Supplementary Material

# Supplementary Methods 1

*Floral scent collection and chemical analysis*

Floral scents from both *N. ustulata* varieties were collected using the dynamic headspace technique and through solvent extracts. In the first approach, adsorbent tubes composed of Chromatoprobe quartz glasses containing 3 mg of a mixture of Tenax™ TA (mesh 60-80) and Carbotrap B (mesh 20-40) (1:1 mixture; both Supelco, Bellefonte, USA) were used to trap the floral scent through the dynamic headspace method. Fresh inflorescences from different plants (n = 1 inflorescence per individual, Table 1) were individually enclosed in a polyester oven bag (5 × 15 cm approx., Toppits®, Cofresco Frischhalteprodukte GmbH & Co., Minden, Germany) for 1 h. Subsequently the emitted floral scents were drawn through the Chromatoprobes by a membrane pump (Rietschle Thomas GmbH., Puchheim, Germany) at a constant flow rate of 300 mL/min. Chromatoprobes were then placed in glass vials and stored in the freezer at -20ºC. Environmental (empty bags) and vegetative (non-floral structures) samples were also collected as negative controls. Floral compounds were those detected either exclusively in floral scent samples or in higher amounts in comparison to the control samples. In the second approach, ten open flowers from an inflorescence (n = 1 inflorescence per individual, Table 1) were cut off above the ovary. They were then washed in glass vials containing 1 mL pentane (99.9%, HPLC grade, Sigma-Aldrich) at darkness during 24 h.

Chromatoprobes samples collected in 2015 were analysed using an Agilent mass spectrometer 5977A (Agilent Technologies, Santa Clara, USA) linked to an Agilent gas chromatograph 7890B equipped with a nonpolar DB-5 capillary column (30 m × 0.25 mm i.d. J&W, Agilent Technologies, Santa Clara, USA) and a cooled injection system (CIS, Gerstel GmbH & Co.KG, 45473 Mülheim an der Ruhr, Germany). A thermal desorption unit (TDU) was used to introduce the chromatoprobes at 10°C. After 1 min, the TDU started heating up at 15°C until it reached 300°C, a temperature at which it rested for 15 min. The liner was cooled to −100°C. After the transfer to the liner, it was heated up at 12°C/min until the temperature reached 290°C, which was maintained for 6 min. The GC oven was initially set up at 50°C, which was maintained for 1 min, and then increased by 4°C/min to 325°C. Mass spectra were taken at 70 eV with a scanning speed of 1 scan/sec from m/z 35 to 450

Chromatoprobes samples collected in 2016 were analysed under a double-focusing VG70/250 SE mass spectrometer (Vacuum Generators Ltd., Hailsham, UK) linked to a HP 6890 gas chromatograph (Hewlett-Packard Co., Palo Alto, USA) equipped with a nonpolar DB5 capillary column (30 m × 0.25 mm i.d. J&W, Agilent Technologies). The injector had been fitted with a ChromatoProbe kit. Helium was used as the carrier gas with a constant linear velocity of 1 mL/min. Chromatoprobes were thermally desorbed at 310°C and, after 1 min, the split valve was opened and the oven temperature increased by 4°C/min from 50ºC to 310°C. Mass spectra were taken at 70 eV with a scanning speed of 1 scan/sec from m/z 35 to 450.

Extracts were analysed by using GC/FID and GC/MS systems. The GC/MS analysis was performed as described above for chromatoprobes sampled in 2016. GC/FID was equipped as described above for the GC/MS but with a flame ionization detector (FID). Hydrogen was used as the GC carrier gas with a constant linear velocity of 2 mL/min. Aliquots of 1 μL sample were injected splitless at an oven temperature of 50°C. After 1 min, the split valve was opened and the oven temperature was increased at a rate of 8°C/min and 10°C/min to 310°C for GC/MS and GC/FID, respectively.

Structure elucidation of individual compounds was performed by comparing the mass spectra in our samples with those of commercial libraries (NIST, ADAMS) and spectra libraries of the Institute of Evolutionary Ecology and Conservation Genomics (Ulm University) and the Department of Biosciences (Paris-Lodron University of Salzburg), and the retention indexes of our compounds with those from the literature (e.g., Adams, 2017). Data were processed and analysed on the Automated Mass Spectral Deconvolution and Identification System - AMDIS - software (National Institute of Standards and Technology, Gaithersburg, USA).

REFERENCES

Adams, R. P. (2017). *Identification of Essential Oil Components by Gas Chromatograph/Mass Spectrometry*. 5th edition. Carol Stream, IL: Allured Publishing Corporation.

