided

17   carbon tape. Although the freeze-drying process makes the perianths slightly shrunken, it does

1   not affect the observation of wax crystals on the perianth surface. The samples were sputtered

2   with gold for 90 sec. (30 mA) using a fine sputter coater (JFC-1200, JEOL Ltd., Tokyo, Japan)

3   and the surface structures were observed using an SEM (TM-3000, Hitachi, Tokyo, Japan).

4   Furthermore, we wiped a portion of the *F. koidzumiana* tepal with hexane-soaked glass wool

5   before freezing, the same treatment as performed in the wax removal behavioural experiment, to

6   compare them to ordinary tepals to determine how the hexane-wiping treatment affected the

7   presence of wax crystals.

8

9   *Bridging experiment*

10   To test whether the slipperiness prevents ants from entering flowers under field conditions, we

11   bridged the slippery flower surfaces with non-slippery material and studied the ant behaviour in

12   situ (*C. lanceolata* in the Sakauchi population; *F. koidzumiana* in the Tsurugi population). In the

13   case of *C. lanceolata*, we fixed a piece of masking tape (J7520, Nitoms, Inc., Tokyo, Japan) to

14   span the base of the inner corolla and the floral stalk. As controls, we fixed the tape only to the

15   floral stalk. For *F. koidzumiana*, we used a bamboo stick pinned to the ground beneath the flower,

16   the top of which was attached to the inner surfaces of the tepals (Fig. S1). As controls, we

17   similarly pinned the sticks but not touching the tepals. For *C. lanceolata*, we marked 83 flowers

1   from 18 individuals, and for *F. koidzumiana*, we marked 80 flowers from 80 individuals in the

2   field. We divided the flowers into the treatment group (43 flowers in *C. lanceolata* and 40

3   flowers in *F. koidzumiana*) and the control group (40 flowers in *C. lanceolata* and 40 flowers in

4   *F. koidzumiana*). We distributed the *C. lanceolata* flowers from each individual evenly into the

5   two groups. We conducted the above treatments in the morning of the first day, and after an

6   acclimatisation time of 2–3 h, we recorded the number and species of ants in each flower every

7   90 min in *C. lanceolata* and every 60 min in *F. koidzumiana* during the daytime, when the ants

8   were active (09:30 to 16:00). The observations spanned two days in both species. When the

9   flowers withered or bridging materials were accidentally detached from the flowers, we stopped

10   recording and only used the data collected before the accident.

11

12   *Ant introduction experiment*

13   Previous studies have reported that nectar-thieving ants attack and deter pollinators, thereby

14   decreasing plant reproductive success (Tsuji *et al.* 2004; Ness 2006; Cembrowski *et al.* 2014).

15   Thus, we artificially introduced ants to *C. lanceolata* flowers in the field to investigate whether

16   ants negatively affect pollination. First, we divided the flowers from each individual evenly into

17   three groups: the 'ant-present' treatment group and two control groups. For the ant-present

1   treatment, we secured a live *F. japonica* ant inside the flower by knotting a thread around the

2   constriction between the middle legs and the hind legs and fixing the other end of the thread to

3   the floral stalk through a hole made on the corolla base (Fig. S1c). For the control flowers, we

4   either only attached the thread ('thread-only' control) or left them untreated ('untreated' control).

5   In 2017, we only tested the ant-present and thread-only groups, thus the untreated group is only

6   represented in the 2018 data. In total, we used 157 flowers from 25 *C. lanceolata* individuals in

7   this experiment (25 flowers in 2017, 132 flowers in 2018).

8   To test the effect of ant presence on pollinator hornets, we recorded their behaviours by

9   video camera for 1–4 h during the daytime (9:00–16:00). Specifically, we recorded the species,

10   visit duration, and visitation frequency of hornets. The visitation frequency was calculated based

11   on the number of visits (without discriminating hornet individuals) over 120 min from the start of

12   the observation. Thus, video recordings shorter than 120 min were not used in our visitation

13   frequency calculations.

14   We then collected all remaining flowers and fruits 19 days after the last flowers had

15   withered and counted the number of fertilised and unfertilised ovules in each fruit, in order to use

16   fruit set and seed set as a proxy for reproductive success. Eight fruits were severely damaged by

17   seed predators and their ovules could not be counted. Hence, we excluded such damaged fruits

1   from the ovule counts. However, if one or more of the three locules remained undamaged, we

2   used data from the undamaged locules.

3

4   *Statistical analysis*

5   All statistical analyses were carried out using the R version 3.5.0 (R Core Team 2018). We

6   compared the slip rates in the wax removal experiment between the treatment and control groups

7   using the McNemar test with a binomial distribution in the case of *C. lanceolata* and Fisher's

8   exact test in the case of *F. koidzumiana*.

