



Specialization for Tachinid Fly Pollination in the Phenologically Divergent Varieties of the Orchid *Neotinea ustulata*

Carlos Martel^{1*†}, Demetra Rakosy^{2,3,4†}, Stefan Dötterl⁵, Steven D. Johnson⁶, Manfred Ayasse⁷, Hannes F. Paulus², L. Anders Nilsson⁸, Hans Mejlón⁸ and Jana Jersáková⁹

¹ Institute of Omic Sciences and Applied Biotechnology, Pontifical Catholic University of Peru, Lima, Peru, ² Department of Evolutionary Biology, Faculty of Life Sciences, University of Vienna, Vienna, Austria, ³ Department of Community Ecology, Helmholtz Centre for Environmental Research - UFZ, Halle, Germany, ⁴ German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany, ⁵ Department of Biosciences, Paris-Lodron University of Salzburg, Salzburg, Austria, ⁶ Centre for Functional Biodiversity, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, ⁷ Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Ulm, Germany, ⁸ Museum of Evolution, Uppsala University, Uppsala, Sweden, ⁹ Faculty of Science, University of South Bohemia, České Budějovice, Czechia

OPEN ACCESS

Edited by:

Johannes Spaethe,
Julius Maximilian University
of Würzburg, Germany

Reviewed by:

Judith Trunschke,
Kunming Institute of Botany, Chinese
Academy of Sciences, China
Giovanni Scopcece,
University of Naples Federico II, Italy

*Correspondence:

Carlos Martel
carlos.martel@pucp.pe

† These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 27 January 2021

Accepted: 14 May 2021

Published: 16 June 2021

Citation:

Martel C, Rakosy D, Dötterl S,
Johnson SD, Ayasse M, Paulus HF,
Nilsson LA, Mejlón H and Jersáková J
(2021) Specialization for Tachinid Fly
Pollination in the Phenologically
Divergent Varieties of the Orchid
Neotinea ustulata.
Front. Ecol. Evol. 9:659176.
doi: 10.3389/fevo.2021.659176

Despite increased focus on elucidating the various reproductive strategies employed by orchids, we still have only a rather limited understanding of deceptive pollination systems that are not bee- or wasp-mediated. In Europe, the orchid *Neotinea ustulata* has been known to consist of two phenologically divergent varieties, neither of which provide rewards to its pollinators. However, detailed studies of their reproductive biology have been lacking. Our study aimed to characterize and understand the floral traits (i.e., morphology, color, and scent chemistry) and reproductive biology of *N. ustulata*. We found that the two varieties differ in all their floral traits; furthermore, while *Neotinea ustulata* var. *ustulata* appears to be pollinated by both bees (e.g., *Anthophora*, *Bombus*) and flies (e.g., *Dilophus*, *Tachina*), var. *aestivalis* is pollinated almost entirely by flies (i.e., *Nowickia*, *Tachina*). Tachinids were also found to be much more effective than bees in removing pollinaria, and we show experimentally that they use the characteristic dark inflorescence top as a cue for approaching inflorescences. Our results thus suggest that while both *N. ustulata* varieties rely on tachinids for pollination, they differ in their degree of specialization. Further studies are, however, needed to fully understand the reproductive strategy of *N. ustulata* varieties.

Keywords: food deception, flower color, flower morphology, floral scent, pollinator efficiency, pollinator specialization

INTRODUCTION

The orchid family is one of the largest plant families in the world, accounting for around 28,000 described species (Willis, 2017; Fay, 2018). At least one third of these species are deceptive, not providing their pollinators with a reward (Renner, 2005; but see Shrestha et al., 2020). Deceptive orchids employ various strategies to lure in their pollinators, which range from the advertisement

of false nectar or pollen sources to the imitation of brood sites or mating partners (Jersáková et al., 2006; Johnson and Schiestl, 2016). Each type of deception is thought to be associated with a particular combination of floral traits, often influenced by their reliance on particular groups of pollinators (Johnson and Schiestl, 2016; Valenta et al., 2017). Conspicuous visual displays coupled with reduced floral scent emission and the attraction of male and female pollinators are traditionally considered an indication of food-deceptive pollination (Galizia et al., 2004; Jersáková et al., 2012, 2016; Johnson and Schiestl, 2016). Less conspicuous visual displays, the exclusive attraction of either male or female insects and the emission of particular scent bouquets are, in contrast, associated with pollination by sexual deception or brood-site mimicry (Vereecken and Schiestl, 2009; Ayasse et al., 2011; Bohman et al., 2016; Martel et al., 2019; but see Streinzer et al., 2010). For many deceptive orchids the investigation of the floral traits employed can provide an accurate estimate of their pollination strategy; however, some orchid species may have complex strategies that are much more difficult to categorize (e.g., Vogel, 1972; Bino et al., 1982; Vöth, 1989; Valterová et al., 2007; Scopece et al., 2009). This seems also to be the case for the European orchid species, *Neotinea ustulata* (L.) R. M. Bateman, Pridgeon and M. W. Chase.

Neotinea ustulata, like up to 81% of orchid taxa occurring in Europe, Asia Minor and North Africa (Paulus, 2005; Delforge, 2006), is deceptive, i.e., does not offer any readily identifiable reward to potential floral visitors (Claessens and Kleynen, 2011). While most of these deceptive orchids rely on either bumblebees or solitary bees for reproduction, *N. ustulata* has been considered to be potentially fly- and beetle-pollinated (Vöth, 1984; van der Cingel, 1995). The evidence for *N. ustulata*'s pollination system has, however, been mostly anecdotal. The identification of its reproductive strategy has been further complicated by the occurrence of two phenologically distinct varieties, the early flowering (from April to June, or even until July) *Neotinea ustulata* var. *ustulata* and the late flowering (from June to August) *N. ustulata* var. *aestivalis* (Kümpel) Tali, M. F. Fay and R. M. Bateman. The first recorded pollination event in *N. ustulata* var. *ustulata* probably dates back to Godfery (1933), who observed two *Tachina* (*Echinomyia*) aff. *magnicornis* (Zett.) flies, one of them with pollinaria attached to its proboscis, visiting flowers in a population from Eastern France. Further records stem from Vöth (1984), who observed several individuals of *T. aff. magnicornis* carrying pollinaria in Austria. While tachinid flies seem to be common visitors, other records include bees (Borsos, 1962), bumblebees (Paulus, 2005; Claessens and Kleynen, 2016), and empidid flies (Paulus, 2005; Claessens and Kleynen, 2016). Pollinator records are even more scarce for *N. ustulata* var. *aestivalis*, for which there have been only observations of beetles, namely *Chrysanthia* sp. (Oedemeridae) (Danesch and Danesch, 1962) and *Vadonia* (*Leptura*) *livida* (F.) (Cerambycidae) (Mrkvicka, 1991), carrying pollinaria on various parts of their head. These records are, however, insufficient to determine the reproductive system of *N. ustulata* as they only account for sporadic and isolated visitation events.

Throughout their range of distribution, the two *N. ustulata* varieties do not usually occur in sympatry (Tali et al., 2004);

var. *ustulata* being mostly found in dry meadows, whereas var. *aestivalis* in both dry and wet meadows (e.g., Molinetum vegetation) (Paulus, pers. obs.). Plants of var. *aestivalis* are also usually taller than those of var. *ustulata* (Haraštová-Sobotková et al., 2005). Despite these differences, the two varieties are genetically undifferentiated (Tali et al., 2006; but see Haraštová-Sobotková et al., 2005) and have the same chromosome number (Tali, 2004), which indicates no association of autopolyploidy with the phenological differences as reported in others plants (e.g., Simón-Porcar et al., 2017). The mechanisms driving their divergence, thus remains unknown. It is possible that both pollinator- and non-pollinator-mediated selection could have played a role in driving the phenological divergence among populations, leading to the evolution of these two varieties. However, testing the role of pollinator as selection mediators in *N. ustulata* requires firstly a better assessment of its pollinators and of the traits involved in pollinator attraction. To this date there are only very few assessments of floral traits of *N. ustulata* varieties (i.e., Haraštová-Sobotková et al., 2005; Schiestl and Cozzolino, 2008; D'auria et al., 2021). Inflorescences of both varieties have a similar color pattern (Paulus, 2005), both having a very dark brown-purple top (hereafter referred as "dark top"). This provides a strong contrast with the remaining part of the inflorescence (Godfery, 1933; Stace, 2010), and may serve as a signal for attracting pollinators; however, this hypothesis so far has not been tested. Perhaps the most unusual trait of *N. ustulata* is its floral scent. Food-deceptive orchids rarely rely upon flower scent for pollinator attraction, this floral trait is, however, essential in sexually deceptive species (Ayasse et al., 2011; Bohman et al., 2016). Schiestl and Cozzolino (2008) were the first to show that the floral scent of *N. ustulata* contains alkenes, which, although uncommon in most flowers, are frequently associated with sexually deceptive ones (Ayasse et al., 2011; Bohman et al., 2016); in particular, *N. ustulata* flowers produce high relative quantities of 11-tricosene. The role of this specific compound in pollinator attraction is so far unknown, but other tricosene isomers are known to act as sex pheromones in the house fly *Musca domestica* (Carlson et al., 1971; Rogoff et al., 1973) or aggregation pheromones in *Drosophila* (Hedlund et al., 1996). At the moment, we do not know if alkenes could be found in both varieties or these are restricted to just one of them. The particular combination of visual signals associated with the chemical composition of the flower scent raises the interesting possibility that *N. ustulata* may rely on intricate signals to attract its pollinators.

In order to understand the reproductive strategy and, eventually, the evolution of *N. ustulata*, we need to accurately quantify the differences in floral traits between its two varieties as well as identify their reproductive biology. Therefore, our study aims are to compare *N. ustulata* varieties in terms of (i) morphological, visual and chemical floral traits, (ii) pollination success and patterns of fruit set, and (iii) pollinator spectra. Furthermore, we aimed to (iv) assess efficiency in pollinaria removal among the principal pollinator guilds, and (v) identify the role of the inflorescence dark top in the attraction of predominant pollinators.

MATERIALS AND METHODS

Study Species

Neotinea ustulata is a perennial tuberous herb, which is typically found on extensively used meadows (Tali et al., 2004). Each plant of this species bears a single inflorescence, which has a burnt appearance when its flowers are in bud; the species is therefore known as the burnt-tip orchid. *Neotinea ustulata* flowers have three semi-fused sepals, which form a hood; the outer side of the sepals (especially in buds) displays a dark reddish to blackish color (Figure 1). The lip is white with dark red spots and is deeply divided into three parts (Figure 1). The flowers have a nectarless spur, which is blunt. *Neotinea ustulata* occurs in central and southern Europe with populations reaching Spain and Greece in the south, England and southern Sweden in the north, and as far east as the Caucasus and Ural Mountains (Tali et al., 2004; Delforge, 2006). Despite its broad geographical range, the species is vulnerable to extinction and the number of populations have been greatly reduced during the last century (Neiland, 2001; Tali et al., 2004; Baumann et al., 2005; Jacquemyn et al., 2005).

The species consists of two varieties, which, while phenologically distinct, do not differ strongly in their vegetative traits (Kümpel and Mrkvicka, 1990; Wucherpfennig, 1992; Haraštová-Sobotková et al., 2005): the early flowering *N. ustulata* var. *ustulata* and the late flowering var. *aestivalis*. *Neotinea ustulata* var. *ustulata* is 10–35 cm tall, rarely larger, and its flowering period ranges from April till June in most parts of Europe (Tali et al., 2004; Baumann et al., 2005), but from June to July at high altitudes in the Alps (Paulus, pers. obs.). The dense, narrow, up to 8 cm long inflorescence is composed on an average of 30 flowers (Tali et al., 2004; Baumann et al., 2005; Haraštová-Sobotková et al., 2005; Figure 1). *Neotinea ustulata* var. *aestivalis* has in turn a somewhat taller stem, of 33 cm long in average, (rarely up to 80 cm; Haraštová-Sobotková et al., 2005) with longer and narrower leaves, and sharply pointed inflorescences (especially in young stage) (Figure 1). The inflorescences have on

average around 40 flowers (Jensen and Pedersen, 1999). Plants of this variety flower from the end of June or July till mid-August (Tali et al., 2004; Baumann et al., 2005; Paulus, pers. obs.). In order to confirm the identity of the two varieties, detailed digital photographs of individuals from our sampling localities were compared against material deposited in diverse herbaria (e.g., CB, BRNM, OHN, PR, PRC, UPS, VN, and W).

Study Sites

Field observations and experiments were carried out in Austria, the Czech Republic, the Slovak Republic and Sweden (Figure 2), during May–June (for details see Supplementary Table 1). In total, thirteen populations of var. *ustulata* and ten populations of var. *aestivalis* were sampled to analyze different aspects of their reproductive biology (Figure 2; for population details see Supplementary Table 1).