**Supplementary Table 1.** Description of *Neotinea* *ustulata* populations and design of sample collection. Numbers within each column indicate the sample size for each item, except for flower visitors and correlation of pollination success with floral display. Numbers within the parenthesis indicate the year in which the locality was sampled.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Population locality  (year) | Site code | Country | Geographical coordinates | Floral morphology | Floral colour | Floral scent (headspace/extracts) | Reproductive success | Flower visitors  (video recordings) | Flower visitors (direct observations) | Relation of display & pollination success | Experiment on dark top | Pollinator efficiency (bee/fly) |
| ***N*. *ustulata* var. *ustulata*** | | | | | | | | | | | | |
| Bårby  (1978-1984, 1999) | U1 | Sweden | 56.5106N, 16.4378E |  |  |  | 52 |  |  | X |  |  |
| Högsrum  (1978-1984, 1999) | U2 | Sweden | 56.7663N, 16.5987E |  |  |  | 35 |  |  | X |  |  |
| Kalkstad  (1978-1984, 1999) | U3 | Sweden | 56.6097N, 16.5240E |  |  |  | 91 |  | X |  |  |  |
| Räpplinge  (1999) | U4 | Sweden | 56.8295N, 16.6657E |  |  |  |  |  |  | X |  |  |
| Sandby  (1978-1984, 1999) | U5 | Sweden | 56.5788N, 16.6378E |  |  |  | 39 |  |  | X |  |  |
| Skogsby  (1999) | U6 | Sweden | 56.6275N, 16.5106E |  |  |  |  |  |  | X |  |  |
| Albrechtice  (2000-2001) | U7 | Czech Republic | 49.2000N, 13.5667E |  |  |  | 170 |  |  |  |  |  |
| Vědlice  (2000-2001, 2007, 2015-2017) | U8 | Czech Republic | 50.5167N, 14.3333E | 30 | 14 | 10/10 | 137 | X | X |  |  | 8/4 |
| Velký Hubenov  (2015-2017) | U9 | Czech Republic | 50.5333N, 14.3833E | 30 | 10 | 9/10 |  | X | X |  |  | 4/2 |
| Devínská Kobyla  (2000-2001) | U10 | Slovak Republic | 48.1833N, 16.9667E |  |  |  | 80 |  |  |  |  |  |
| Hohe Tauern, Mitteralm  (2006, 2010, 2013) | U11 | Austria | 47.1486N, 12.8161E |  |  |  |  |  | X |  |  |  |
| Vienna, Gießhübl  (1999, 2010) | U12 | Austria | 48.0989N, 16.2206E |  |  |  |  |  | X |  |  |  |
| Vienna, Perchtolsdorf  (1999, 2010) | U13 | Austria | 48.1267N, 16.2465E |  |  |  |  |  | X |  | 16 |  |
| ***N*. *ustulata* var. *aestivalis*** | | | | | | | | | | | | |
| Čertoryje  (1999) | A1 | Czech Republic | 48.8467N, 17.4081E |  |  |  |  |  |  | X |  |  |
| Drahy  (1999, 2015-2017) | A2 | Czech Republic | 48.9219N, 17.6391E | 23 | 10 | 5/11 |  | X | X | X |  |  |
| Hovězí  (2001) | A3 | Czech Republic | 49.3165N, 18.0534E |  |  |  |  |  | X |  |  |  |
| Jazevčí  (2007, 2015-2017) | A4 | Czech Republic | 48.8747N, 17.5701E | 15 | 10 | -/10 |  | X | X |  |  |  |
| Ježůvka  (2000) | A5 | Czech Republic | 49.3485N, 18.0110E |  |  |  | 60 |  | X |  |  |  |
| Losový  (2000) | A6 | Czech Republic | 49.3185N, 18.0939E |  |  |  | 18 |  |  |  |  |  |
| Zahrady pod Hájem  (1999) | A7 | Czech Republic | 48.8833N, 17.5167E |  |  |  |  |  | X | X |  |  |
| Ružomberok 1  (2001) | A8 | Slovak Republic | 49.0500N, 19.3000E |  |  |  | 32 |  | X |  |  |  |
| Ružomberok 2  (2001) | A9 | Slovak Republic | 49.0333N, 19.3000E |  |  |  | 42 |  |  |  |  |  |
| Vienna, Lainzer Tiergarten  (2009-2010, 2020) | A10 | Austria | 48.1668N, 16.2068E |  |  |  |  |  | X |  | 4 |  |

**Supplementary Table 2.** Eigenvectors of the first two principal components (PC1 and PC2) of the PCA of floral morphology traits in *Neotinea ustulata*

|  |  |  |
| --- | --- | --- |
| **Morphological floral trait** | **PC1** | **PC2** |
| Hood opening width | -0.834 | 0.226 |
| Hood opening height | -0.487 | -0.534 |
| Spur opening width | -0.759 | 0.130 |
| Spur length | -0.823 | 0.214 |
| Distance from column to spur opening | -0.591 | -0.566 |
| Lip length | -0.682 | -0.304 |
| Maximum spur width | -0.742 | 0.182 |
| Width at the spur bottom | -0.702 | 0.289 |