9   To analyse the results of the bridging experiment and the ant-introduction experiment, we

10   assessed the generalised linear model (GLM) using the *glm* function and the generalised linear

11   mixed model (GLMM) using the *glmer* function in the lme4 R package (Bates *et al.* 2015). To

12   compare the frequencies of the ant-present records for each flower treatment, we used GLMM

13   with a binomial distribution and individual ID as a random effect for *C. lanceolata,* and GLM

14   with a binomial distribution for *F. koidzumiana*. We compared the durations between different

15   treatments using GLMM with a gamma distribution, and year, individual ID (nested within year),

16   and flower ID (nested within individual ID) as random effects. To compare visitation frequencies,

17   we used GLMM with a Poisson distribution, treatments and flower sex stage as explanatory

1   variables, and year and individual ID (nested within year) as random effects. This is because the

2   sex stage of the flower has a large effect on the visitation frequency (male-stage flowers are more

3   attractive), and models including flower sex stage yielded the lowest Akaike information criterion

4   (AIC) values. To compare the fruit set (proportion of flowers that developed as fruits), we used

5   GLMM with a binomial distribution, the fruit set of each flower (1 or 0) as a response variable,

6   and the individual ID as a random effect. To compare the seed set (proportion of ovules that

7   developed as seeds) we used GLMM with a binomial distribution, the seed set of each ovule (1 or

8   0) as a response variable, and the individual ID and flower ID (nested within individual ID) as

9   random effects.

10

11   **RESULTS**

12   *Epicuticular wax crystals and slipperiness*

13   In the behavioural assays, ants were significantly less likely to slip on hexane-wiped flowers than

14   on control flowers in both *C. lanceolata* and *F. koidzumiana* (Fig. 2A, $P < 0.05$). The slippery

15   surfaces of the flowers of the two plant species—the abaxial surface and distal adaxial surface of

16   *C. lanceolata* and both sides of the tepal of the *F. koidzumiana* flowers—were densely covered

17   by epicuticular wax crystals (Fig. 2B, C, S2). The crystals of *C. lanceolata* consisted of clusters

1   of platelets and threads. Although both types were observed on adaxial and abaxial surfaces,

2   platelets dominated on adaxial surfaces. The crystals of *F. koidzumiana* were composed of

3   polygonal rodlets. The classification of wax crystal morphology followed Barthlott *et al.* (1998).

4   In contrast, the basal adaxial surface of the *C. lanceolata* flower, which was not slippery, was not

5   covered by wax crystals. This difference can be adumbrated by the naked eye: the slippery

6   surface has a matt appearance, but the non-slippery surface is distinctly shiny. The boundary

7   between the slippery and non-slippery surfaces is visible as a distinct change in pigment

8   coloration (Fig. 2B, SEM image of the boundary is shown in Figure S2). After wiping with

9   hexane, these crystals were clearly removed from the *F. koidzumiana* tepals, which appeared

10   slightly shrunken (Fig. 2C).

11

12   *Ants enter flowers via non-slippery bridges*

13   Nectar-thieving ants observed in the bridging experiment included *N. flavipes* (4.0 ± 3.5

14   individuals per flower, n = 31), *Camponotus umematsui* (1.2 ± 0.44, n = 17) and *F. japonica* (1.0

15   ± 0.0, n = 5) in *C. lanceolata*, and *F. japonica* (1.0 ± 0.21, n = 23), *L. japonicus* (1.5 ± 0.79, n =

16   23), and *N. flavipes* (1.6 ± 0.88, n = 9) in *F. koidzumiana* (mean ± SD). In both *C. lanceolata* and

17   *F. koidzumiana*, bridged flowers received ants more often than control flowers (*C. lanceolata*,

16

1   Fig. 3A, GLMM, Wald test, $P < 0.001$; *F. koidzumiana*, Fig. 3B, GLM, Wald test, $P \ll 0.001$).

2   Whereas only 10% of *C. lanceolata* flowers and only 5.1% of *F. koidzumiana* flowers in the

3   control group received ants at least once, the percentage increased to 28% in *C. lanceolata* and

4   45% in *F. koidzumiana* when the flowers were bridged.

5

6   *Ant effects on pollinators*

7   In total, 306 pollinator visitations were recorded during a cumulative total of 177.2 video

8   recording hours, 268 of which were by *Vespa simillima xanthoptera*, 34 by *V. analis*, and 4 by

9   *Vespa* hornets that could not be identified due to unclear images. After excluding records that

10   were too obscure for us to measure visit durations, we used 269 visitations (147 ant-present, 94

11   thread-only, and 28 untreated flowers) in the analysis. After alighting on a flower, hornets

12   collected nectar from the five nectar spurs as they moved within the flower, with their dorsal side

13   facing the stylar column (thus contacting the pollen and the stigma; Fig. 1C, Supplementary

14   Movie S3). However, when the hornets came into contact with ants in ant-present flowers, they

15   stepped back abruptly and left the flowers (Supplementary Movie S4). As a result, the hornets

16   stayed at ant-present flowers for significantly shorter durations than at thread-only flowers (Fig.

17   3C; GLMM, Tukey's test, $P = 0.039$), but not at untreated flowers ($P = 0.16$). The mean visit

17

1   duration in ant-present flowers was approximately 65% that of the two control flowers (6.6 s in

2   ant-present, 10.1 s in thread-only, and 10.2 s in untreated flowers). There was no significant

3   difference in visitation frequency among treatments (Fig. 3D; GLMM, Tukey's test, $P = 0.73$ for

4   control vs. ant-present, $P = 0.63$ for thread-only vs. ant-present, and $P = 0.97$ for thread-only vs.