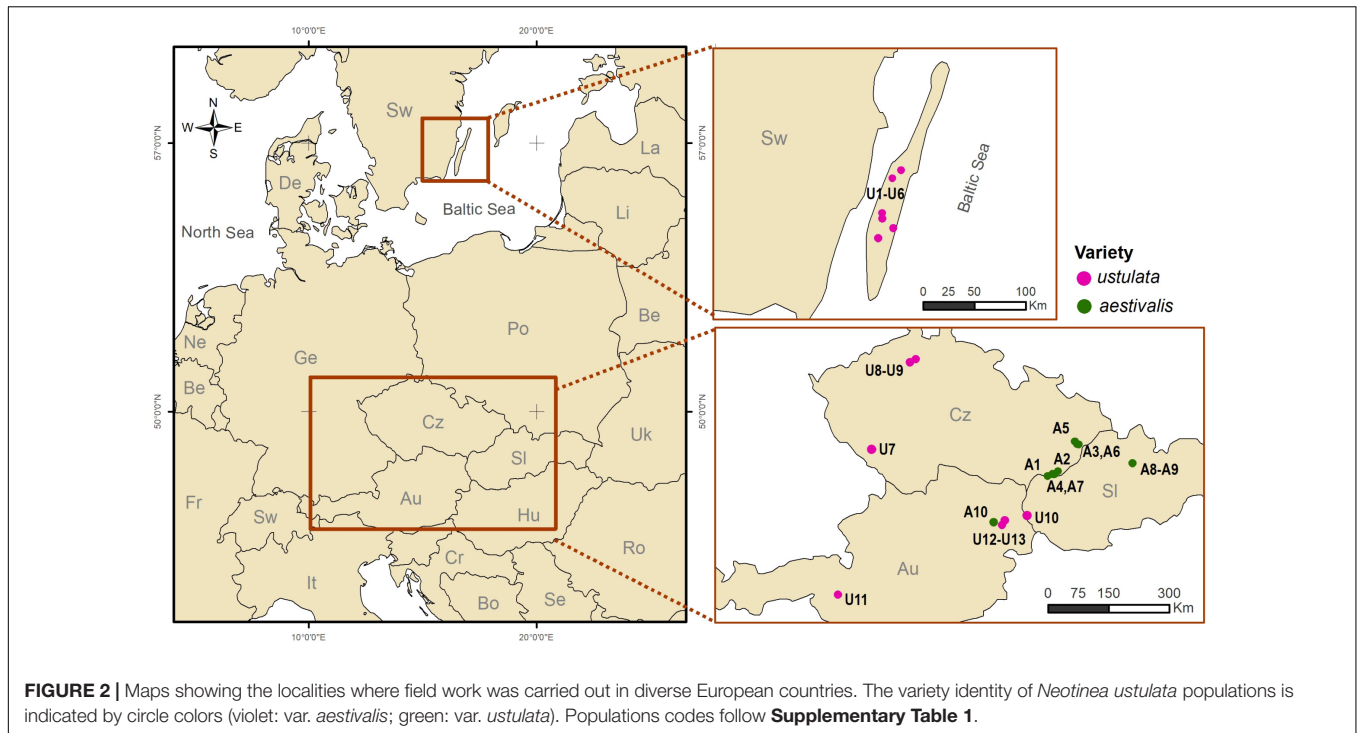
Morphological, Visual, and Chemical Floral Traits

Morphology

We sampled inflorescences of *N. ustulata* var. *ustulata* and var. *aestivalis* from two populations each (i.e., 30, 30, 23, and 15 inflorescences from U8, U9, A2, and A4, respectively, see Supplementary Table 1 for population codes). From the middle of each inflorescence, we picked a single, fully opened and yet unpollinated flower. Flowers were placed in 70% ethanol (Sigma Aldrich, Munich, Germany) and stored until further usage. Individual flowers were mounted on a styrofoam base and then photographed from three different angles with the aid of a Dino-Lite digital microscope (AnMo Electronics Corporation, Taiwan). Size calibration and distance measurements were performed with the in-house software DinoCapture (AnMo Electronics Corporation, Taiwan). Eight floral traits (i.e., hood opening width, hood opening height, spur opening width, spur width at 3/4 spur length, width at spur bottom, spur length, distance from column to the spur opening, and lip length) were measured.



FIGURE 1 | The burnt tip orchid *Neotinea ustulata*. (A) Habitus of *N. ustulata*. (B) Inflorescence from a var. *ustulata* plant. (C) Inflorescence from a var. *aestivalis* plant. Pictures by J. Jersáková.



To identify which morphological traits, if any, are responsible for differences among varieties of *N. ustulata*, we performed a principal component analysis (PCA) using the *factoextra* package (Kassambara and Mundt, 2020) of the R software (R version 3.6.1; R Core Team., 2017). Morphological data were scaled and normalized prior to conducting the PCA. To explore differences in morphology between the two varieties, we performed a permutational multivariate analysis of variance (PERMANOVA) with the raw morphological data. We constructed a matrix based on Euclidean distances to run the PERMANOVA with 10,000 permutations, considering “variety” as fixed factor and “population” as nested factor in “variety.” PERMANOVA was performed using the *vegan* package (Oksanen et al., 2019) of R.

Color

The spectral reflectance was measured for 44 flowers (one flower per plant) from two populations per *N. ustulata* variety (i.e., 14, 10, 10, and 10 flowers from U8, U9, A2, and A4, respectively). We specifically measured the reflectance of the dark top, outer sepals and lips. All floral reflectance measurements were performed using an Ocean optics UV-VIS Spectrometer JAZ and an Ocean Optics DT-mini light source (200–1,100 nm; Dunedin, FL, United States), calibrated with a white reflection standard (WS-1-SL, Ocean Optics). Readings were taken through a fiber-optic reflection probe (UV/VIS 400 μ m) held at 45° ca. 5 mm from the surface of the object. The visual similarity between varieties, as perceived by their visitors, was determined by plotting their reflectance spectra as loci in bee- and fly-specific color spaces, assuming adaptation to foliage background (Chittka and Kevan, 2005). Selection of these two insect models was based on our pollinator list (see section “Results”). To model bee

color vision, we used the color hexagon (Chittka, 1992), based on the spectral sensitivities of *Apis mellifera* (L.). To model fly color vision, we used Vorobyev and Osorio, 1998) receptor noise model. This method uses spectral sensitivities and the relative numbers of each fly photoreceptor type to calculate the perceived color contrast between objects. We did not use Troje’s (1993) fly categorical color vision model because it was already shown to be unreliable when contrasted with experimental data (e.g., Jersáková et al., 2012; Renoult et al., 2014; An et al., 2018). Based on color loci, we also run a PERMANOVA, considering “variety” and “inflorescence part” as fixed factors and “population” as a nested factor in variety, to test for differences in spectral reflectance between varieties while considering population origin. The visual modeling and PERMANOVA were performed using the *pavo* (Maia et al., 2021) and *vegan* packages of R, respectively.

Floral Scent

Highly volatile compounds of the floral scents of both *N. ustulata* varieties were collected using dynamic headspace techniques (i.e., 10, 9, and 5 flowers from U8, U9, and A2, respectively), whereas semi- and low-volatile compounds were collected through solvent extracts (i.e., 10, 10, 11, and 10 flowers from U8, U9, A2, and A4, respectively). Samples were analyzed by a gas-chromatograph and gas chromatograph/mass spectrometer using a DB-5 non-polar column (for detailed description of the analytical techniques see **Supplementary Methods 1**). Chemical identification was carried out by comparing the mass spectra of pure compounds with the ones of commercial libraries (NIST, ADAMS) or of our own libraries, and the retention indexes of detected compounds with those from the literature (e.g., Adams, 2017). Mass spectra were processed using the

Automated Mass Spectral Deconvolution and Identification System—AMDIS—software (National Institute of Standards and Technology, Gaithersburg, United States). Differences in floral scent bouquets of both varieties were analyzed by comparing the relative proportions of compounds identified in our two type of samples. We calculated the Bray-Curtis similarity index to determine semi-quantitative differences in odor bouquets. Then, we performed a PERMANOVA with 10,000 permutations to test for differences in scent bouquets between the two varieties while considering population origin. A non-metric multidimensional scaling (NMDS) was performed to graphically display the (dis)similarities in floral scent patterns among samples. PERMANOVA and NMDS were performed using the *vegan* package and displayed graphically using the *ggplot2* package (Wickham et al., 2020) of R.

Reproductive Success Patterns

During 6 years (i.e., 1978, 1981, 1983, 1984, 2000, and 2001), we examined seven populations of *N. ustulata* var. *ustulata* and four populations of var. *aestivalis* for fruit production (see **Supplementary Table 1** for population details). We recorded the total number of flowers and position of fruits within inflorescences for each inflorescence sampled. The differences in the number of flowers, the number of fruits produced and the proportion of flowers that set fruits between the *N. ustulata* varieties were tested by means of generalized linear mixed-models (GLMMs) with Poisson model distribution with log link function for number of flowers and fruits, and binomial model distribution with logit link function for the proportion of flowers that set fruits; “population” was set as a random factor.

In deceptive systems, it is expected to find position effects on fruit production, as first open flowers are more likely to be visited by yet unexperienced insects than late flowers; however, it is unknown if this pattern generalized and also happens in *N. ustulata*. Therefore, we checked whether the development of fruits is affected by the relative position of the flower in the inflorescence by dividing each inflorescence into three sections (i.e., bottom, middle, and top), calculated mean reproductive success for each section, and then performed a GLMM with a binomial model distribution and log link functions, and conducted Tukey *post hoc* tests to compare pairs of means; “plant individual” was set as a random factor. GLMMs and *post hoc* tests were carried out using the *lme4* (Bates et al., 2019) and *emmeans* packages (Lenth et al., 2020), respectively, in R.

Diversity of Pollinator Guilds and Their Efficiency

Flower Visitors, Pollinators, and Their Overall Efficiency

Insect visitors on *N. ustulata* flowers were recorded by video footages (73 and 37 h of footage on *N. ustulata* var. *ustulata* and var. *aestivalis*, respectively) and complemented by direct observations in our study sites (see **Supplementary Table 1** for population details). Floral visitor identity and identification of visitors as pollinators were assessed based on both direct observations and video recordings; whereas frequency of visits

and floral visitor behavior on the inflorescence were assessed based on the video footage only. Visitors were identified to the order and family level, and when possible up to genus/species level. To test for differences in the flower visitor composition, a G-test of independence comparing the frequency of species within orders between varieties was performed using the *DescTools* package (Signorell, 2020) of R. Representatives of each floral visiting species were collected using entomological nets in parallel to the video recordings in search of attached pollinaria and for insect identification. We also checked for presence and placement of pollinaria in insects feeding on rewarding flowers of plants co-occurring with *N. ustulata*, such as species of *Aegopodium* L. (Apiaceae), *Crataegus* L. (Rosaceae), *Dorycnium* Mill. (Fabaceae) and *Euonymus* L. (Celastraceae). To corroborate the pollinaria identity, they were compared with pollinaria directly collected from *N. ustulata* individuals. Insects were considered pollinators based on both the presence of attached pollinaria and evidence of pollinaria removal (assessed from the video footage or direct observations).

Furthermore, we also calculate male (i.e., pollinaria removed/total flowers \times 100) and female (i.e., pollinia deposited/total flowers \times 100) reproductive success, and pollen transfer efficiency (i.e., ratio of pollinia deposited to pollinaria removed \times 100; Nunes et al., 2016), which can be linked to specialization. We performed a GLMM with Poisson model distribution and log link function to estimate the effects of variety on pollen transfer efficiency and with “population” set as a random factor. GLMM was carried out using the *lme4* package of R.

Efficiency in Pollinaria Removal Between Pollinator Guilds

We compared the pollinaria removal efficiency between the tachinid fly *Tachina fera* (L.) and the bee *Anthophora plumipes* (Pallas), in order to identify whether pollinators of different guilds are equally efficient. Our set up was as follows: one inflorescence of *N. ustulata* var. *ustulata* with pollinaria present in all its open flowers was placed in a plastic container (100 \times 50 mm i.d., Greiner Bio-One GmbH, Frickenhausen, Germany) in which a single individual of either *T. fera* ($n = 6$) or *A. plumipes* ($n = 12$) was introduced. We recorded the removal of pollinaria in up to three attempts (i.e., insertion of the proboscis in the flower) of each *T. fera* and *A. plumipes*. We performed a GLMM with binomial model distribution and logit link function to test for removal efficiency between pollinators. We also conducted Tukey *post hoc* tests to compare pairs of means. Our GLMM for repeated measures, compound symmetry was used as covariance pattern model, included “pollinator guild” and “attempt number” as fixed factors and “insect individual” as a random factor. GLMM and *post hoc* tests were performed using the *lme4* and *emmeans* packages in R.