**Supplementary Table 3.** Chemical compounds, their retention index (KI) and their relative proportion (mean ± SD) in floral headspace samples of *N*. *ustulata* varieties. Compounds listed according to their KI, calculated from retention times in relation to those of a series of n-alkanes separated on a non-polar DB-5 capillary column. Relative proportions of compounds with more than 5% are shown in bold. Samples were classified by population (Supplementary Table 1).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Compound** | **KI** | **var. *ustulata*** | | **var. *aestivalis*** |
| **U8 (n = 9)** | **U9 (n = 10)** | **A2 (n = 5)** |
| **α-thujene**a | 919.82 | 0.06 ± 0.18 | 0.02 ± 0.06 | 0.42 ± 0.41 |
| **α-pinene**b | 926.29 | **10.53 ± 15.78** | **19.90 ± 21.77** | **7.18 ± 9.48** |
| **sabinene**b | 966.58 | 0.36 ± 0.58 | 2.05 ± 4.80 | 3.54 ± 3.51 |
| **β-pinene**b | 970.99 | 1.04 ± 2.20 | 0.86 ± 1.05 | 0.07 ± 0.16 |
| **β-myrcene**b | 985.11 | **13.86 ± 16.14** | **14.34 ± 5.39** | **12.83 ± 5.17** |
| **δ-2-carenea** | 1000.95 | 0.00 ± 0.00 | 0.34 ± 0.41 | 0.00 ± 0.00 |
| **Unidentified 1** | 1004.15 | 1.33 ± 2.42 | 0.64 ± 1.59 | 1.76 ± 3.93 |
| **p-cymene**a | 1021.50 | 0.50 ± 1.37 | 0.01 ± 0.02 | 0.00 ± 0.00 |
| **limonene**b | 1025.92 | 2.90 ± 7.67 | **16.48 ± 10.91** | **16.88 ± 7.82** |
| **eucalyptol**b | 1028.89 | **7.41 ± 10.12** | **6.86 ± 13.88** | **14.11 ± 21.54** |
| **(*Z*)-β-ocimene**b | 1032.30 | 0.12 ± 0.24 | 0.05 ± 0.14 | 2.63 ± 5.68 |
| **benzeneacetaldehyde**b | 1038.31 | 1.76 ± 2.51 | 0.01 ± 0.04 | 0.00 ± 0.00 |
| **(*E*)-β-ocimene**b | 1042.88 | **8.52 ± 15.93** | **8.23 ± 18.24** | 4.64 ± 9.94 |
| **γ-terpinene**b | 1060.12 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.20 ± 0.29 |
| **(*Z*)-sabinene hydrate**a | 1067.19 | 0.00 ± 0.00 | 0.13 ± 0.22 | 0.09 ± 0.21 |
| **m-cymenene**a | 1082.03 | 0.08 ± 0.19 | 0.03 ± 0.09 | 0.00 ± 0.00 |
| **(*E*)-linalool oxide (furanoid)**b | 1087.90 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.28 ± 0.37 |
| **p-cymenene**a | 1088.38 | 0.30 ± 0.38 | 0.08 ± 0.26 | 0.00 ± 0.00 |
| **α-pinene oxidea** | 1095.42 | 0.07 ± 0.13 | 0.01 ± 0.03 | 0.00 ± 0.00 |
| **linalool**b | 1098.61 | 3.12 ± 5.63 | **18.80 ± 30.51** | **14.32 ± 20.62** |