5   control treatments). Fruit and seed sets were calculated based on 53 fruits resulting from 123

6   flowers, but no differences were detected among the three treatments (GLMM, Tukey's test;

7   Table S1; fruit set, $P = 0.41$ for control vs. ant-present, $P = 0.86$ for thread-only vs. ant-present, P

8    $= 0.72$ for thread-only vs. control treatments; seed set, $P = 0.29$ for control vs. ant-present, $P =$

9   0.77 for thread-only vs. ant-present, $P = 0.70$ for thread-only vs. control treatments).

10

11   **DISCUSSION**

12   *Slippery perianth surfaces covered with wax crystals deter nectar-thieving ants*

13   The wax removal experiment showed that the hexane-wiping treatment eliminates the slippery

14   properties of the perianths to ants (Fig. 2A). Our SEM observations indicated that the slippery

15   surfaces of *C. lanceolata* and *F. koidzumiana* flowers are densely covered with epicuticular wax

16   crystals, whereas no such crystals were observed on the non-slippery area (basal adaxial surface

17   of *C. lanceolata*) (Fig. 2B, C). On the other hand, there is no difference between the cell shapes

1   of slippery and non-slippery areas; they are both convex or flat in *C. lanceolata* and flat in *F.*

2   *koidzumiana* (Figure S2). Furthermore, the crystals were removed by hexane-wiping treatment

3   (Fig. 2C). Although we cannot fully rule out the possibility that the hexane-wiping treatment

4   destroyed the epidermal cell surface structure, rather than epicuticular wax per se, a clear

5   association between the presence/absence of wax crystals on slippery and non-slippery portions

6   of *C. lanceolata* corolla indicates that epicuticular wax crystals are most likely responsible for the

7   slippery properties of *C. lanceolata* and *F. koidzumiana* floral surfaces to ants. A number of

8   mutually non-exclusive mechanisms by which wax crystals decrease insect attachment have been

9   proposed: the roughness of the surface decreases the real contact area between insect tarsal

10   attachment devices and plant surfaces; easily detachable crystals contaminate the adhesive pads

11   of insects; wax crystals adsorb the pad secretion that reinforces attachment; wax crystals dissolve

12   in pad secretion and thereby thicken the fluid layer (Gorb and Gorb 2002, 2006). We could not

13   determine which of the above mechanisms is responsible in the two studied plant species;

14   however, Gorb and Gorb (2006) reported that platelet or rodlet crystals effectively contaminate

15   the pads of the beetle *Chrysolina fastuosa* (Coleoptera: Chrysomelidae). Thus, contamination

16   effect is one possible mechanism.

1   The bridging experiment suggested that the slipperiness prevents ants from entering

2   flowers, explaining why ants are rarely found in the flowers of these species under natural

3   conditions. When ants are observed on flowers in the wild, such flowers are usually attached to

4   surrounding foliage, which acts as a bridge allowing ants to bypass the slippery area (K. Takeda,

5   personal observation). Although wax crystals may also have other functions, such as controlling

6   transpiration, gas-exchange, or surface temperature (e.g. Jeffree 1986; Barthlott 1990; Barthlott

7   and Neinhuis 1997), slippery perianths function as an effective means of deterring ants from

8   flowers in these plants.

9   Importantly, the slipperiness of the flowers does not necessarily prevent entry by

10   legitimate pollinators (hornets in *C. lanceolata* and andrenid bees in *F. koidzumiana*). This may

11   be due to the presence of 'footholds' in these flowers. In *C. lanceolata*, there are non-slippery

12   areas toward the base of the inner corolla (Fig. 2B). In *F. koidzumiana*, there are hair-like

13   processes on the edges of the nectaries and on the edges of the outer tepals (Fig. 2C). When

14   hornets visit *C. lanceolata* flowers, they approach flowers flying, grab the fringes of the petals

15   with their claws, which have a rough texture (Fig. 2B), and then reach the non-slippery area with

16   their forelegs (Supplementary Movie S3). Pollinator hornets are large enough (*V. analis*, 24.2 ±

17   1.7 mm, n = 3; *V. simillima xanthoptera*, 19.8 ± 0.60 mm, n = 5; means ± SE) to stride over the

20

1   slippery area of the distal adaxial surface (ca. 10 mm), whereas ants cannot, even when they

2   reach the fringe, owing to their small body size (less than 7 mm). Thus, slippery areas and

3   footholds may act in concert to effectively filter out ants while accepting legitimate pollinators.

4

5   *Presence of ants in flowers affects pollinator behaviour*

6   In *C. lanceolata*, hornets were disturbed by the presence of ants inside the flowers and

7   consequently remained at flowers containing ants for shorter durations (Fig. 3C). These results

8   are consistent with those of previous studies showing that nectar-thieving ants deter pollinators

9   (Tsuji *et al.* 2004; Ness 2006; Cembrowski *et al.* 2014). Visitation frequency of pollinator

10   hornets did not differ between treatments (Fig. 3D), suggesting that the presence of ants does not

11   have long-lasting effects on the foraging pattern of the pollinators.