Role of Visual Traits in Pollination Success

In 1999 we sampled inflorescences from eight populations from Sweden and Czech Republic belonging to both varieties (see **Supplementary Table 1** for population details). They

were examined for inflorescence display and dark top display. *Neotinea ustulata* inflorescences have a cylindrical arrangement and, therefore, the inflorescence display was calculated as its two-dimensional projection (i.e., the area of a rectangle: length \times width of inflorescence); while the dark top has a circular shape and, therefore, dark top display was approximated as the area of a circle (i.e., $\pi \times \{\text{width of dark top}/2\}^2$). We also assessed the number of flowers with removed pollinaria, deposited pollinia and flowers where pollination events occur; they were then transformed to proportions by dividing them by the number of open flowers. We performed GLMMs with binomial model distribution and logit link function to estimate the effects of visual display on the probability of a flower to be pollinated. Our models included “inflorescence display,” “dark top display” and “variety” as fixed effects, and “population” as random factor. GLMMs were performed using the *lme4* package in R.

Role of Dark Top in Fly Pollinator Attraction

We tested the importance of the dark top in the attraction of tachinids since it might be an attractant to flies (Godfery, 1933; van der Cingel, 1995). *Tachina* flies have been previously considered the main pollinators of *N. ustulata* (Vöth, 1984; Trunschke et al., 2017), and their importance as pollinators is supported by our own observations (see section “Results”). Experiments were carried out with both varieties. For var. *ustulata*, four inflorescence pairs were selected for experiments. For each trial, two inflorescences of equal height and similar phenological state were placed less than 10 cm apart. Each inflorescence pair was first observed for about 30 min, during which the number of tachinids attracted by each inflorescence was recorded. After the initial observations the dark top of one of the two inflorescences was arbitrarily cut off. The inflorescence pairs (i.e., one intact, one without the dark top) were then observed again for the same amount of time and the number of tachinids which landed and/or probed the flowers was recorded. In case of var. *aestivalis*, only two inflorescence pairs were selected, but the experimental setup was similar to that described for var. *ustulata*, with two exceptions: (i) For the second trial, the cut off dark top of var. *aestivalis* was placed below the selected plant, in such a way that it was hidden from the view to approaching flies. This setup ensured that olfactory cues released by the dark top were still present when the dark top was cut off. (ii) An additional trial was carried out, in which the dark top was reattached to the plant. Then, the inflorescence pairs were offered again to tachinids. This trial controlled for any effect of the damage (e.g., green leaf volatiles after plant tissue damage) to the inflorescence on their attractiveness to *Tachina* flies. We performed GLMMs with negative binomial model distribution and log link function to test for differences in the attractiveness of manipulated and non-manipulated inflorescences within each variety. Our GLMMs for repeated measures incorporated a compound symmetry covariance matrix included “treatment stage” and “manipulation condition” as fixed factors, “plant individual” as the subject for repeated measures before and after the manipulation, and “number of visits” as the response variable.

GLMMs were performed using the *MASS* package (Ripley et al., 2020) in R.

RESULTS

Comparison of Morphological, Visual, and Chemical Floral Traits Morphology

The flowers of *N. ustulata* var. *ustulata* are larger in all measured traits in comparison to var. *aestivalis* (Table 1). In our PCA, the first two PCs accounted for 62.1% of the total variance (PC1: 50.5%, PC2: 11.6%). All the morphological floral traits loaded negatively on the PC1 (Supplementary Table 2), which separates var. *ustulata* (negative scores) from var. *aestivalis* (positive scores) (Figure 3). Indeed, the PERMANOVA analysis revealed that the floral morphology differed significantly between var. *ustulata* and var. *aestivalis* [Pseudo- $F_{(1, 97)} = 81.12, p < 0.0001$], and between populations [Pseudo- $F_{(2, 97)} = 5.32, p < 0.001$].

Color

The dark top and the lightly colored lip plotted apart in both the fly and bee color models, indicating that these two parts of the flower appear highly contrasting, and are therefore easily discriminated by bees as well as flies. Loci of the individual color measurements of the dark top ($n = 44$) plotted, except by one case, in the UV-blue color category within the bee hexagon and also grouped together within the fly tetrahedron (Figure 4). Similarly, color loci of the lip ($n = 44$) clustered together within both the bee hexagon and the fly tetragon (Figure 4). The color loci of the hood ($n = 44$) were more broadly distributed, spanning the space from the dark top loci to the lip loci (Figure 4). In terms of color information, the dark top and hood may often be perceived as uncolored against the leaf background by bees based on color hexagon distances. The color loci of different inflorescence parts were different, independently of variety, as our PERMANOVA analysis confirmed [Pseudo- $F_{(7, 131)} = 52.64, p < 0.0001$]. Furthermore, PERMANOVA analyses also indicated that there are statistical differences in the spectral reflectance between *N. ustulata* varieties [Pseudo- $F_{(1, 131)} = 9.28, p < 0.01$] and within varieties among populations [Pseudo- $F_{(3, 131)} = 2.81, p < 0.05$].

TABLE 1 | Measurements (in mm) of the floral morphology traits in *Neotinea ustulata*.

Morphological floral trait	var. <i>ustulata</i> (mean \pm SD)	var. <i>aestivalis</i> (mean \pm SD)
Hood opening width	4.20 \pm 0.79	2.28 \pm 0.57
Hood opening height	2.23 \pm 0.67	1.90 \pm 0.44
Spur opening width	0.66 \pm 0.09	0.42 \pm 0.13
Spur width at 3/4 spur length	1.11 \pm 0.19	0.88 \pm 0.18
Width at the spur bottom	0.64 \pm 0.08	0.52 \pm 0.09
Spur length	2.40 \pm 0.32	1.83 \pm 0.22
Distance from column to spur opening	1.67 \pm 0.24	1.48 \pm 0.19
Lip length	3.19 \pm 0.58	2.53 \pm 0.56

Mean and standard deviation values for each trait and variety are shown.

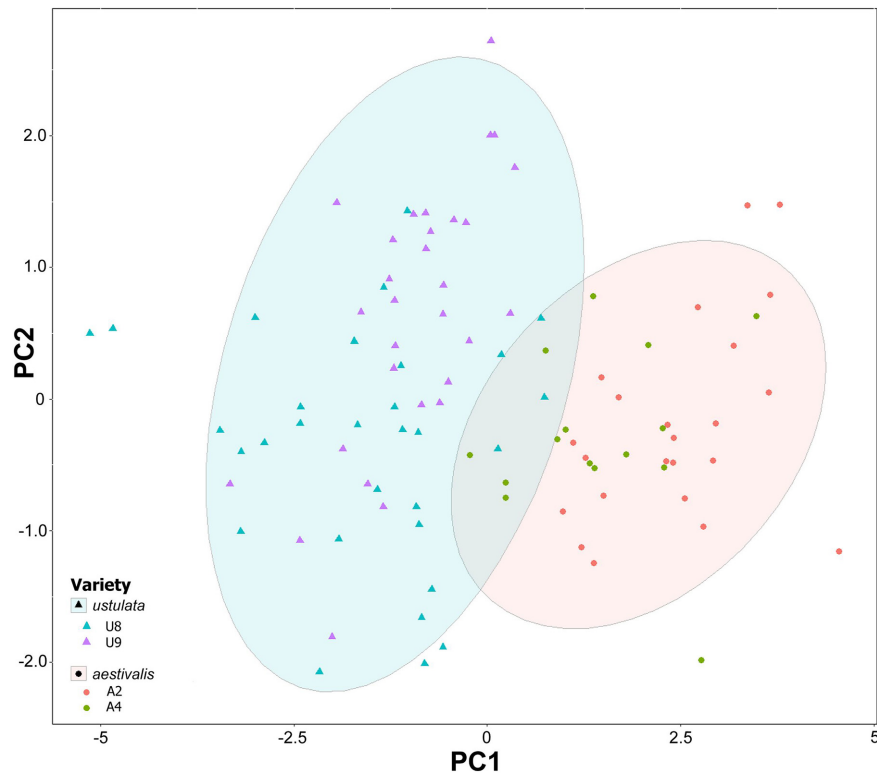


FIGURE 3 | Plotting of *Neotinea ustulata* samples based on floral morphology traits in PC1 and PC2. Different symbols represent different varieties and ellipses display 95% confidence area of each variety. Different symbol colors represent different populations. Population codes as in **Supplementary Table 1**.

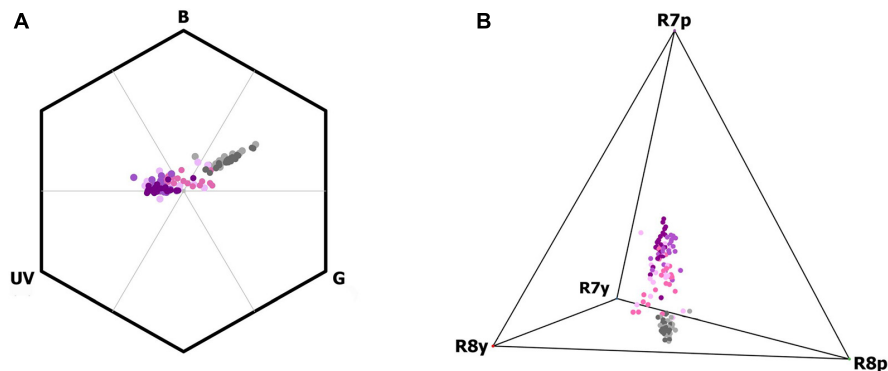
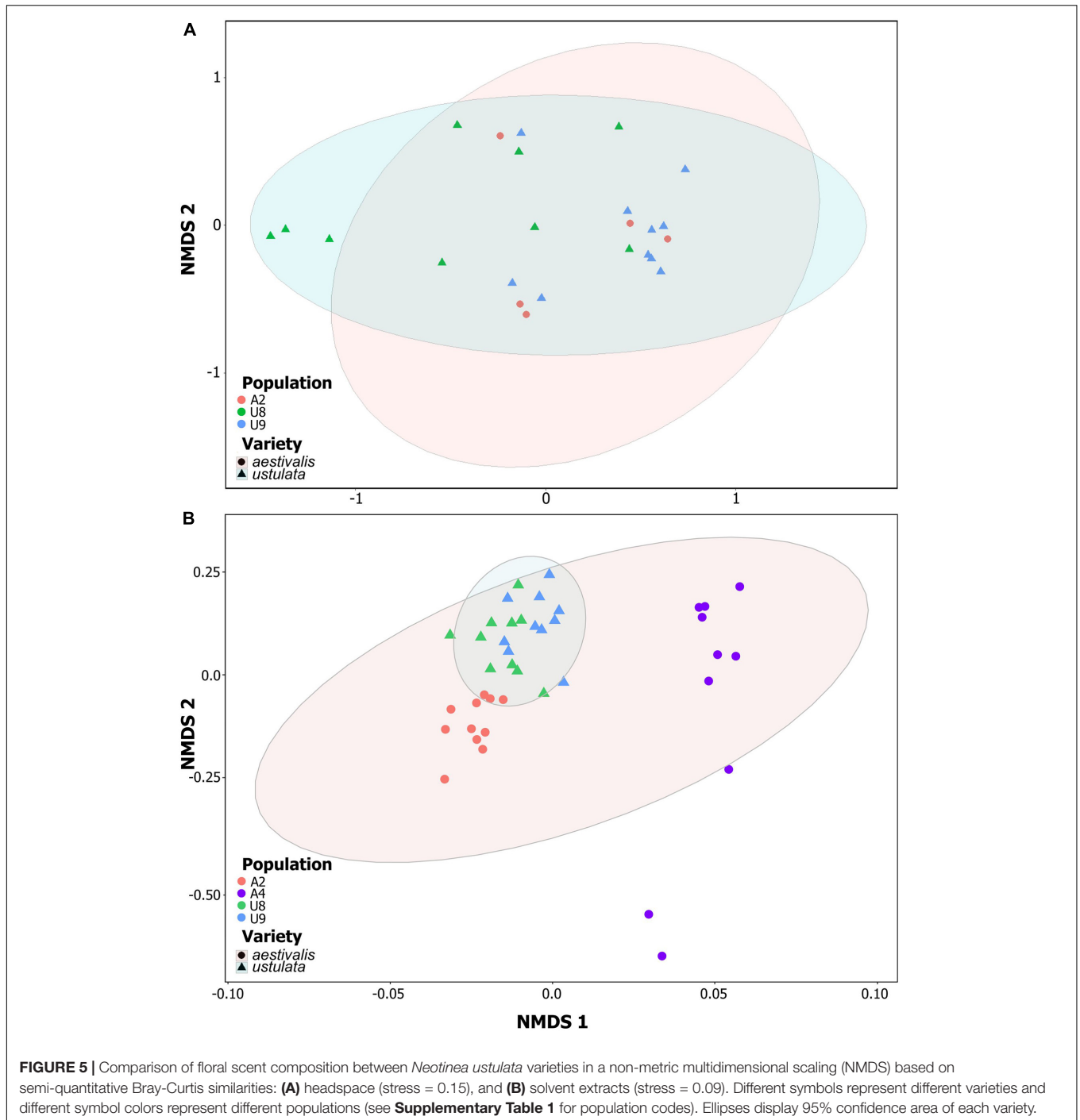


FIGURE 4 | Color loci (circles) of different inflorescence parts of *Neotinea ustulata* varieties (purple: dark top; pink: hood; gray: lip) plotted within the vision color models of (A) bees (bee-hexagon) and (B) flies (fly-tetrahedron). Var. *ustulata*: dark-colored tones (i.e., light purple, light pink, light gray); var. *aestivalis*: light-colored tones (i.e., dark purple, dark pink, dark gray). Bee (UV, B, G) and fly (R7p, R7y, R8p, R8y) receptors are shown.