| **2-phenylethyl alcohol**b | 1107.64 | **18.36 ± 17.04** | 0.49 ± 1.81 | **6.11 ± 5.69** |
| **Unidentified 2** | 1121.26 | 2.13 ± 3.58 | 0.98 ± 3.11 | 0.35 ± 0.79 |
| **α-campholenal**a | 1124.70 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.09 ± 0.13 |
| ***allo*-ocimene**b | 1125.67 | 0.86 ± 1.23 | 1.18 ± 1.42 | 0.97 ± 2.04 |
| **(*3E,5E*)-2,6-dimethyl-1,3,5,7-octatetraene**a\* | 1136.70 | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.89 ± 4.23 |
| ***neoallo*-ocimene**b | 1138.46 | 0.49 ± 0.89 | 0.12 ± 0.27 | 0.23 ± 0.51 |
| **verbenol**a | 1141.90 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.05 ± 0.12 |
| **pinocarvone**a | 1157.85 | 0.14 ± 0.21 | 0.04 ± 0.05 | 0.13 ± 0.15 |
| **Unidentified 3** | 1180.34 | 1.02 ± 1.88 | 0.15 ± 0.28 | 0.00 ± 0.00 |
| **α-terpineol**b | 1192.39 | 0.97 ± 1.58 | **7.11 ± 16.18** | **8.44 ± 14.63** |
| **verbenone**b | 1202.86 | 1.06 ± 2.44 | 0.23 ± 0.28 | 0.59 ± 0.86 |
| **Unidentified 4** | 1205.96 | 0.64 ± 1.04 | 0.02 ± 0.07 | 0.00 ± 0.00 |
| **Shisofuran**a | 1206.28 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.18 ± 0.35 |
| **Unidentified 5** | 1240.99 | 0.03 ± 0.09 | 0.12 ± 0.21 | 0.00 ± 0.00 |
| **Unidentified 6** | 1242.81 | 1.49 ± 2.52 | 0.00 ± 0.00 | 0.39 ± 0.55 |
| **2-phenethyl acetate**b | 1249.60 | 2.55 ± 3.98 | 0.14 ± 0.19 | 0.41 ± 0.60 |
| **Unidentified 7** | 1331.66 | 2.30 ± 3.45 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **neryl acetate**b | 1355.99 | **11.65 ± 18.01** | 0.05 ± 0.16 | 0.00 ± 0.00 |
| **geranyl acetateb** | 1376.01 | 0.25 ± 0.57 | 0.04 ± 0.11 | 0.00 ± 0.00 |
| **β-cubebene**a | 1382.89 | 0.71 ± 1.22 | 0.14 ± 0.21 | 0.07 ± 0.15 |
| **Unidentified 8** | 1395.39 | 1.55 ± 3.84 | 0.20 ± 0.44 | 0.00 ± 0.00 |
| **(*E*)-β-farnesene**b | 1453.47 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.23 ± 0.25 |
| ***allo*-aromandendrene**a | 1455.79 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.69 ± 0.94 |
| **Unidentified 9** | 1460.95 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.22 ± 0.21 |
| **Unidentified 10** | 1461.14 | 1.07 ± 1.47 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **δ-cadinene**a | 1511.78 | 0.78 ± 2.30 | 0.16 ± 0.36 | 0.00 ± 0.00 |