12   In the present study, we could not confirm the negative effect of ants on plant

13   reproductive success (seed set and fruit set; Table S1). However, we consider that this result does

14   not necessarily mean that ants in flowers do not have any negative effect. Due to technical

15   limitations, we used only one ant in each flower in this experiment, but ant harassment would

16   likely have a larger effect on pollinator behaviour when flowers are occupied by more than one

17   ant, as is the general pattern in bridging experiments. Introduction of several ants in each flower

1   may be ideal to replicate ant-occupied flowers in natural condition. In addition to the direct effect

2   of ants on pollinator behaviour, depletion of nectar in flowers with ants (Fritz and Morse 1981)

3   may further reduce the visit duration or visitation frequency of pollinators and thus affect

4   pollination success, although we did not evaluate this effect in the present study. Ants may also

5   negatively affect pollination by decreasing the performance of pollen due to antibiotic substances

6   secreted by ant bodies (Galen and Butchart 2003). Further field experiments are needed in order

7   to confirm whether the slippery perianths of *C. lanceolata* and *F. koidzumiana* are adaptive by

8   preventing floral entry by ants.

9

10   *Function of slippery petals*

11   A number of studies have proposed that slippery plant surfaces covered with epicuticular wax

12   crystals act as an ant deterrent mechanism. For example, von Marilaun (1878) reported that the

13   stems of *Salix* trees are covered with wax, which may make the stems slippery and deter

14   nectar-thieving ants. Harley (1991) reported that plants in two Lamiaceae genera, *Hypenia* and

15   *Eriope*, have waxy stems that ants are unable to walk on, and proposed the term 'greasy pole

16   syndrome' for the combination of characteristics that seems to prevent ants from climbing stems

17   (Harley 1991). Several other studies have also reported that wax crystals on the stems of various

1   plants can prevent ants from climbing them (Eigenbrode 2004; Whitney *et al.* 2009*b*; Gorb and

2   Gorb 2011, 2017).

3   While the examples mentioned above are restricted to wax on stems (Kerner von

4   Marilaun 1878; Harley 1991; Whitney *et al.* 2009*b*; Gorb and Gorb 2011, 2019) or bracts (Kerner

5   von Marilaun 1878), waxy perianths have rarely been reported (Barthlott 1990) apart from trap

6   flowers (e.g., *Aristolochia* (Aristolochiaceae), *Coryanthes* (Orchidaceae) and *Cypripedium*

7   (Orchidaceae)(Gerlach and Schill 1989; Bänziger *et al.* 2005; Antonelli *et al.* 2009; Oelschlägel

8   *et al.* 2009)). However, Bräuer *et al.* (2017) reported that the perianths of *Lapageria rosea*

9   (Philesiaceae) and *Platycodon grandifloras* (Campanulaceae) have dense wax crystals, and are

10   slippery to insects (honeybees and greenbottle flies). They mentioned that such waxy perianths,

11   like in *C. lanceolata* and *F. koidzumiana*, may deter nectar-thieving ants or other floral

12   antagonists (Bräuer *et al.* 2017).

13   Apart from wax, floral slipperiness caused by flat perianth epidermal cells has also been

14   proposed to deter nectar-robbing bees (Ojeda *et al.* 2012, 2016; Papiorek *et al.* 2014; Moyroud

15   and Glover 2016). Ojeda *et al.* (2016) examined perianth epidermal structures of related plant

16   species pairs and found that bird-pollinated flowers were more likely to have flat cells on their

17   petal surfaces compared to bee-pollinated relatives, which often have conical cells. Conical cells

1   are proposed to increase the roughness of petal surface and make it easier for pollinators to grip

2   the floral surface (Whitney *et al.* 2009*a*; Alcorn *et al.* 2012). Thus, the flat cells in bird-pollinated

3   flowers may hinder landing by nectar-robbing bees (Ojeda *et al.* 2016) , although recent

4   comparative analyses showed no clear relationship between perianth epidermal cell shape and

5   pollinator type (Coiro and Barone Lumaga 2018; Kraaij and Kooi 2019).

6   Despite the history of studies proposing that slippery plant surfaces may deter unwanted

7   floral visitors, this function has not been explicitly tested by field experiments. Previous studies

8   were based either on behavioural and structurally related biomechanical experiments under

9   laboratory condition (Whitney *et al.* 2009*a*; Alcorn *et al.* 2012; Bräuer *et al.* 2017; Gorb and

10   Gorb 2019), ocular observation (Kerner von Marilaun 1878; Harley 1991) or comparative

11   analysis of perianth surface structures between related plant species (Ojeda *et al.* 2012, 2016;

12   Papiorek *et al.* 2014; Costa *et al.* 2017; Coiro and Barone Lumaga 2018; Kraaij and Kooi 2019).

13   To our knowledge, this is the first study to experimentally demonstrate that slippery plant

14   surfaces prevent floral access by unwanted visitors (ants) in the field. Recent studies on floral

15   defence showed that there is a trade-off between defence and attraction of pollinators (e.g.,

16   Barlow *et al.* 2017). If pollinators can discriminate and remember the slipperiness of petal surface

17   (Kevan and Lane 1985; Whitney *et al.* 2009*a*; Alcorn *et al.* 2012), slippery flowers receive fewer

1   antagonistic visitors, but may simultaneously attract fewer pollinators. To elucidate the effect of

2   slipperiness on floral choice by pollinators and the ecological conditions leading to the evolution

3   of floral defence based on slipperiness, further experimental studies, especially those conducted

4   in the field, are needed.