Floral Scent

We detected 44 floral volatiles in the headspace samples and 54 chemical compounds in the floral extracts of *N. ustulata* with the majority of the compounds present in both varieties (see **Supplementary Tables 3, 4**). Headspace samples were dominated by terpenes (e.g., eucalyptol, limonene, linalool, β -myrcene, (*E*)- β -ocimene), although benzenoids (e.g., 2-phenylethyl alcohol) were also identified (see **Supplementary Table 3**); whereas extracts were dominated

by alkanes (e.g., pentacosane, heptacosane) and alkenes (e.g., tricosene and heptacosene isomers), but also aldehydes (see **Supplementary Table 4**). The (dis)similarity of floral scent composition between *N. ustulata* var. *ustulata* and var. *aestivalis* is shown in **Figure 5**. Although there were differences in floral scent patterns among populations of the same varieties [PERMANOVA headspace samples: Pseudo- $F_{(1, 23)} = 4.72$, $p < 0.01$; PERMANOVA extract samples: Pseudo- $F_{(2, 40)} = 12.65$, $p < 0.0001$], we did not



find any significant differences between the two varieties when comparing headspace samples [Pseudo- $F_{(1, 23)} = 1.48$, $p = 0.171$]. Significant differences were only found when comparing the composition of the floral extracts of the two varieties [Pseudo- $F_{(1, 40)} = 37.33$, $p < 0.0001$]. *Neotinea ustulata* var. *ustulata* could thus be distinguished from var. *aestivalis* through the low- or semi-volatile compounds emitted, but not by the more volatile fraction of the flower scent.

Pollination Success and Patterns of Fruit Set

Differences in the number of flowers between varieties were significant (var. *aestivalis*: mean \pm SD: 40.89 ± 13.57 ; var. *ustulata*: mean \pm SD: 33.34 ± 11.37 ; $\chi^2 = 4.83$, $df = 1$, $p < 0.05$; **Figure 6**), while differences in the number of fruits approached statistical significance (var. *aestivalis*: mean \pm SD: 9.82 ± 9.32 ; var. *ustulata*: mean \pm SD: 3.93 ± 5.01 ; $\chi^2 = 3.59$,

$df = 1$, $p = 0.058$; **Figure 6**); however, no differences were found comparing the proportion of flowers that set fruits (var. *aestivalis*: mean \pm SD: 0.22 ± 0.19 ; var. *ustulata*: mean \pm SD: 0.13 ± 0.17 ; $\chi^2 = 1.41$, $df = 1$, $p = 0.24$; **Figure 6**). The number of flowers per inflorescence had a positive impact on the probability to set at least one fruit ($\chi^2 = 6.23$, $df = 2$, $p < 0.05$), and the estimated probability to set a fruit increased 4% for each 10 flowers.

The probability to set a fruit varied significantly with the position of a flower within the inflorescence ($\chi^2 = 30.88$, $df = 2$, $p < 0.0001$; **Supplementary Figure 1**), but not the variety ($\chi^2 = 1.54$, $df = 1$, $p = 0.21$) or the interaction of variety and position ($\chi^2 = 1.71$, $df = 2$, $p = 0.42$; **Supplementary Figure 1**). *Post hoc* tests comparing the fruit set among the sections within each variety showed that compared to the top section, probability to set fruit was higher in the bottom (*aestivalis*: $z = 4.040$, $p < 0.001$; *ustulata*: $z = 3.995$, $p < 0.001$) and middle sections (*aestivalis*: $z = 2.882$, $p < 0.05$; *ustulata*: $z = 2.915$, $p < 0.05$); while no differences were found when comparing bottom and middle sections (*aestivalis*: $z = 1.556$, $p = 0.63$; *ustulata*: $z = 1.210$, $p = 0.83$; **Supplementary Figure 1**).

Pollinator Guilds and Their Efficiency in Pollinaria Removal

Flower Visitors, Pollinators, and Their Overall Efficiency

Inflorescences of both varieties were visited by a broad range of insect taxa within the Coleoptera, Diptera, Hymenoptera and Lepidoptera. Diptera and Hymenoptera were more frequently observed in our video recordings and together accounted for around 90% of the total insect visits (i.e., 415 insect visitors) to both *N. ustulata* varieties (**Figure 7** and see **Supplementary Table 5** for details of insect identity and frequency). Based on the same video recordings, the frequencies of the attracted

insect orders significantly differed between var. *aestivalis* and var. *ustulata* ($G = 71.68$, $df = 3$, $p < 0.0001$; **Figure 7**). *Anthophora plumipes* and *Lasioglossum* sp. bees were particularly frequent on *N. ustulata* var. *ustulata*, and *T. aff. magnicornis* flies were most abundant on var. *aestivalis* (**Supplementary Table 5**). Despite the high number of insect visits, only three *Anthophora* bees carrying pollinaria of *N. ustulata* var. *ustulata* and one *T. aff. magnicornis* removing pollinaria of var. *aestivalis* were observed in our videos. By direct observations, we recorded some *Tachina* individuals visiting the flowers and carrying pollinaria (**Supplementary Table 6**), thereby acting as pollinators of *N. ustulata* var. *ustulata* and var. *aestivalis*. While bees almost exclusively landed on the white part of the inflorescence moving upwards in their search for a reward, *Tachina* flies always settled briefly (for 1–4 s.) on the dark top of the inflorescence; then they searched for potential nectar in the uppermost freshly open flowers and introduced their head to reach the base of the flower spur with their body in downright position (**Figure 8**). The pollinaria then became attached on the ventral side of the lower part of the proboscis (**Figure 8**). Identification of tachinid specimens revealed that they belong to three morphologically similar species, *Tachina fera*, *T. magnicornis* and *Nowickia ferox*. Both taxa pollinate flowers of *N. ustulata* var. *ustulata*, while var. *aestivalis* was only observed to be pollinated by *T. magnicornis* and *N. ferox* (**Supplementary Table 6**). Beside tachinids, solitary bees and bumblebees acted as regular pollinators, whereas beetles and other flies were highly occasional (see **Supplementary Table 6** and **Supplementary Figure 2**).

Differences in female success between both varieties (var. *aestivalis*: mean \pm SD: 6.73 ± 15.57 , $n = 86$; var. *ustulata*: 20.80 ± 22.74 , $n = 172$) were marginally significant ($\chi^2 = 3.787$, $df = 1$, $p = 0.052$). However, we did not find differences in male success ($\chi^2 = 0.444$, $df = 1$, $p = 0.51$) and pollen transfer efficiency ($\chi^2 = 0.477$, $df = 1$, $p = 0.49$) between var. *aestivalis* (male success:

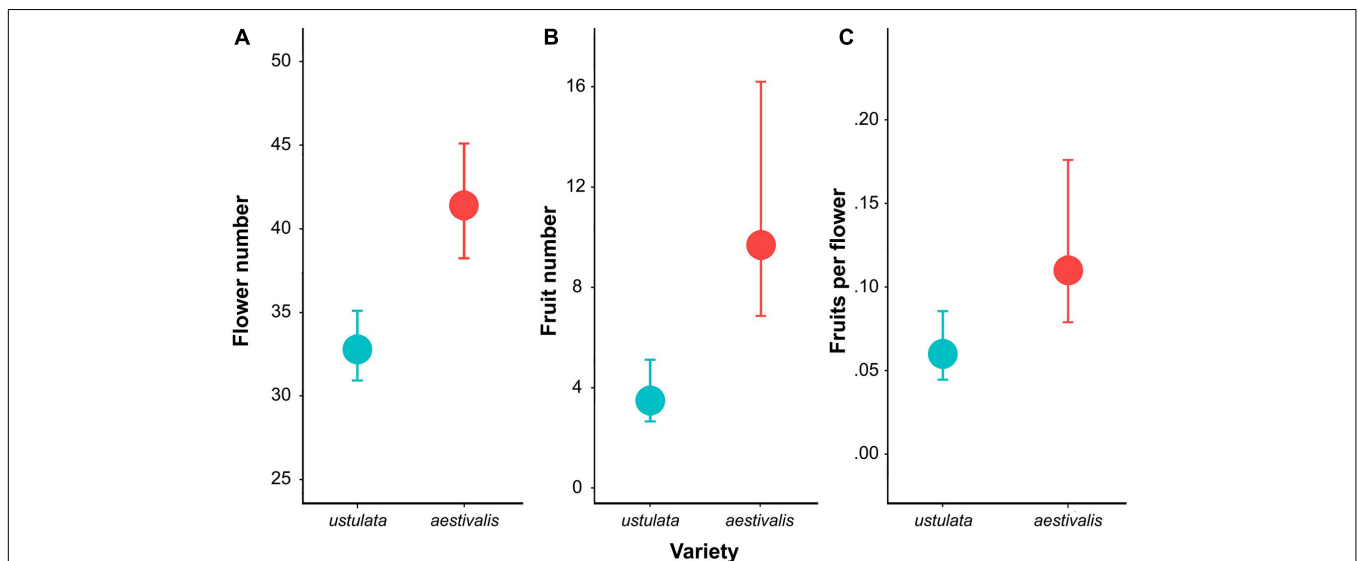
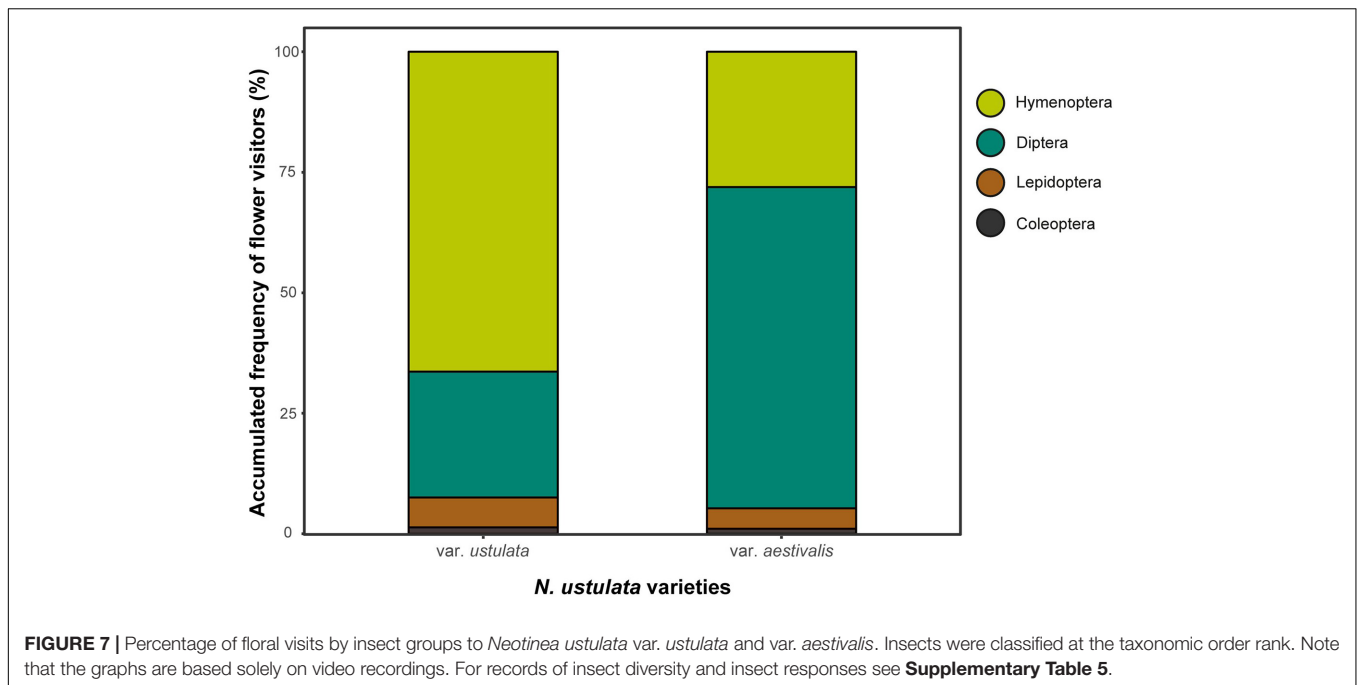


FIGURE 6 | Floral traits related to reproductive success: (A) the number of flowers, (B) the number of fruits, and (C) proportion of flowers that set fruit between *Neotinea ustulata* var. *ustulata* and var. *aestivalis*. Back-transformed means and standard errors are shown.



mean \pm SD: 23.03 ± 20.28 , $n = 86$; pollen transfer efficiency: mean \pm SD: 55.14 ± 237.41 , $n = 70$) and var. *ustulata* (male success: mean \pm SD: 24.29 ± 19.81 , $n = 172$; pollen transfer efficiency: mean \pm SD: 83.07 ± 81.82 , $n = 151$).