\* This compound can be an artifact produced from ocimene (see Kaiser, 1993).

a Identification based on MS match with library entries and published RI.

b Identification based on authentic standards.

**Supplementary Table 4.** Chemicals, their retention index (KI) and their relative proportion (mean ± SD) in floral extracts of *N*. *ustulata* varieties. Alkene compounds were tentatively identified by injection of known alkenes and double bond position was confirmed by derivatization of samples (Buser *et al*., 1983; Dunkelblum et al., 1985). Compounds listed according to their KI, calculated from retention times in relation to those of a series of n-alkanes separated on a non-polar DB-5 capillary column. Relative proportions of compounds with more than 5% are shown in bold. Samples were classified by population (see Supplementary Table 1).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Chemical compound** | **KI** | **var. *ustulata*** | | **var. *aestivalis*** | |
| **U8 (n = 10)** | **U9 (n = 10)** | **A2 (n = 11)** | **A4 (n = 10)** |
| **nonanala** | 1103.64 | 0.71 ± 0.28 | 0.8 ± 0.26 | 1.07 ± 0.39 | 0.03 ± 0.04 |
| **undecanala** | 1306.16 | 1.06 ± 0.39 | 1.08 ± 0.35 | 1.57 ± 0.7 | 1.77 ± 0.67 |
| **dodecanala** | 1407.53 | 0.8 ± 0.43 | 0.86 ± 0.49 | 0.25 ± 0.2 | 0.27 ± 0.18 |
| **tetradecanala** | 1611.12 | 0.33 ± 0.19 | 0.24 ± 0.12 | 0.97 ± 0.45 | 0.1 ± 0.42 |
| **Unidentified 1** | 2018.29 | 0.15 ± 0.07 | 0.20 ± 0.07 | 0.14 ± 0.05 | 0.17 ± 0.1 |
| **(*Z*)-9-heneicoseneb** | 2071.32 | 0.04 ± 0.02 | 0.01 ± 0.04 | 0.01 ± 0.01 | 0 ± 0 |
| **(*Z*)-7-heneicoseneb** | 2075.35 | 0.04 ± 0.01 | 0.04 ± 0.02 | 0.05 ± 0.08 | 0.02 ± 0.04 |
| **Unidentified 2** | 2093.63 | 0.08 ± 0.03 | 0.07 ± 0.02 | 0.05 ± 0.01 | 0.45 ± 0.22 |
| **heneicosanec** | 2100.00 | 0.34 ± 0.07 | 0.49 ± 0.14 | 0.27 ± 0.06 | 0.43 ± 0.05 |
| **Unidentified 3** | 2123.21 | 1.16 ± 0.69 | 0.55 ± 0.25 | 0.03 ± 0.01 | 2.18 ± 1.39 |
| **Unidentified 4** | 2128.52 | 2.5 ± 1.08 | 1.16 ± 0.68 | 0 ± 0 | 4.8 ± 1.72 |
| **Unidentified 5** | 2161.53 | 0.32 ± 0.1 | 0.25 ± 0.07 | 0.00 ± 0.01 | 0.35 ± 0.18 |
| **docosaneb** | 2200.00 | 0.28 ± 0.06 | 0.46 ± 0.25 | 0.19 ± 0.08 | 0.35 ± 0.09 |
| **Unidentified 6** | 2222.22 | 0.83 ± 0.32 | 0.88 ± 0.34 | 0.83 ± 0.35 | 1.14 ± 0.33 |
| **(*Z*)-11-tricosenec** | 2267.55 | **8.81 ± 6.7** | **20.64 ± 7.64** | 2.61 ± 1.97 | 3.78 ± 2.42 |
| **(*Z*)-9-tricosenec** | 2270.65 | 0.86 ± 0.4 | 1.02 ± 0.54 | 0.41 ± 0.19 | 0.84 ± 0.21 |
| **(*Z*)-7-tricoseneb** | 2277.08 | 0.37 ± 0.18 | 0.55 ± 0.19 | 0.13 ± 0.07 | 0.07 ± 0.07 |
| **(*Z*)-5-tricoseneb** | 2284.52 | 0.03 ± 0.03 | 0.08 ± 0.04 | 0.02 ± 0.01 | 0.03 ± 0.03 |
| **(*Z*)-3-tricoseneb** | 2289.46 | 0.05 ± 0.02 | 0.12 ± 0.07 | 0.01 ± 0.01 | 0.18 ± 0.09 |
| **tricosaneb** | 2300.00 | **8.78 ± 1.6** | **12.29 ± 2.82** | **5.30 ± 0.96** | **8.86 ± 1.59** |
| **(*Z*)-11-tetracosenec** | 2371.13 | 0.13 ± 0.08 | 0.07 ± 0.04 | 0.01 ± 0.01 | 0.26 ± 0.18 |
| **(*Z*)-9-tetracosenec** | 2384.57 | 0.12 ± 0.05 | 0.11 ± 0.04 | 0.11 ± 0.05 | 0.2 ± 0.07 |