5

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1   **SUPPLEMENTARY DATA**

2   Supplementary data consist of the following. Details of the wax removal experiment. Figure S1:

3   Experimental set-up of the bridging experiment and the ant-introduction experiment. Figure S2:

4   Additional SEM image of petal surface. MovieS1: *Camponotus japonicus* trying to walk on the

5   abaxial surface of a *Codonopsis lanceolata* flower. MovieS2: *Nylanderia flavipes*, *Aphaenogaster*

6   *famelica* and *Formica japonica* trying to walk on the adaxial surface of a *C. lanceolata* flower.

7   MovieS3: Visitation behaviour of the pollinator hornet to a *C. lanceolata* flower. MovieS4:

8   Disturbed visitation in an ant-present flower in *C. lanceolata.*

9

10   **FUNDING INFORMATION**

12

13   **ACKNOWLEDGEMENTS**

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13
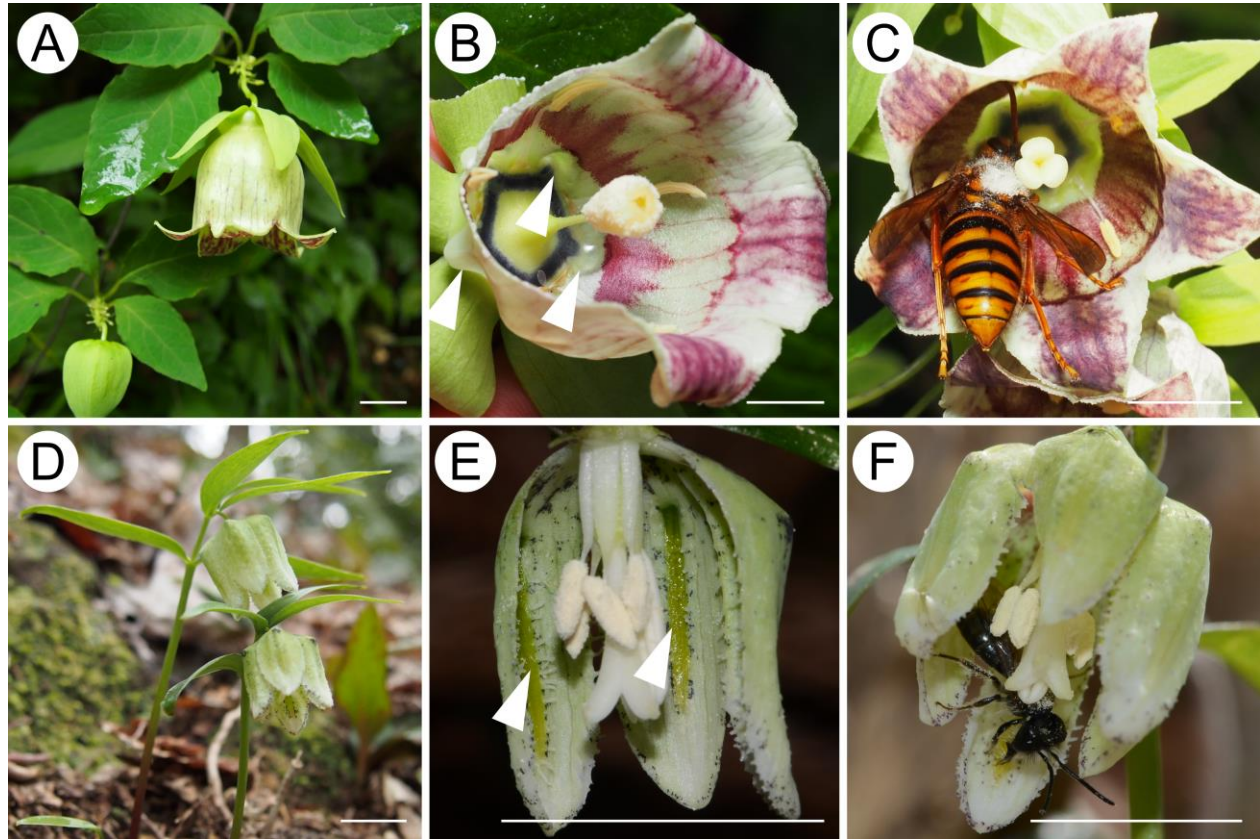
Figure 1. Flowers and pollinators of *Codonopsis lanceolata* (A–C) and *Fritillaria koidzumiana* (D–F)(bar = 10mm). (A) General appearance. (B) Longitudinal section of a flower showing nectar droplets in nipple-shaped spurs (white arrowheads). (C) Pollinator: *Vespa simillima xanthoptera* collecting floral nectar at the base of the flower. Note that its dorsal thorax is dusted with pollen and touches the stigma. (D) General appearance. (E) Longitudinal section of the flower showing band-shaped nectaries on the tepals (white arrowheads). (F) Pollinator: *Andrena* bee visiting a flower with pollen on dorsal thorax. The pollinator manoeuvres itself into the bell-shaped flower.
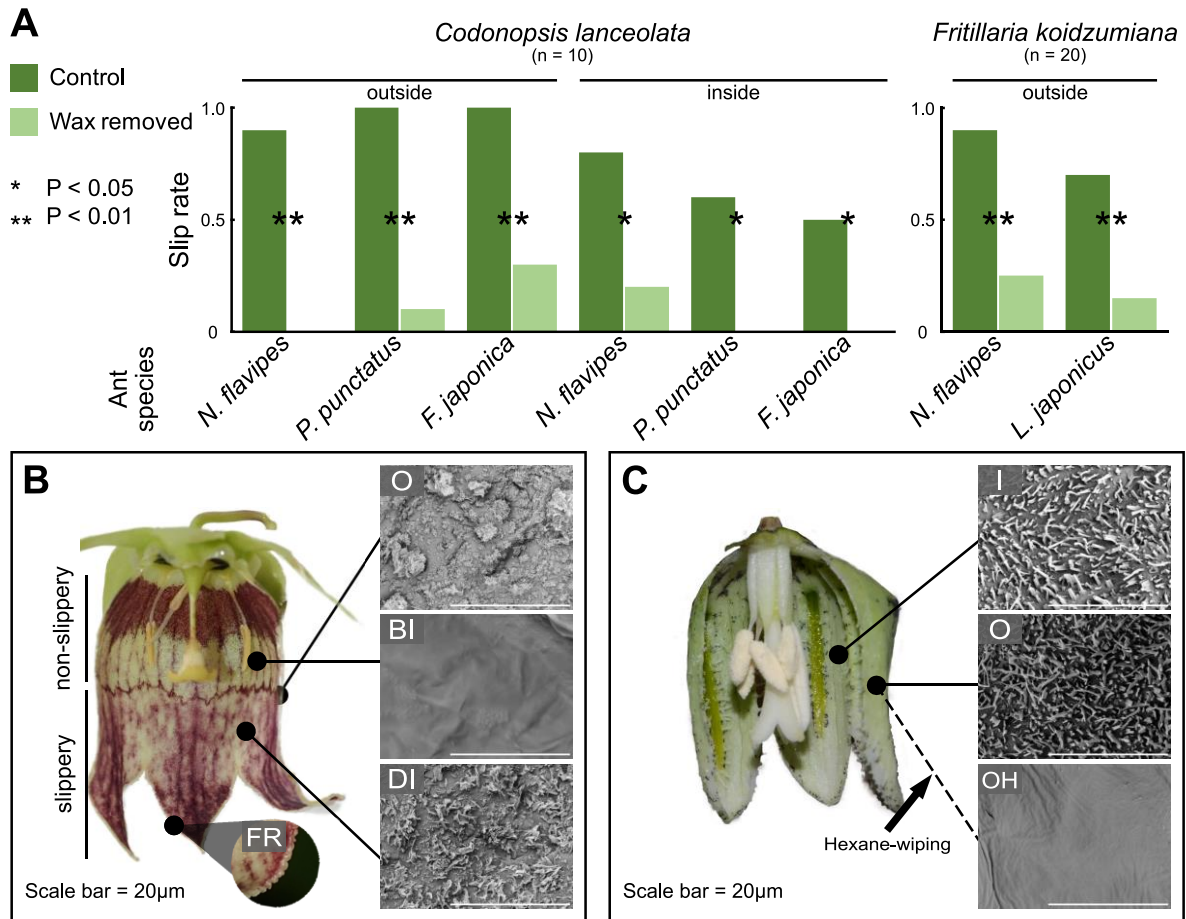
Figure 2. Impact of epicuticular wax crystals on the attachment of freely walking ants. (A) Slip