Efficiency in Pollinaria Removal Between Pollinator Guilds

While all tachinids but one, which probed the flower with the body in an upright position, removed pollinaria at their first attempt, some bees did not remove pollinaria even after three attempts. When testing the efficiency of pollinaria removal, we found that pollinator type had significant effects on pollinaria removal ($\chi^2 = 7.01$, $df = 1$, $p < 0.01$; **Figure 9**) but no effects of number of attempts on pollinaria removal success ($\chi^2 = 2.34$, $df = 2$, $p = 0.31$; **Figure 9**). *Post hoc* tests indicated that the efficiency of pollinaria removal was significantly higher within each attempt for *T. fera* compared to *A. plumipes* ($z = -2.850$, $p \leq 0.05$; **Figure 9**).

The Role of Visual Traits in Pollination Success

Inflorescence display was significantly larger in *N. ustulata* var. *aestivalis* (mean \pm SD: 758.1 ± 320.1 mm²) than in var. *ustulata* (mean \pm SD: 543.9 ± 325.4 mm²; $\chi^2 = 62.71$, $df = 1$, $p < 0.0001$); whereas dark top display did not differ between *N. ustulata* var. *ustulata* (mean \pm SD: 121.0 ± 35.8 mm²) and var. *aestivalis* (mean \pm SD: 123.0 ± 31.5 mm²; $\chi^2 = 0.20$, $df = 1$, $p = 0.655$). We also did not find an effect of the dark top display on the probability of pollinaria removal ($\chi^2 = 0.54$, $df = 1$, $p = 0.464$), pollinia deposition ($\chi^2 = 2.30$, $df = 1$, $p = 0.130$) and pollination event ($\chi^2 = 1.29$, $df = 1$, $p = 0.255$). Although we also did not detect an effect of the total inflorescence display on the probability of pollinaria removal ($\chi^2 = 2.11$, $df = 1$, $p = 0.15$), effect of inflorescence display on pollinia deposition approached

statistical significance ($\chi^2 = 3.27$, $df = 1$, $p = 0.070$) and was marginally non-significant for pollination events ($\chi^2 = 3.74$, $df = 1$, $p = 0.053$).

Role of Dark Top in Fly Pollinator Attraction

In our experiment with manipulation of the inflorescence dark top, there were significant effects of the treatment stage ($\chi^2 = 5.68$, $df = 1$, $p < 0.05$), the inflorescence type (i.e., selected for dark top excision or not; $\chi^2 = 10.29$, $df = 1$, $p < 0.01$) and the interaction treatment stage \times inflorescence type ($\chi^2 = 15.97$, $df = 1$, $p < 0.001$) on the number of *Tachina* aff. *fera* visits to var. *ustulata* (**Figure 10**). In the case of var. *aestivalis*, we did not find significant effects of the treatment stage on the number of *Nowickia ferox* visits ($\chi^2 = 0.017$, $df = 2$, $p = 0.99$), however the inflorescence type ($\chi^2 = 10.27$, $df = 1$, $p < 0.01$) and the interaction treatment stage \times inflorescence type ($\chi^2 = 6.98$, $df = 1$, $p < 0.05$) had significant effects on pollinator visits (**Figure 10**). *Post hoc* tests indicated that inflorescences with the dark top had significantly more visits than inflorescences without it ($z = 3.507$, $p < 0.01$; **Figure 9**), but no other pair-wise differences were found.

DISCUSSION

Despite being widely distributed, *Neotinea ustulata* is one of the most enigmatic European orchids in terms of its reproductive biology. Our study reveals that morphological (e.g., larger flowers in var. *ustulata*), visual (e.g., spectral reflectance pattern) and chemical (e.g., pattern of semi- and low- volatile compounds) floral traits distinguish *N. ustulata* var. *ustulata* from var. *aestivalis*. Our observations across different locations in Europe also support some early records that *N. ustulata* is mainly

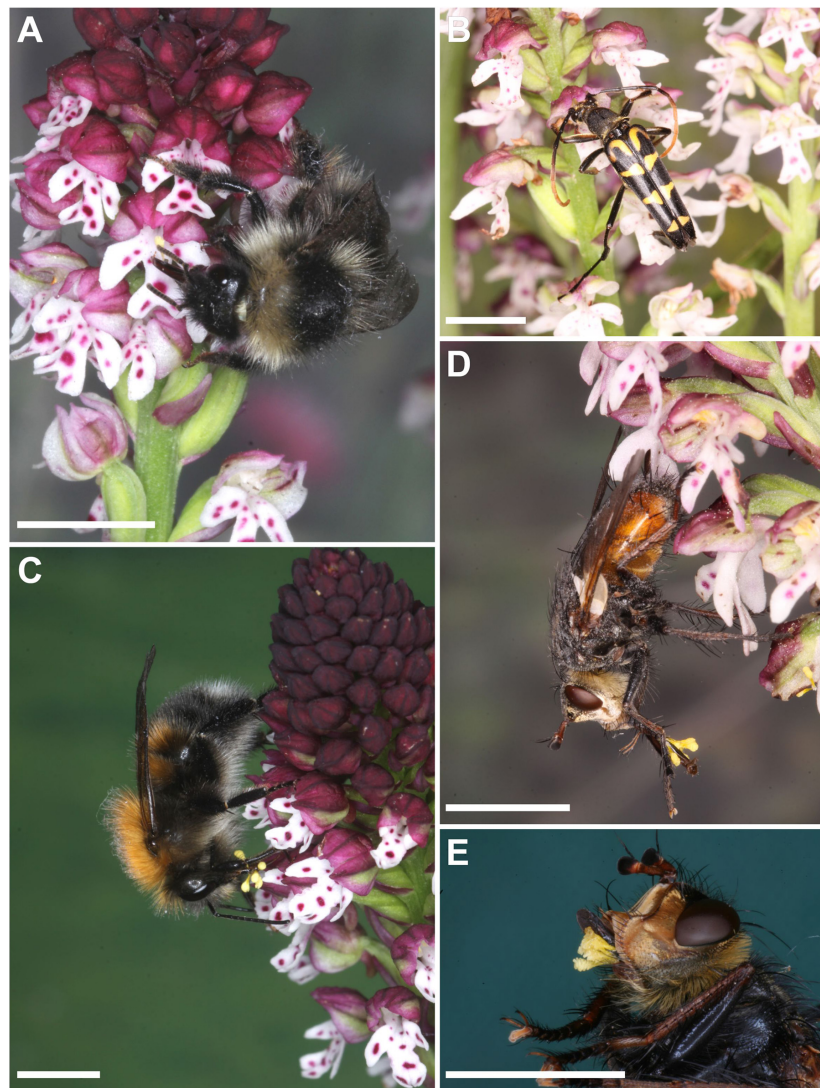


FIGURE 8 | Pollinators of *Neotinea ustulata*: **(A)** *Bombus mucidus*, **(B)** *Leptura annularis*, **(C)** *Bombus hypnorum*, **(D)** *Tachina fera*, **(E)** *Tachina fera* close-up. They were recorded visiting flowers of *N. ustulata* var. *ustulata* **(A,B,D,E)** and var. *aestivalis* **(C)**. White bars represent scale of 5 mm. Photographs by H. Paulus.

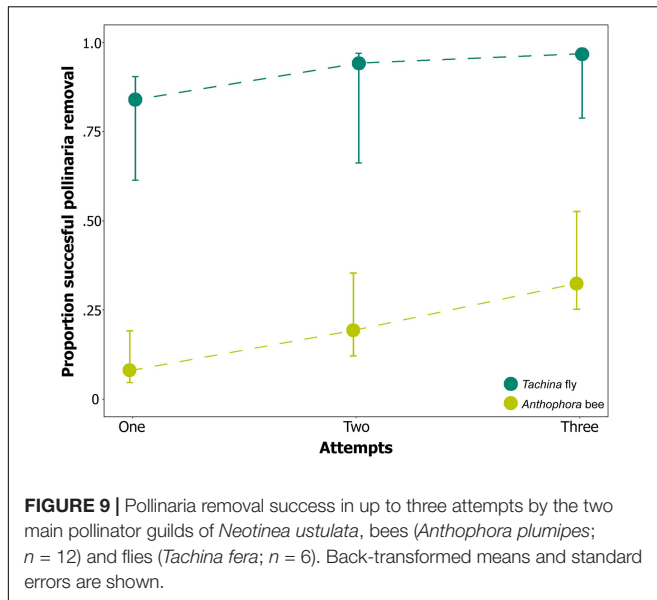
pollinated by tachinid flies (Godfery, 1933; Vöth, 1984). However, the relative importance of tachinids differs between the two varieties, playing a predominant role as pollinator for the late flowering var. *aestivalis*. The complexity of floral traits and pollinator spectra of *N. ustulata* may indicate a complex, and not yet fully understood, food-deceptive mechanism.

Morphological, Visual, and Chemical Differences Between *N. ustulata* Varieties

Morphological Cues

Flower morphology can play a key role in pollination by promoting reproductive isolation, particularly in highly specialized pollination systems (Schiestl and Schlüter, 2009). For example, in the sexually deceptive orchid genus *Chiloglottis* R.

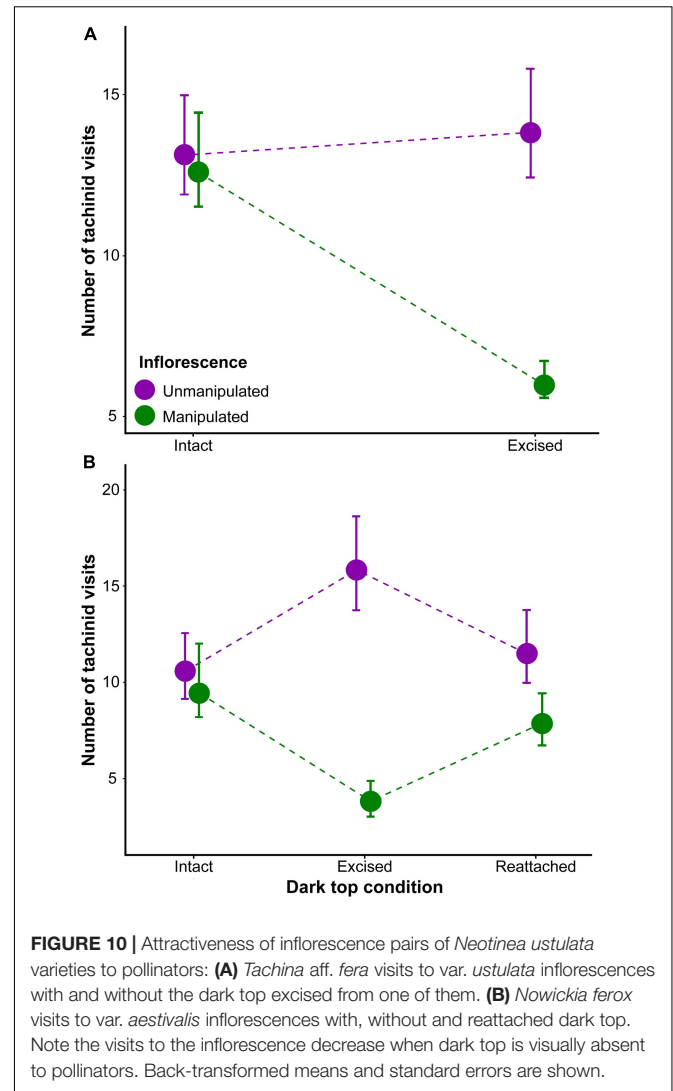
Br., differences in floral morphology have been shown to be enough to achieve pollinator specificity in species relying on the same chemical attractant (de Jager and Peakall, 2016). Variations in floral size can also affect morphological fit and, therefore, pollen/pollinia transfer by their respective pollinators (Anderson and Johnson, 2008; Solís-Montero and Vallejo-Marín, 2017; de Jager and Peakall, 2019). Even relatively subtle differences in flower structures can have an impact on floral isolation such as the position of pollinaria in the moth pollinated *Platanthera bifolia* (L.) Rich. and *P. chlorantha* (Custer) Rchb., which are then attached on different parts of the pollinators (Nilsson, 1983; Schiestl and Schlüter, 2009). Although the differences in size among floral structures of *N. ustulata* var. *ustulata* and var. *aestivalis* were usually quite small, they are consistent even at the population level, and suggest that the two varieties might be morphologically adapted to different pollinator spectra, although



phenotypic plasticity and non-pollinator selection should not be ruled out (Caruso et al., 2019; Sletvold, 2019). For instance, the width of the hood, the length of the lip and the length of the spur could all act as filters for potential pollinators. In fact, a previous study has already shown that the spur length of *N. ustulata* could be under pollinator-mediated selection (Trunschke et al., 2017). The role of flower traits for ensuring mechanical fit has been documented in diverse plant groups (e.g., Steiner and Whitehead, 1990; Muchhala, 2007; Anderson and Johnson, 2008; Pauw et al., 2009), including orchids (e.g., Chapurlat et al., 2015; Gögler et al., 2015; Rakosy et al., 2017; de Jager and Peakall, 2019). The smaller dimensions of *N. ustulata* var. *aestivalis* flowers could thus favor tachinids as pollinators and, conversely, larger flowers with deeper spur could favor a wider range of pollinators and improve pollinaria transfer. Assessment of mechanical fitting and behavioral experiments are necessary to test the role of morphology in *N. ustulata* pollinator filtering.