| **Unidentified 7** | 2394.14 | 0.10 ± 0.03 | 0.18 ± 0.1 | 0.04 ± 0.01 | 0.08 ± 0.04 |
| **tetracosaneb** | 2400.00 | 1.20 ± 0.29 | 1.13 ± 0.21 | 1.32 ± 0.35 | 1.69 ± 0.40 |
| **Unidentified 8** | 2425.74 | 1.22 ± 0.4 | 1.11 ± 0.25 | 1.24 ± 0.4 | 1.71 ± 0.41 |
| **Unidentified 9** | 2457.13 | 0.04 ± 0.01 | 0.02 ± 0.01 | 0.0 ± 0.01 | 0.12 ± 0.1 |
| **(*Z*)-11-pentacosenec** | 2466.04 | 2.22 ± 1.01 | 2.71 ± 2.01 | **7.25 ± 4.23** | **5.1 ± 4.37** |
| **(*Z*)-9-pentacosenec** | 2470.52 | 2.05 ± 0.8 | 1.77 ± 0.46 | 0.92 ± 0.25 | 3.4 ± 4.27 |
| **(*Z*)-7-pentacoseneb** | 2477.94 | 0.33 ± 0.16 | 0.39 ± 0.19 | 0.37 ± 0.24 | 0.48 ± 0.73 |
| **Unidentified 10** | 2481.79 | 0.02 ± 0.05 | 0.04 ± 0.09 | 0 ± 0 | 0.12 ± 0.08 |
| **(*Z*)-5-pentacoseneb** | 2486.72 | 0.14 ± 0.05 | 0.24 ± 0.11 | 0.13 ± 0.08 | 0.10 ± 0.11 |
| **(*Z*)-3-pentacoseneb** | 2493.10 | 0.01 ± 0.02 | 0.04 ± 0.03 | 0.04 ± 0.01 | 0.13 ± 0.14 |
| **pentacosaneb** | 2500.00 | **11.37 ± 2.35** | **9.47 ± 3.43** | **15.49 ± 1.62** | **17.49 ± 1.7** |
| **Unidentified 11** | 2589.89 | 1.14 ± 0.62 | 1.57 ± 0.92 | 0.88 ± 0.53 | 1.02 ± 0.88 |
| **hexacosaneb** | 2600.00 | 1.0 ± 0.13 | 0.97 ± 0.17 | 1.13 ± 0.22 | 1.19 ± 0.15 |
| **Unidentified 12** | 2629.47 | 1.07 ± 0.54 | 1.58 ± 0.8 | 0.61 ± 0.13 | 0.75 ± 0.22 |
| **(*Z*)-13-heptacosenec** | 2661.55 | 0.01 ± 0.01 | 0.03 ± 0.03 | 0.01 ± 0.01 | 0.04 ± 0.04 |
| **(*Z*)-11-heptacosenec** | 2666.14 | 0.64 ± 0.16 | 0.58 ± 0.15 | 0.58 ± 0.17 | 0.59 ± 0.28 |
| **(*Z*)-9-heptacosenec** | 2672.13 | **5.93 ± 1.48** | 4.74 ± 1.09 | 3.22 ± 0.94 | 3.43 ± 1.50 |
| **(*Z*)-7-heptacosenec** | 2679.46 | 1.06 ± 0.4 | 0.77 ± 0.25 | 0.55 ± 0.19 | 0.79 ± 0.94 |
| **Unidentified 13** | 2690.26 | 0.94 ± 0.28 | 1.04 ± 0.35 | 0.67 ± 0.27 | 0.58 ± 0.39 |
| **heptacosaneb** | 2700.00 | **8.27 ± 0.96** | **7.1 ± 2.1** | **12.96 ± 2.05** | **10.44 ± 1.8** |
| **(*Z*)-9-octacosenec** | 2772.81 | 0.09 ± 0.02 | 0.09 ± 0.03 | 0.11 ± 0.04 | 0.08 ± 0.06 |
| **(*Z*)-3-octacoseneb** | 2793.71 | 0.69 ± 0.65 | 0.33 ± 0.11 | 1.21 ± 0.73 | 0.82 ± 0.18 |
| **octacoseneb** | 2800.00 | 0.72 ± 0.12 | 0.69 ± 0.17 | 1.32 ± 0.19 | 0.48 ± 0.51 |
| **(*Z*)-11-nonacosenec** | 2870.52 | 2.95 ± 0.97 | 2.2 ± 0.63 | 2.06 ± 0.76 | 2.37 ± 2.32 |
| **(*Z*)-9-nonacosenec** | 2876.23 | **13.11 ± 2.81** | **8.32 ± 2.23** | **11.78 ± 2.99** | **6.97 ± 3.88** |
| **(*Z*)-7-nonacosenec** | 2882.27 | 2.5 ± 0.91 | 1.65 ± 0.79 | 1.68 ± 0.48 | 1.78 ± 2.59 |
| **Unidentified 14** | 2895.08 | 0.65 ± 0.23 | 0.48 ± 0.29 | 0.45 ± 0.32 | 0.58 ± 1.41 |
| **nonacosaneb** | 2900.00 | 3.95 ± 0.71 | 3.57 ± 1.19 | **8.01 ± 1.25** | 3.86 ± 1.5 |
| **triacontaneb** | 3000.00 | 0.19 ± 0.05 | 0.18 ± 0.07 | 0.34 ± 0.08 | 1.61 ± 4.59 |
| **(*Z*)-11-hentriacontenec** | 3077.54 | **6.69 ± 1.97** | 3.53 ± 1.19 | **8.61 ± 2.35** | 3.96 ± 1.5 |
| **(*Z*)-9-hentriacontenec** | 3083.75 | 0.39 ± 0.1 | 0.28 ± 0.17 | 0.52 ± 0.16 | 0.19 ± 0.1 |
| **hentriacontaneb** | 3100.00 | 1.48 ± 0.40 | 1.14 ± 0.34 | 2.46 ± 0.45 | 0.83 ± 0.25 |