rates of ants in the behavioural assays on *Codonopsis lanceolata* and *Fritillaria koidzumiana*

perianths. The dark green bars show the slip rate on the control perianths, and the light green bars

show the same on the wax-removed perianths (wax removed by wiping with hexane). The sizes

of the ants used in the experiment are: *Nylanderia flavipes*, 2 mm; *Pristomyrmex punctatus*, 3

mm; *Lasius japonicus*, 3 mm; *Formica japonica*, 7 mm. (B, C) Longitudinal sections of the

flowers and corresponding scanning electron microscopy (SEM) images of the perianths of *C*.

1   *lanceolata* (B) and *F. koidzumiana* (C). Arrowheads show the hairy processes on *F. koidzumiana*

2   tepals. O, outer (abaxial) surface of perianths; BI, basal inner (adaxial) surface; DI, distal inner

3   (adaxial) surface; FR, rough structure on the fringe of corolla; I, inner (adaxial) surface; O, outer

4   (abaxial) surface; OH, outer (abaxial) surface wiped with hexane.

5
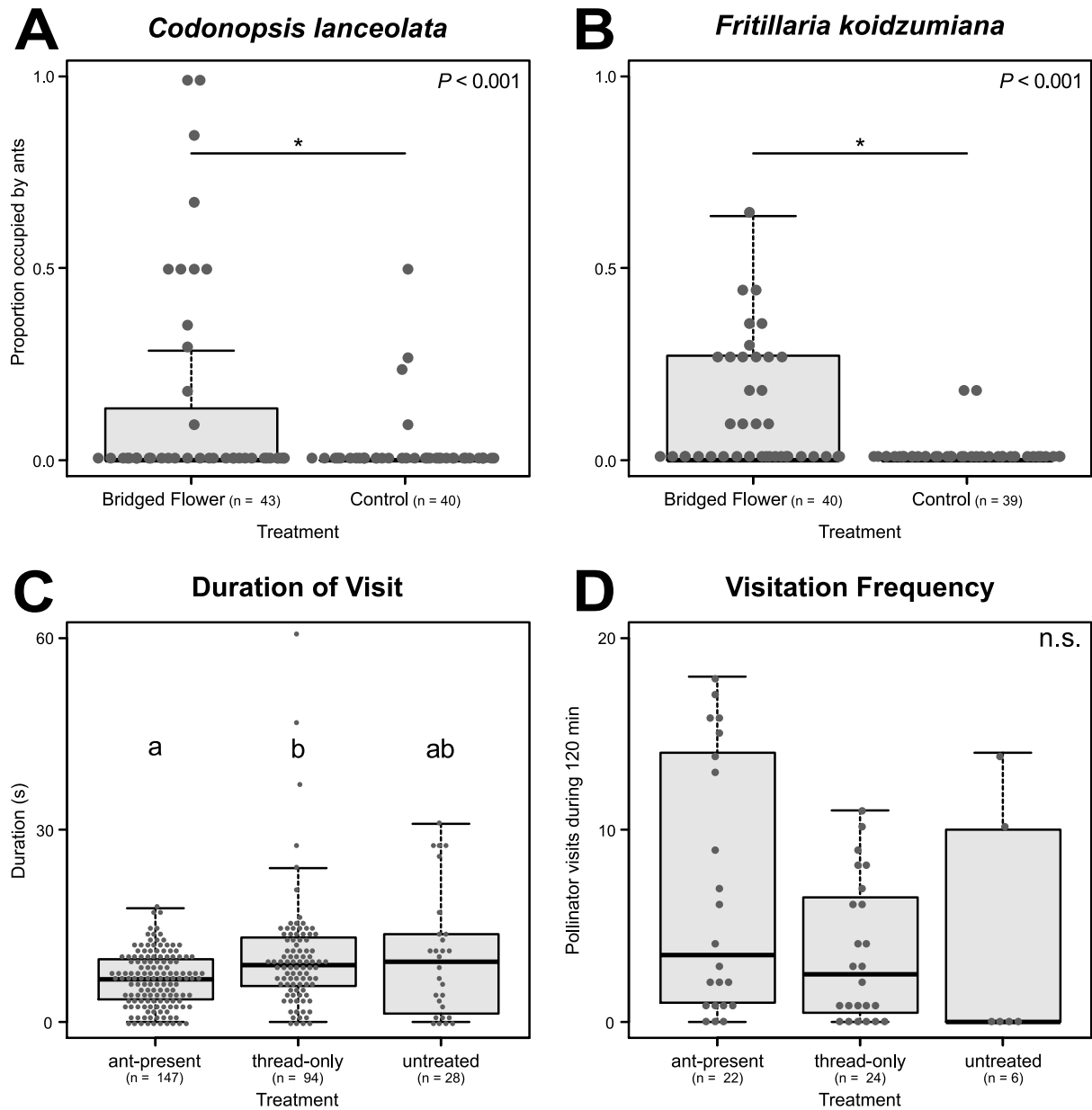
1

2   Figure 3. Results of the field experiments. (A–B) Frequencies of the ant-present records for each

3   flower treatment in the bridging experiment. (A) *Codonopsis lanceolata*. (B) *Fritillaria*

4   *koidzumiana*. The asterisks indicate significant differences according to Wald tests following a

5   generalised linear mixed model (GLMM) or generalised linear model (GLM) ($P < 0.05$). (C-D)

1   Effect of nectar-thieving ants on pollinator behaviour. (C) Duration of pollinator visits to

2   *Codonopsis lanceolata* flowers in the ant-introduction experiment. The alphabets indicate

3   significant differences according to Tukey's test following generalised linear mixed model

4   (GLMM, $P < 0.05$). (D) Visitation frequency of pollinators to *C. lanceolata* flowers during the

5   120-min observation period in the ant-introduction experiment.

6

1   Table 1 Reproductive success of flowers in each treatment in the ant-present experiment.

Treatment	Fruit set	Seed set (mean ± SD)	
Untreated	0.46 (n=52)	0.59 ± 0.22	(n=18)
Thread-only	0.43 (n=37)	0.51 ± 0.29	(n=14)
Ant-present	0.38 (n=34)	0.47 ± 0.27	(n=13)

2

3

1　**Supplemental data**

2　*Details of behavioural assays*

3　The origin of the sample is mentioned in Material and Method in the main text. Flowers were

4　kept in *Ziploc* bags (S. C. Johnson & Sons, Inc., US) until the assays. The assays were conducted

5　less than four hours after the sampling, when flowers were kept fresh. To test the slipperiness of

6　the adaxial surface of the corolla, we placed single ants at the bottom of upward-facing flowers

7　positioned on the ground and recorded whether they slipped off while climbing up the flowers.

8　To test the abaxial surface of the flowers, we placed ants at the summit of downward-facing

9　flowers and recorded whether they slipped off while walking down the flowers. Ants were

10　recorded as 'slipped' either when they fell off or could not walk down the flower in 120 s. This is

11　because ants often stayed still without attempting to walk further on the slippery zone, most of

12　which frequently groomed their feet. This behaviour was not observed on non-slippery surfaces.

13　In the case of *F. koidzumiana*, the flowers were not detached from plants. We placed ants at the

14　summit of downward-facing flowers and recorded whether they slipped off while walking down

15　the flowers in 120 s.