Visual Cues

Floral color is one of the main traits used by pollinators to identify both rewarding flowering plants and their deceptive mimics (Reverté et al., 2016). While all pollinators are able to respond to flower color signals, their preference for particular color hues and their ability to discriminate between them varies among groups (Reverté et al., 2016). Color reflectance patterns were structured at the population within and between varieties of *N. ustulata*. Although these differences can be related to phenotypic plasticity and non-pollinator-mediated selection (Ellis and Johnson, 2009; Caruso et al., 2019; Sletvold, 2019), pollinator preferences could be also involved. For instance, pollinator preferences have shaped daisy communities, which function as fly pollination specialist (Ellis et al., 2021). Variation in color hue among *N. ustulata* populations can be related to pollinator detectability and environmental conditions such as background context (e.g., coloration of surrounding plants and



soil). Independently of the population or variety origin, the stark chromatic contrast between the dark top and the white lips can be discriminated by both bees and flies, and its presence may be result of pollinator selection (see below). This is supported by the occurrence of bright top forms (e.g., light pink or white top) in *N. ustulata* inflorescences, which are not stable and appear from time to time in different populations (Foley, 1990). Color pattern and color contrast have been shown to be important in pollinator attraction (Zhang et al., 2017), and the dark top coloration would play a similar role (see below). Experiments are needed to test the role of color pattern in the pollination success of *N. ustulata* varieties.

Chemical Cues

Both *N. ustulata* varieties produced a rather complex floral scent and the scent bouquets of *N. ustulata* individuals were highly variable. This pattern is not restricted to *N. ustulata* populations from Czech Republic since the floral scent of a var. *ustulata* plant from Sweden was similarly dominated by

α -pinene, β -myrcene, limonene, eucalyptol, and (*E*)- β -ocimene (Kaiser, unpublished data). A study based on a plant from Italy also reported a complex bouquet including eucalyptol and D-limonene (i.e., D'auria et al., 2021); although we are cautious with the identity of some compounds also reported by the authors as they seem to be environmental contaminants. Differences between *N. ustulata* varieties were only detected when comparing compounds with low- and semi-volatility; however, larger scale sampling would be required to confirm that there are not differences between varieties when comparing highly volatile compounds. Differences in hydrocarbon compounds, which were predominant among compounds with lower volatility, could induce differential responses of pollinators once they approach or land on the flowers. Indeed, the function of these compounds, especially alkenes, has been intensively investigated in sexually deceptive orchids (Ayasse et al., 2011; Bohman et al., 2016). In fact, it is extremely unusual for food-deceptive species to produce such a high proportion of alkenes (Schiestl and Cozzolino, 2008), and in particular the production of (*Z*)-11-tricosene, which is extremely rare even among sexually deceptive plants but has been recorded in some tachinids (Martel et al., unpublished data). Therefore, alkenes and other low-volatile compounds could play a major role at short-range attraction in *N. ustulata* by eliciting certain behaviors in tachinid pollinators, while highly volatile compounds and visual cues remain probably the most important stimuli for long-range attraction. Further physiological and behavioral analyses are needed to elucidate the differential importance of *N. ustulata* floral scent in pollination.

Reproductive Success in *N. ustulata* Varieties

As typical for deceptive orchids, fruit set was rather low in most populations of *N. ustulata*. Reproductive success of *N. ustulata* was found to be higher than previously reported (i.e., Neiland, 2001; Harder and Johnson, 2008), but matches with those of some food-deceptive orchids (Gill, 1989; Fritz and Nilsson, 1994; Scopece et al., 2009, 2010; Trunschke et al., 2017). Contrary to previous reports (i.e., Tali et al., 2004; Haraštová-Sobotková et al., 2005), we did find differences in the characters associated with reproductive success between varieties. Furthermore, *N. ustulata* var. *aestivalis* was found to be slightly more successful than var. *ustulata*. Nevertheless, independently of the variety, the bottom and middle section of the inflorescence developed more fruits than the top section. This is consistent with gradual acropetalous opening of *N. ustulata* inflorescences and with tachinid behavior, as they always visit the freshest flowers on the top, but as flies habituate and more buds open, later opening flowers get less visits. This pattern is similar to those recorded in food-deceptive plants pollinated by bumblebees, which contrary to tachinids usually pursue the inflorescence from the bottom toward the top (Nilsson, 1980, 1983, 1984; Fritz, 1990; Jersáková and Kindlmann, 1998) and in sexually deceptive plants pollinated by bees and wasps, which learn to avoid deceitful plants (Peakall, 1990; Peakall and Handel, 1993; Ayasse et al., 2000). Thus, reproductive success pattern of *N. ustulata* indicates that pollinators are initially attracted to visit the plants, but then learn to avoid them.

Pollinator Spectra in *N. ustulata* Varieties

Diverse insect taxa visited flowers of both *N. ustulata* varieties, but only a few acted as true pollinators and the two varieties differ in their main pollinators. Both *Anthophora* and *Tachina* are able to remove pollinaria of *N. ustulata* var. *ustulata* with their proboscis, but *Tachina* flies were more efficient in doing so. Furthermore, while the tachinids were unable to detach the pollinaria from their proboscis, we frequently observed *Anthophora* bees grooming them off. Our observations of *N. ustulata* var. *ustulata* are in line with previous records in which bees and flies have been recorded (Paulus, 2005; Claessens and Kleynen, 2016). In contrast, our observations do not support the assumption that *N. ustulata* var. *aestivalis* is adapted to beetle pollination (see Danesch and Danesch, 1962; Mrkvicka, 1991; van der Cingel, 1995), though beetles can also act as occasional pollinators. While we observed a broad range of beetles, bees and even butterflies visiting the flowers of var. *aestivalis*, only *Nowickia* and *Tachina* were consistently observed removing pollinaria and occasionally *Bombus* spp. Thus, *N. ustulata* var. *aestivalis* may have become more specialized for fly pollination than var. *ustulata* and this adaptation could be triggered by differences in availability of insects through space and time of flowering, and the higher efficiency of tachinids in transferring pollinia compared to bees. Despite the difference in pollinator spectra, pollen transfer efficiency was similar between both varieties, which would also be linked to their similar degrees of specialization or adaptation to fly pollinators. Our tachinid collection from Austrian and Czech populations suggests that the two *N. ustulata* varieties have one species of *Nowickia* and two of *Tachina* as main pollinators (i.e., *N. ustulata* var. *ustulata* is pollinated by *T. fera* and *T. magnicornis*, and var. *aestivalis* by *T. magnicornis* and *N. ferox*); however, differences in tachinid attraction can also be due to differences in species availability throughout the year and not specific adaption to one of them. *Tachina fera* and *T. magnicornis* are morphologically very similar, can co-occur and can be easily misidentified without a close inspection by a trained person. Although previous reports indicated that *T. magnicornis* flies were visiting *N. ustulata* var. *ustulata* flowers (e.g., Vöth, 1984; Paulus, 2005), some of those flies could have actually been *T. fera* (see **Supplementary Table 6**), which is more abundant and common than *T. magnicornis* (Paulus, pers. obs.). Fly pollination is rather rare among European orchids (Jersáková et al., 2016) and, to the best of our knowledge, no tachinid species has been recorded as the main pollinator of any European orchid. Tachinids have been previously reported in only a couple of specialized pollination systems such as those of *Schizochilus* (van der Niet et al., 2010) and *Telipogon* (Martel et al., 2016). Independently of the identity of their pollinators, both varieties have a similar male success and pollen transfer efficiency, but different female success. Hence, based on pollen transfer efficiency, pollinators of both varieties seem to be very efficient in delivering pollinia (i.e., a ratio of pollinia deposited to pollinia removed above 0.50). The pollinator efficiency is linked to the pollinator constancy as well as pollinator identity (Peter and Johnson, 2009). We therefore suggest that the reported efficiency may be related to tachinids, which seem to be more constant and are more efficient in removing pollinaria.

Role of the Dark Top in *N. ustulata*

We have reliably demonstrated that the dark top is an attractant signal of *N. ustulata* as inflorescences bearing the dark top received significantly more visits of tachinids than inflorescences without it. This increasing attractiveness can be result of a combined effect of color, shape and size of the dark top (i.e., visual stimuli). These findings appear to support the theory put forward almost 90 years ago by Godfrey (1933), which considered that the dark top may be an adaptation to attract fly pollinators. The dark top of *N. ustulata* is quite unique among European orchids and although other species can also present a dark top (e.g., *Orchis purpurea* Huds.), they seem to have a far weaker contrast against the lips. Dark spots have also arisen in diverse plant groups such as Apiaceae, Asteraceae, Geraniaceae, Iridaceae, Liliaceae, Papaveraceae and Ranunculaceae (Dafni et al., 1990; Westmoreland and Muntan, 1996; Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010). It was suggested that dark, contrasting spots in flowers and inflorescences might even act as an insect mimic as demonstrated in some beetle- and fly-pollinated plants (e.g., Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010); however, at this point, it is unknown how tachinids are perceiving the contrasting dark top of *N. ustulata*. Although dark spots in flowers promotes pollinator attraction in some plants (e.g., Johnson and Midgley, 1997; van Kleunen et al., 2007; Goulson et al., 2009; Ellis and Johnson, 2010), it does not necessarily involve enhancement of fruit set (e.g., Westmoreland and Muntan, 1996; Johnson and Midgley, 1997; Ellis and Johnson, 2010). However, as dark spots promote visits of the main pollinators, their presence is likely to impact the fruit set of *N. ustulata*. *In situ* manipulation experiments would be needed to know how tachinids are perceiving the contrasting dark and its impact on pollination success.

Pollination Strategy of *N. ustulata*

Neotinea ustulata varieties are visited by generalist insects that seek for nectar and pollen (e.g., bees and flies), and the lip is bright white as in other food-deceptive plants (Johnson and Schiestl, 2016). However, other floral traits such as a strong and complex scent including alkenes, the evolutionary innovation of a dark top on the inflorescence and the predominance of tachinid male visitors (Martel et al., unpublished data) suggest that *N. ustulata* may have evolved a complex food-deceptive pollination system. Based on previous studies (i.e., Vogel, 1972; Bino et al., 1982; Valterová et al., 2007; Scopece et al., 2009), it seems that some orchid species potentially classified as food-deceptive have also evolved signals not usually associated with this strategy (e.g., some kind of chemical mimicry of their pollinators) and their pollinators are gender biased (i.e., attract predominantly female or male pollinators). This may also occur in *N. ustulata* as most abundant alkenes have been also detected in *Tachina* flies (Martel et al., unpublished data). Most floral visitors of *N. ustulata* are only seeking food, but tachinids would in addition perceive alkenes as a signal of other yet unknown resources. For instance, alkenes are key in the chemical mimicry and pollinator attraction of the sexually deceptive *Telipogon*

peruvianus T.Hashim. to its tachinid specialist pollinator (Martel et al., 2019). In other complex pollination systems, the fly-pollinated *Gorteria diffusa* Thunb. (Asteraceae) attracts flies seeking food, but male flies are also sexually attracted and try to copulate with the black petal ornamentation (Ellis and Johnson, 2010); similarly, some populations of the beetle-pollinated *Luisia teres* Gaudich attracts nectar-feeding females and male beetles, whereas others sexually attract males (Sugiura et al., 2021). Hence, the combination of traits present in *N. ustulata* indicates that this orchid would exploit not only food-based signals but also sexual ones (e.g., gender-specific pheromones). To address this issue further studies should identify physiologically active scent compounds and carry out behavioral bioassays.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CM, DR, MA, HP, and JJ conceived the study. CM, DR, SD, SJ, MA, HP, and JJ designed the study. CM, DR, HP, LN, HM, and JJ collected the data. CM, DR, SD, SJ, and JJ analyzed the data. CM, DR, and JJ drafted the first version of the manuscript. CM wrote the final version. All authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

This work was partially funded by DAAD and MŠMT to MA and JJ (DAAD-16-13/2016-2017 and 7AMB16DE003, respectively).