aIdentification based on MS match with library entries and published RI.

bIdentification based on authentic standards.

cIdentification based on authentic standards and identification of double bond position by derivatization.

**Supplementary Table 5.** Floral visitors’ identity, their behaviour and number of visits to each variety of *Neotinea* *ustulata* based on video recordings. Behaviour pattern (%) was calculated from the total number of insect visits per variety landing pattern (%) was calculated from the number of visits per floral visitor taxon.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Floral visitor taxon†** | **Behaviour pattern (%)** | | | **Landing pattern (%)** | | **Number of visits** |
| **Approach** | **Landing** | **Probing** | **Dark top** | **White part** |
| ***Neotinea ustulata* var. *ustulata*** | | | | | | |
| **Coleoptera** |  |  |  |  |  | **3** |
| Coleoptera sp. | 0.4 | 0.9 | 0.0 | 50 | 50 | 3 |
| **Diptera** |  |  |  |  |  | **59** |
| Anthomyiidae sp. | 0.0 | 0.9 | 1.3 | 0 | 100 | 5 |
| Bombyliidae sp. | 0.4 | 0.0 | 1.8 | 0 | 100 | 5 |
| Diptera sp. 1 | 0.9 | 10.2 | 4.9 | 50 | 50 | 36 |
| Diptera sp. 2 | 0.4 | 0.0 | 0.0 | 0 | 0 | 1 |
| Muscidae sp. | 0.0 | 0.9 | 0.4 | 70 | 30 | 3 |
| Syrphidae sp. | 2.2 | 0.0 | 0.0 | 0 | 0 | 5 |
| Stratiomyidae sp. | 0.0 | 0.4 | 0.4 | 50 | 50 | 2 |
| *Tachina* aff. *fera* | 0.0 | 0.4 | 0.4 | 100 | 0 | 2 |
| **Hymenoptera** |  |  |  |  |  | **150** |
| *Andrena* sp. | 0.4 | 0.0 | 0.4 | 0 | 100 | 2 |
| *Anthophora plumipes* | 11.9 | 0.0 | 13.3 | 0 | 100 | 57 |
| Apidae sp. 1 | 0.0 | 0.0 | 0.4 | 0 | 100 | 1 |
| Apidae sp. 2 | 0.4 | 0.0 | 0.0 | 0 | 0 | 1 |
| *Apis mellifera* | 0.0 | 0.0 | 0.4 | 0 | 100 | 1 |
| *Bombus* sp*.* | 0.4 | 0.0 | 0.4 | 0 | 100 | 2 |
| *Halictus* sp*.* | 0.9 | 1.3 | 0.4 | 0 | 100 | 6 |
| *Halictus/Lasioglossum* sp*.* | 0.0 | 2.7 | 0.4 | 0 | 100 | 7 |
| Hymenoptera sp. | 0.9 | 0.0 | 0.4 | 0 | 100 | 3 |
| *Lasioglossum* sp*.* | 0.4 | 0.0 | 0.0 | 0 | 100 | 1 |
| *Sphecodes* sp*.* | 7.1 | 14.6 | 8.4 | 0 | 100 | 68 |
| Vespidae sp. | 0.0 | 0.4 | 0.0 | 0 | 0 | 1 |
| **Lepidoptera** |  |  |  |  |  | **14** |
| *Anthocharis cardamines* | 0.4 | 0.0 | 0.0 | 0 | 0 | 1 |
| Lepidoptera sp. | 0.9 | 0.0 | 0.4 | 0 | 100 | 3 |
| *Pieris rapae* | 0.9 | 0.0 | 0.0 | 0 | 0 | 2 |
| Noctuidae sp. 1 | 1.8 | 0.9 | 0.4 | 30 | 70 | 7 |
| Noctuidae sp. 2 | 0.0 | 0.4 | 0.0 | 0 | 100 | 1 |
| **Absolute events (n)** | **70** | **77** | **79** | **27** | **129** | **226** |
|  | | | | | | |
| ***Neotinea ustulata* var*. aestivalis*** | | | | | | |
| **Coleoptera** |  |  |  |  |  | **2** |
| Coleoptera sp. | 0.5 | 0.0 | 0.0 | 0 | 0 | 1 |
| Curculionidae sp. | 0.0 | 0.0 | 0.5 | 0 | 0 | 1 |
| **Diptera** |  |  |  |  |  | **126** |
| Diptera sp. | 0.5 | 0.0 | 0.0 | 0 | 0 | 1 |
| Syrphidae sp. | 1.1 | 0.0 | 0.5 | 0 | 100 | 3 |
| *Tachina* aff*. magnicornis* | 4.8 | 38.6 | 21.2 | 100 | 0 | 122 |
| **Hymenoptera** |  |  |  |  |  | **53** |
| *Andrena sp.* | 0.0 | 0.5 | 0.0 | 0 | 100 | 1 |
| *Apis mellifera* | 1.6 | 0.0 | 0.0 | 0 | 0 | 3 |
| Apidae sp. | 0.5 | 0.0 | 0.0 | 0 | 0 | 1 |
| *Bombus* sp. | 1.1 | 0.0 | 0.0 | 0 | 0 | 2 |
| *Lasioglossum* sp*.* | 4.2 | 2.6 | 12.7 | 0 | 100 | 37 |
| Megachilidae sp. | 0.0 | 0.0 | 0.5 | 0 | 100 | 1 |
| Symphyta sp. | 1.1 | 1.1 | 1.6 | 0 | 100 | 7 |
| Vespidae sp. | 0.5 | 0.0 | 0.0 | 0 | 0 | 1 |
| **Lepidoptera** |  |  |  |  |  | **8** |
| *Brenthis hecate* | 0.5 | 0.0 | 0.5 | 100 | 0 | 2 |
| *Euphydrias maturna* | 0.5 | 0.0 | 0.0 | 0 | 0 | 1 |
| Lepidopterasp. | 0.0 | 0.0 | 0.5 | 100 | 0 | 1 |
| *Maniola jurtina* | 0.5 | 0.0 | 0.5 | 0 | 100 | 2 |
| *Melanargia galathea* | 1.1 | 0.0 | 0.0 | 0 | 0 | 2 |
| **Absolute events (n)** | **35** | **81** | **73** | **115** | **38** | **189** |