16

1

2   Figure S1

3   Experimental set-up of the bridging experiment (a, b) and the ant-present experiment (c) (bar =

4   10mm). (a) The slippery zone of the *Codonopsis lanceolata* flower bridged with masking tape.

5   (b) The flower of *Fritillaria koidzumiana* bridged with a bamboo stick. (c) The ant *Formica*

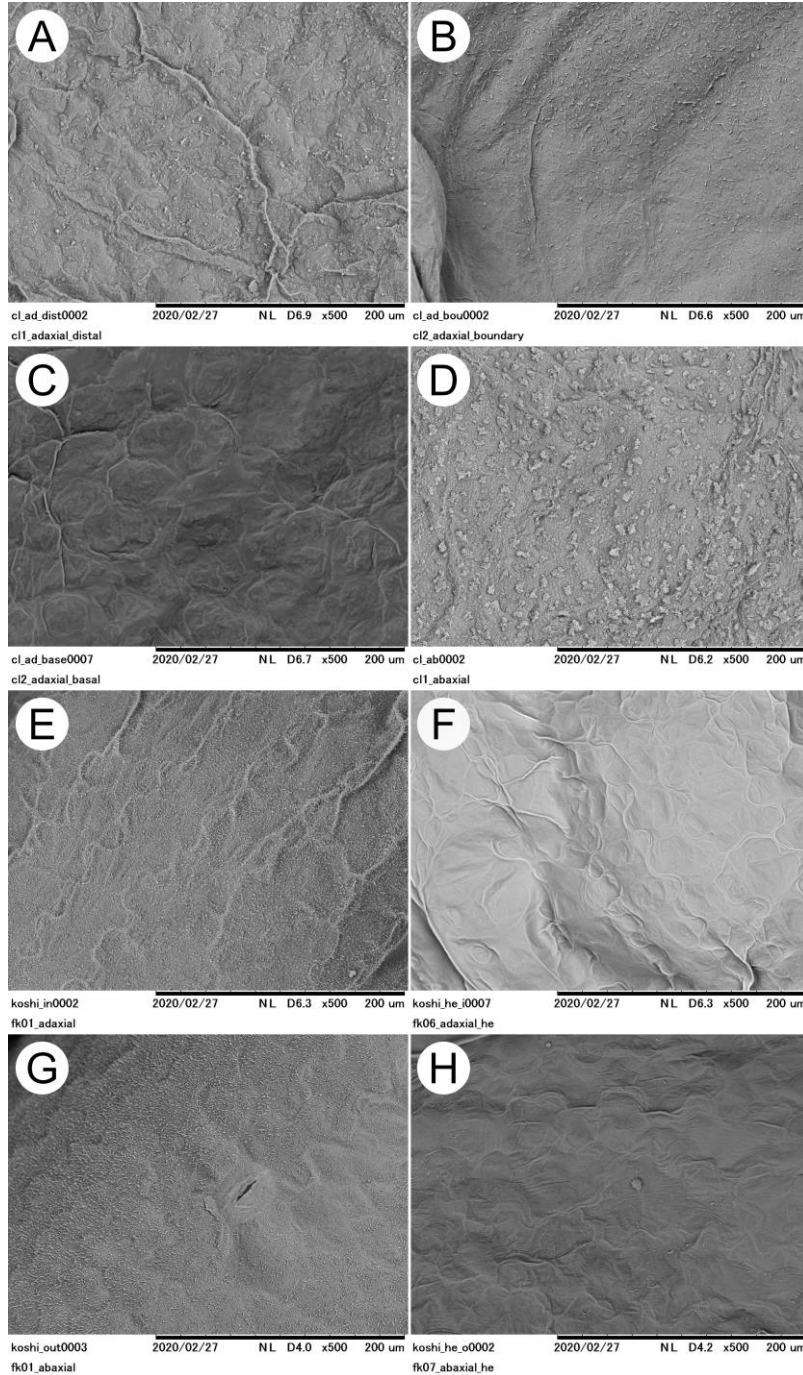6   *japonica* fixed with thread inside the corolla of *C. lanceolata.*

7

Figure S2

Additional scanning electron microscopy (SEM) images of the perianths of *Codonopsis lanceolata* (a–d) and *Fritillaria koidzumiana* (e–h) under lower magnification.

(a) Distal adaxial surface of *C. lanceolata* petal; (b) boundary of slippery and non-slippery surfaces of *C. lanceolata* petal. The slippery surface is on the upper half, and the non-slippery surface is on the lower half; (c) basal adaxial surface of *C.*

15

16 *lanceolata* petal; (d) abaxial surface of *C. lanceolata* petal; (e) adaxial surface of *F. koidzumiana*

17 tepal; (f) adaxial surface of *F. koidzumiana* tepal after hexane-wiping; (g) abaxial surface of *F.*

1   *koidzumiana* tepal; (h) abaxial surface of *F. koidzumiana* tepal after hexane wiping.

1   Supplemental movies

2   S1: *Camponotus japonicus* trying to walk on the outer surface of a *Codonopsis lanceolata* flower.

3   S2: *Nylanderia flavipes*, *Aphaenogaster famelica* and *Formica japonica* trying to walk on the

4   inner surface of a *C. lanceolata* flower. S3: Visitation behaviour of the pollinator hornet to a *C.*

5   *lanceolata* flower S4: Disturbed visitation in an ant-present flower in *C. lanceolata*.

6