ACKNOWLEDGMENTS

We thank Jaromír Vaňhara, Peter Sehnal, and Hans-Peter Tschorsnig for tachinid determination; Ivana Jongepierová, Karel Fajmon, Vlastimil Vlačiha, Dano Dítě for locations of *N. ustulata* populations. We also thank Casper van der Kooi and Ewan Bodenham for the invitation and support to publish our study in this special issue. Roxana Rojas kindly prepared the map shown in Figure 2. The reviewers make very helpful comments that improved an earlier version of the manuscript. DAAD and MŠMT supported this study by a shared Grant to JJ (7AMB16DE003) and MA (DAAD-16-13/2016-2017). CM thanks the Max Planck Partner Group for providing financial supports at PUCP.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.659176/full#supplementary-material>

REFERENCES

- Adams, R. P. (2017). *Identification of Essential Oil Components by Gas Chromatograph/Mass Spectrometry*, 5th Edn. Carol Stream, IL: Allured Publishing Corporation.
- An, L., Neimann, A., Eberling, E., Algora, H., Brings, S., and Lunau, K. (2018). The yellow specialist: dronefly *Eristalis tenax* prefers different yellow colours for landing and proboscis extension. *J. Exp. Biol.* 221:jeb184788. doi: 10.1242/jeb.184788
- Anderson, B., and Johnson, S. D. (2008). The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution* 62, 220–225. doi: 10.1111/j.1558-5646.2007.00275.x
- Ayasse, M., Schiestl, F. P., Paulus, H. F., Löfstedt, C., Hansson, B. S., Ibarra, F., et al. (2000). Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: how does flower-specific variation of odour signals influence reproductive success? *Evolution* 54, 1995–2006. doi: 10.1111/j.0014-3820.2000.tb01243.x
- Ayasse, M., Stokl, J., and Francke, W. (2011). Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* 72, 1667–1677. doi: 10.1016/j.phytochem.2011.03.023
- Bates, D., Maechler, M., Bolker, B., Walker, S., Bojesen Christensen, R. H., Singmann, H., et al. (2019). *lme4: linear mixed-effects models using 'eigen' and s4. R package version 1.1-21*. Available Online at: <https://cran.r-project.org/package=lme4/>.
- Baumann, H., Blatt, H., Kretzschmar, H., and Eccarius, W. (2005). "Die Orchideentaxa," in *Die Orchideen Deutschlands*, eds H. Blatt, W. Eccarius, and H. Kretzschmar (Uhlstädt-Kirchhasel: Verlag Arbeitskreise Heimische Orchideen), 223–685.
- Bino, R. J., Dafni, A., and Meeuse, A. D. J. (1982). The pollination ecology of *Orchis galilaea* (Bornm. et Schulze) Schltr. (Orchidaceae). *New Phytol.* 90, 315–319. doi: 10.1111/j.1469-8137.1982.tb03263.x
- Bohman, B., Flematti, G. R., Barrow, R. A., Pichersky, E., and Peakall, R. (2016). Pollination by sexual deception - it takes chemistry to work. *Curr. Opin. Plant Biol.* 32, 37–46. doi: 10.1016/j.pbi.2016.06.004
- Borsos, O. (1962). Geobotanische monographie der orchideen der pannonischen und karpatischen Flora VI. *Sec. Biol.* 5, 27–61.
- Carlson, D. A., Mayer, M. S., Silhacek, D. L., James, L. D., Beroza, M., and Bierl, B. A. (1971). Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science* 174, 76–77. doi: 10.1126/science.174.4004.76
- Caruso, C. M., Eisen, K. E., Martin, R. A., and Sletvold, N. (2019). A meta-analysis of the agents of selection on floral traits. *Evolution* 73, 4–14. doi: 10.1111/evo.13639
- Chapurlat, E., Ågren, J., and Sletvold, N. (2015). Spatial variation in pollinator-mediated selection on phenology, floral display and spur length in the orchid *Gymnadenia conopsea*. *New Phytol.* 208, 1264–1275. doi: 10.1111/nph.13555
- Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. *J. Comp. Physiol. A* 170, 533–543. doi: 10.1007/BF00199331
- Chittka, L., and Kevan, P. G. (2005). "Flower colour as advertisement," in *Practical Pollination Biology*, eds A. Dafni, P. G. Kevan, and B. C. Husband (Cambridge, ON: Enviroquest), 157–196.
- Claessens, J., and Kleynen, J. (2011). *The Flower of the European Orchid. Form and Function*. Geulle: Jean Claessens and Jacques Kleynen Publishers.
- Claessens, J., and Kleynen, J. (2016). *Orchidées d'Europe - Fleur et Pollinisation*. Mèze: Biotope éditions.
- Dafni, A., Bernhardt, P., Schmida, A., Ivri, Y., Greenbaum, S., O'Toole, C., et al. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. *Israel J. Bot.* 39, 81–92. doi: 10.1080/0021213X.1990.10677134
- Danesch, E., and Danesch, O. (1962). *Orchideen Europas, Mitteleuropa*. Bern: Hallwag Verlag.
- D'auria, M., Lorenz, R., Mecca, M., Racioppi, R., Romano, V. A., and Viggiani, L. (2021). The scent of *Neotinea* orchids from basilicata (southern Italy). *Nat. Prod. Res.* 2021, 1–3. doi: 10.1080/14786419.2020.1871343
- de Jager, M. L., and Peakall, R. (2016). Does morphology matter? An explicit assessment of floral morphology in sexual deception. *Funct. Ecol.* 30, 537–546. doi: 10.1111/1365-2435.12517
- de Jager, M. L., and Peakall, R. (2019). Experimental examination of pollinator-mediated selection in a sexually deceptive orchid. *Ann. Bot.* 123, 347–354. doi: 10.1093/aob/mcy083
- Delforge, P. (2006). *Orchids of Europe, North Africa and the Middle East*. London: A and C Black Publishers Ltd.
- Ellis, A. G., Anderson, B., and Kemp, J. E. (2021). Geographic mosaics of fly pollinators with divergent color preferences drive landscape-scale structuring of flower color in daisy communities. *Front. Plant Sci.* 12:617761. doi: 10.3389/fpls.2021.617761
- Ellis, A. G., and Johnson, S. D. (2009). The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (Asteraceae). *Am. J. Bot.* 96, 793–801. doi: 10.3732/ajb.0800222
- Ellis, A. G., and Johnson, S. D. (2010). Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *Am. Nat.* 176, E143–E151. doi: 10.1086/656487
- Fay, M. F. (2018). Orchid conservation: how can we meet the challenges in the twenty-first century? *Bot. Stud.* 9:16. doi: 10.1186/s40529-018-0232-z
- Foley, M. J. Y. (1990). The current distribution and abundance of *Orchis ustulata* L. in southern England. *Watsonia* 18, 37–48.
- Fritz, A., and Nilsson, L. A. (1994). How pollinator-mediated mating varies with population size in plants. *Oecologia* 100, 451–462. doi: 10.1093/beheco/arih147
- Fritz, A. L. (1990). Deceit pollination of *Neotinea spitzelii* (Orchidaceae) on the island of Gotland in the Baltic: a suboptimal system. *Nord. J. Bot.* 9, 577–587. doi: 10.1111/j.1756-1051.1990.tb00548.x
- Galizia, C. G., Kunze, J., Gumbert, A., Borg-Karlson, A.-K., Sachse, S., Markl, C., et al. (2004). Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. *Behav. Ecol.* 16, 159–168.
- Gill, D. E. (1989). "Fruiting failure, pollinator inefficiency and speciation in orchids," in *Speciation and its Consequences*, eds D. Otte, and J. A. Endler (Sunderland, MA: Sinauer), 458–481.
- Godfrey, M. J. (1933). *Monograph and Iconograph of Native British Orchidaceae*. Cambridge: Cambridge University Press.
- Gögler, J., Stöckl, J., Cortis, P., Beyrle, H., Rosaria, M., Lumaga, B., et al. (2015). Increased divergence in floral morphology strongly reduces gene flow in sympatric sexually deceptive orchids with the same pollinator. *Evol. Ecol.* 29, 703–717. doi: 10.1007/s10682-015-9779-2
- Goulson, D., McGuire, K., Munro, E. E., Adamson, S., Colliar, L., Park, K. J., et al. (2009). Functional significance of the dark central floret of *Daucus carota* (Apiaceae) L.; is it an insect mimic? *Plant Species Biol.* 24, 77–82. doi: 10.1111/j.1442-1984.2009.00240.x
- Haraštová-Sobotková, M., Jersáková, J., Kindlmann, P., and Čurn, L. (2005). Morphometric and genetic divergence among populations of *Neotinea ustulata* (Orchidaceae) with different flowering phenologies. *Folia Geobot.* 40, 385–405. doi: 10.1007/BF02804287
- Harder, L. D., and Johnson, S. D. (2008). Function and evolution of aggregated pollen in angiosperms. *Int. J. Plant Sci.* 169, 59–78. doi: 10.1086/523364
- Hedlund, K., Bartel, R. J., Dicke, M., and Vet, L. E. M. (1996). Aggregation pheromones of *Drosophila* immigrants, *D. phalerata*, and *D. subobscura*. *J. Chem. Ecol.* 22, 1835–1844. doi: 10.1007/BF02028507
- Jacquemyn, H., Brys, R., Hermy, M., and Willems, J. O. (2005). Does nectar reward affect rarity and extinction probabilities of orchid species? An assessment using historical records from Belgium and the Netherlands. *Biol. Conserv.* 121, 226–257. doi: 10.1016/j.biocon.2004.05.002
- Jensen, J. M., and Pedersen, H. A. (1999). Ny lokalitet for Bakke-Gögehurt (*Orchis ustulata*) - med noter om artens fanologiske og morfologiske variation. *Flora og fauna* 105, 29–36.
- Jersáková, J., Johnson, S. D., and Kindlmann, P. (2006). Mechanisms and evolution of deceptive pollination in orchids. *Biol. Rev.* 81, 219–235. doi: 10.1017/S1464793105006986
- Jersáková, J., Jürgens, A., Smilauer, P., and Johnson, S. D. (2012). The evolution of floral mimicry: identifying traits that visually attract pollinators. *Funct. Ecol.* 26, 1381–1389. doi: 10.1111/j.1365-2435.2012.02059.x
- Jersáková, J., and Kindlmann, P. (1998). Patterns of pollinator-generated fruit set in *Orchis morio* (Orchidaceae). *Folia Geobot.* 33, 377–390. doi: 10.1007/BF02803641
- Jersáková, J., Spaethe, J., Streinzer, M., Neumayer, J., Paulus, H., Dötterl, S., et al. (2016). Does *Traunsteinera globosa* (the globe orchid) dupe its pollinators