**Supplementary Table 6.** Pollinator assessment based on the observation frequency and presence of pollinaria on insect visitors.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Insect taxon** | | **Gender** | **Percentage of individuals with pollinaria (sample size)1** | **Countries where pollinators were recorded2** |
| ***Neotinea ustulata* var. *ustulata*** | | | | |
| **Coleoptera** | |  |  |  |
| Coleoptera sp. 1 | | ? | 0.0 (1) | Czech Republic |
| Coleoptera sp. 2 | | ? | 0.0 (3) | Czech Republic |
| *Leptura annularis* | | ? | 100 (1) | Austria |
| **Diptera** | |  |  |  |
| Bombyliidae sp. | | ? | 0.0 (9) | Czech Republic |
| Calliphoridae sp. | | ? | 0.0 (3) | Czech Republic |
| Bibionidae: *Dilophus* sp. | | ♀ | \* | Sweden |
| Diptera sp. | | ? | 0.0 (1) | Czech Republic |
| Empididae sp. | | ? | 0.0 (4) | Czech Republic |
| *Peleteria rubescens* | | ♂ | 100.0 (5) | Sweden |
| Syrphideae sp. | | ? | 0.0 (2) | Czech Republic |
| *Tachina. fera* | | ♂/♀ | \* / 8.5 (59) | Austria, Czech Republic |
| *Tachina. magnicornis* | | ♂ | † / 100.0 (2) | Austria, Sweden |
| **Hymenoptera** | |  |  |  |
| *Anthophora plumipes* | | ♀ | 15.4 (26) | Czech Republic |
| *Anthophora aestivalis* | | ♂ | 100 (1) | Austria |
| Apidae sp. 1 | | ♀? | 100.0 (1) | Czech Republic |
| Apidae sp. 2 | | ♀? | 50.0 (2) | Czech Republic |
| Apidae sp. 3 | | ♀? | 0.0 (1) | Czech Republic |
| Apidae sp. 4 | | ♀? | 100.0 (1) | Czech Republic |
| Apidae sp. 5 | | ♀? | 0.0 (1) | Czech Republic |
| Apidae sp. 6 | | ♀? | 0.0 (5) | Czech Republic |
| Apidae sp. 7 | | ♀? | 0.0 (1) | Czech Republic |
| *Apis mellifera* | | ♀ (worker) | 50.0 (4) | Czech Republic |
| *Eucera pollinosa* | | ♂ | 0.0 (1) | Austria |
| *Bombus lucorum* | | ♂ | 100 (2) | Austria |
| *Bombus mucidus* | | ♀ | 100 (1) | Austria |
| *Bombus pascuorum* | | ♀ (worker) | 100 (9) | Austria |
| *Bombus pratorum* | | ♂ | 100 (4) | Austria |
| *Bombus ruderarius* | | ♀ (queen) | 100 (2) | Austria |
| *Bombus rupestris* | | ♀ | † | Austria |
| *Bombus terrestris* | | ♀ (queen/worker) | † / 0.0 (6) | Austria, Czech Republic |
| *Bombus vestalis* | | ♀ | 100 (4) | Austria |
| *Osmia rufohirta* | | ♀? | 100 (1) | Austria |
| Vespidae sp. | | ♀? | 0.0 (4) | Czech Republic |
| **Lepidoptera** | |  |  |  |
| Lepidoptera sp. 1 | | ? | 0.0 (1) | Czech Republic |
| Lepidoptera sp. 2 | | ? | 0.0 (1) | Czech Republic |
| Lepidoptera sp. 3 | | ? | 0.0 (1) | Czech Republic |
| Lepidoptera sp. 4 | | ? | 0.0 (5) | Czech Republic |
|  |  | | | |
| ***Neotinea ustulata* var. *aestivalis*** | | | | |
| **Coleoptera** | |  |  |  |
| *Rhagonycha* *fulva* | | ? | 100.0 (1) | Czech Republic |
| **Diptera** | |  |  |  |
| *Nowickia ferox* | | ♂ | † | Austria |
| *Tachina magnicornis* | | ♂/♀ | \* / 23.9 (71) | Austria, Czech Republic |
| **Lepidoptera** | |  |  |  |
| Lepidoptera sp. 1 | | ? | 0.0 (1) | Czech Republic |
| **Hymenoptera** | |  |  |  |
| Apidae sp. 1 | | ♀? | 0.0 (1) | Czech Republic |
| Apidae sp. 2 | | ♀? | 0.0 (2) | Czech Republic |
| *Bombus hypnorum* | | ♂ | 100 (3) | Austria |

1 Numbers are based on observations, when more than one observation per country, they were separated by a slash and order follows country’s name.

2 Pollinator observations for Austrian and Sweden populations date back 1998 until 202020, but the frequency of observations was not always registered.

\* Specimens were neither collected nor counted, but dozens were observed carrying pollinaria of *N*. *ustulata* var. *ustulata*.

† Frequency of observed insects was non-recorded, but some specimens were observed with pollinaria attached.

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**Supplementary Figure 1.** Pattern of fruit set (in percentage) in relation to its position within the inflorescence of *Neotinea ustulata* var. *ustulata* and var. *aestivalis*. Points indicate means and vertical lines indicate standard errors.

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**Supplementary Figure 2.** Bumblebee floral visitors of *N*. *ustulata* var. *ustulata* in Gießhübl, Austria: *Bombus* *pascuorum* (upper left and right), *B*. *pratorum* (bottom left) and *B*. *vestalis* (bottom right). Photographs by H. Paulus.