- through generalized food deception or mimicry? *Bot. J. Linn. Soc.* 180, 269–294. doi: 10.1111/boj.12364
- Johnson, S. D., and Midgley, J. J. (1997). Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. *Am. J. Bot.* 84, 429–436. doi: 10.2307/2446018
- Johnson, S. D., and Schiestl, F. P. (2016). *Floral mimicry*. Oxford: Oxford University Press.
- Kassambara, A., and Mundt, F. (2020). *factoextra: extract and visualize the results of multivariate data analyses. R package version 1.0.7*. Available Online at: <https://cran.r-project.org/package=factoextra/>.
- Kümpel, H., and Mrkvicka, A. C. (1990). Untersuchungen zur Abtrennung der *Neotinea ustulata* L. subsp. *aestivalis* (Kümpel) Kümpel and Mrkvicka. *Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ.* 22, 306–324.
- Lenth, R., Singmann, H., Love, J., Buerkner, P., and Herve, M. (2020). *emmeans: estimated marginal means, aka least-squares means. R package version 1.4.8*. Available Online at: <http://cran.r-project.org/package=emmeans/>
- Maia, R., White, T., Gruson, H., Endler, J., Eliason, C., and Bitton, P.-P. (2021). *pavo: perceptual analysis, visualization and organization of spectral colour data. R package version 2.4.0*. Available Online at: <https://cran.r-project.org/package=pavo/>.
- Martel, C., Cairampoma, L., Stauffer, F. W., and Ayasse, M. (2016). *Telipogon peruvianus* (Orchidaceae) flowers elicit pre-mating behaviour in Eudejeania (Tachinidae) males for pollination. *PLoS One* 11:e0165896. doi: 10.1371/journal.pone.0165896
- Martel, C., Francke, W., and Ayasse, M. (2019). The chemical and visual bases of the pollination of the Neotropical sexually deceptive orchid *Telipogon peruvianus*. *New Phytol.* 223, 1989–2001. doi: 10.1111/nph.15902
- Mrkvicka, A. C. (1991). Bestäuber, Chromosomenzahl und weitere Beobachtungen zu *Neotinea ustulata* L. subsp. *aestivalis* (Kümpel) Kümpel and Mrkvicka. *Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ.* 23, 331–338.
- Muchhala, N. (2007). Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *Am. Nat.* 169, 494–504.
- Neiland, R. M. (2001). “Neotinea: ecology,” in *Genera Orchidacearum 2. Orchidoideae*, Part 1, eds A. M. Pridgeon, P. J. Cribb, M. W. Chase, and F. N. Rasmussen (Oxford, UK: Oxford University Press), 320–323.
- Nilsson, L. A. (1980). The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). *Bot. Not.* 133, 367–385.
- Nilsson, L. A. (1983). Processes of isolation and introgressive interplay between *Platanthera bifolia* (L.) Rich. and *Platanthera chlorantha* (Custer) Reichb. (Orchidaceae). *Bot. J. Linn. Soc.* 87, 325–350. doi: 10.1111/j.1095-8339.1983.tb00997.x
- Nilsson, L. A. (1984). Anthecology of *O. morio* (Orchidaceae) and its outpost in the North. *Nova Acta Regiae Soc. Sci. Upsal.* 3, 167–180.
- Nunes, C. E. P., Amorim, F. W., Mayer, J. L. S., and Sazima, M. (2016). Pollination ecology of two species of *Elleanthus* (Orchidaceae): novel mechanisms and underlying adaptations to hummingbird pollination. *Plant Biol.* 18, 15–25. doi: 10.1111/plb.12312
- Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: community ecology package. R package version 2.5-6*. Available Online at: <https://cran.r-project.org/package=vegan/>.
- Paulus, H. F. (2005). “Zur Bestäubungsbiologie der Orchideen,” in *Die Orchideen Deutschlands*, eds H. Blatt, W. Eccarius, and H. Kretschmar (Uhlstädt-Kirchhasel: Verlag Arbeitskreise Heimische Orchideen), 98–140.
- Pauw, A., Stofberg, J., and Waterman, R. J. (2009). Flies and flowers in Darwin's race. *Evolution* 63, 268–279. doi: 10.1111/j.1558-5646.2008.00547.x
- Peakall, R. (1990). Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. *Funct. Ecol.* 4, 159–167. doi: 10.2307/2389335
- Peakall, R., and Handel, S. N. (1993). Pollinators discriminate among floral heights of a sexually deceptive orchid: implications for selection. *Evolution* 47, 1681–1687. doi: 10.1111/j.1558-5646.1993.tb01260.x
- Peter, C. I., and Johnson, S. D. (2009). Reproductive biology of *Acrolophia cochlearis* (Orchidaceae): estimating rates of cross-pollination in epidendroid orchids. *Ann. Bot.* 104, 573–581. doi: 10.1093/aob/mcn218
- R Core Team. (2017). *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.
- Rakosy, D., Cuervo, M., Paulus, H. F., and Ayasse, M. (2017). Looks matter: changes in flower form affect pollination effectiveness in a sexually deceptive orchid. *J. Evol. Biol.* 30, 1978–1993. doi: 10.1111/jeb.13153
- Renner, S. S. (2005). “Rewardless flowers in the angiosperms and the role of insect cognition in their evolution,” in *Plant-Pollinator Interactions: from Specialization to Generalization*, eds N. M. Waser, and J. Olerton (Chicago, IL: University of Chicago Press), 123–144.
- Renoult, J. P., Valido, A., Jordano, P., and Schaefer, H. M. (2014). Adaptation of flower and fruit colours to multiple, distinct mutualists. *New Phytol.* 201, 678–686. doi: 10.1111/nph.12539
- Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower colour preferences but flowers with similar colours do not attract similar pollinators. *Ann. Bot.* 118, 249–257. doi: 10.1093/aob/mcw103
- Ripley, B., Venables, B., Bates, D. M., Hornik, K., Gebhardt, A., and Firth, D. (2020). *MASS: support functions and datasets for Venables and Ripley's MASS. R package version 7.3-51.6*. Available Online at: <http://cran.r-project.org/package=MASS/>
- Rogoff, W. M., Gretz, G. H., Jacobson, M., and Beroza, M. (1973). Confirmation of (Z)-9-tricosene as a sex pheromone of the housefly. *Ann. Entomol. Soc. Am.* 66, 739–741. doi: 10.1093/aesa/66.4.739
- Schiestl, F. P., and Cozzolino, S. (2008). Evolution of sexual mimicry in the orchid subtribe Orchidinae: the role of preadaptations in the attraction of male bees as pollinators. *BMC Evol. Biol.* 8:27. doi: 10.1186/1471-2148-8-27
- Schiestl, F. P., and Schlüter, P. M. (2009). Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annu. Rev. Entomol.* 54, 425–446. doi: 10.1146/annurev.ento.54.110807.090603
- Scopece, G., Cozzolino, S., Johnson, S. D., and Schiestl, F. P. (2010). Pollination efficiency and the evolution of specialized deceptive pollination systems. *Am. Nat.* 175, 98–105. doi: 10.1086/648555
- Scopece, G., Juillet, N., Müller, A., Schiestl, F. P., and Cozzolino, S. (2009). Pollinator attraction in *Anacamptis papilionacea* (Orchidaceae): a food or a sex promise? *Plant Species Biol.* 24, 109–114. doi: 10.1111/j.1442-1984.2009.00244.x
- Shrestha, M., Dyer, A. G., Dorin, A., Ren, Z. X., and Burd, M. (2020). Rewardlessness in orchids: how frequent and how rewardless? *Plant Biol.* 22, 555–561. doi: 10.1111/plb.13113
- Signorell, A. (2020). *DescTools: tools for descriptive statistics. R package version 0.99.36*. Available Online at: <http://cran.r-project.org/package=DescTools/>
- Simón-Porcar, V. I., Silva, J. L., Meeus, S., Higgins, J. D., and Vallejo-Marín, M. (2017). Recent autopolyploidization in a naturalized population of *Mimulus guttatus* (Phrymaceae). *Bot. J. Linn. Soc.* 185, 189–207. doi: 10.1093/botlinnean/box052
- Sletvold, N. (2019). The context dependence of pollinator-mediated selection in natural populations. *Int. J. Plant Sci.* 180, 934–943. doi: 10.1086/705584
- Solis-Montero, L., and Vallejo-Marín, M. (2017). Does the morphological fit between flowers and pollinators affect pollen deposition? An experimental test in a buzz-pollinated species with anther dimorphism. *Ecol. Evol.* 7, 2706–2715. doi: 10.1002/ece3.2897
- Stace, C. A. (2010). *New Flora of the British Isles*, 3rd Edn. Cambridge: Cambridge University Press.
- Steiner, K. E., and Whitehead, V. B. (1990). Pollinator adaptation to oil-secreting flowers-*Rediviva* and *Diascia*. *Evolution* 44, 1701–1707. doi: 10.2307/2409348
- Streinzer, M., Ellis, T., Paulus, H. F., and Spaethe, J. (2010). Visual discrimination between two sexually deceptive *Ophrys* species by a bee pollinator. *Arthropod-Plant Int.* 4, 141–148. doi: 10.1007/s11829-010-9093-4
- Sugiura, N., Matsumura, S., and Yokota, M. (2021). Beetle pollination of *Luisia teres* (Orchidaceae) and implications of a geographic divergence in the pollination system. *Plant Species Biol.* 36, 52–59. doi: 10.1111/1442-1984.12294
- Tali, K. (2004). *Species structure of Neotinea ustulata. Doctoral thesis*. Estonia: Institute of Botany and Ecology, University of Tartu.
- Tali, K., Fay, M. F., and Bateman, R. M. (2006). Little genetic differentiation across Europe between early-flowering and late-flowering populations of the rapidly declining orchid *Neotinea ustulata*. *Biol. J. Linn. Soc.* 87, 13–25. doi: 10.1111/j.1095-8312.2006.00550.x
- Tali, K., Foley, M. J. Y., and Kull, T. (2004). *Orchis ustulata* L. *J. Ecol.* 92, 174–184. doi: 10.1111/j.1365-2745.2004.00858.x
- Trunschke, J., Sletvold, N., and Ågren, J. (2017). Interaction intensity and pollinator-mediated selection. *New Phytol.* 214, 1381–1389. doi: 10.1111/nph.14479

- Valenta, K., Nevo, O., Martel, C., and Chapman, C. A. (2017). Plant attractants: integrating insights from pollination and seed dispersal ecology. *Evol. Ecol.* 31, 249–267. doi: 10.1007/s10682-016-9870-3
- Valterová, I., Kunze, J., Gumbert, A., Luxová, A., Liblikas, I., Kalinová, B., et al. (2007). Male bumble bee pheromonal components in the scent of deceit pollinated orchids; unrecognized pollinator cues? *Arthropod-Plant Int.* 1, 137–145. doi: 10.1007/s11829-007-9019-y
- van der Cingel, N. A. (1995). *An Atlas of Orchid Pollination: European orchids*. Rotterdam: A. A. Balkema.
- van der Niet, T., Jürgens, A., and Johnson, S. D. (2010). Pollinators, floral morphology and scent chemistry in the southern African orchid genus *Schizochilus*. *S. Afr. J. Bot.* 76, 726–738. doi: 10.1016/j.sajb.2010.07.004
- van Kleunen, M., Nanni, I., Donaldson, J. S., and Manning, J. C. (2007). The role of beetle marks and flower colour on visitation by monkey beetles (*Hopliini*) in the greater cape floral region, South Africa. *Ann. Bot.* 100, 1483–1489. doi: 10.1093/aob/mcm256
- Vereecken, N. J., and Schiestl, F. P. (2009). On the roles of colour and scent in a specialized floral mimicry system. *Ann. Bot.* 104, 1077–1084. doi: 10.1093/aob/mcp208
- Vogel, S. (1972). Pollination von *Orchis papilionacea* L. in den Schwarmbahnen von *Eucera tuberculata* F. *Jahresber. Naturw. Ver. Wuppertal* 25, 67–74.
- Vorobyev, M., and Osorio, D. (1998). Receptor noise as a determinant of colour thresholds. *Proc. R. Soc. Lond. B Biol. Sci.* 265, 351–358. doi: 10.1098/rspb.1998.0302
- Vöth, W. (1984). *Echinomyia magnicornis* Zett. Bestäuber von *Neotinea ustulata* L. *Die Orchidee* 35, 189–192.
- Vöth, W. (1989). Die Bestäuber von *Orchis papilionacea* L. (Orchidaceae). *Linzer Biol. Beitr.* 21, 391–404.
- Westmoreland, D., and Muntan, C. (1996). The influence of dark central florets on insect attraction and fruit production in Queen Anne's lace (*Daucus carota* L.). *Am. Midl. Nat.* 135, 122–129. doi: 10.1111/nph.13555
- Wickham, H., Chang, W., Henry, L., Pedersen, T. L., Takahashi, K., Wilke, C., et al. (2020). *ggplot2: create elegant data visualisations using the grammar of graphics. R package version 3.3.0*. Available Online at: <https://cran.r-project.org/package=ggplot2/>.
- Willis, K. J. (2017). *State of the World's Plants 2017*. London: Royal Botanic Gardens, Kew.
- Wucherpfennig, W. (1992). Spätblühende sippen von *Orchis ustulata* L. *Ber. Bayer. Bot. Ges.* 63, 33–35.
- Zhang, C., Vereecken, N. J., Wang, L., Tian, B., Dafni, A., Yang, Y., et al. (2017). Are nectar guide colour changes a reliable signal to pollinators that enhances reproductive success? *Plant Ecol. Divers.* 10, 89–96. doi: 10.1080/17550874.2017.1350763
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- The reviewer, GS, declared a past co-authorship with several of the authors, CM, MA, to the handling editor.

Copyright © 2021 Martel, Rakosy, Dötterl, Johnson, Ayasse, Paulus, Nilsson, Mejlon and Jersáková. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.