Corrected: Author Correction

**OPEN**

# Diversity of nectar amino acids in the *Fritillaria* (Liliaceae) genus: ecological and evolutionary implications

Katarzyna Roguz[1*], Andrzej Bajguz[2], Magdalena Chmur[2], Agnieszka Gołębiewska[2], Agata Roguz[3] & Marcin Zych[1*]

Nectar is considered to be a primary food reward for most pollinators. It mostly contains sugars, but also has amino acids. The significance of the concentration and composition of amino acids in nectar is often less understood than that of its volume, sugar concentration and composition. However, there is a trend towards a broader approach in ecological research, which helps to understand nectar properties in an ecological context. The genus *Fritillaria*, exhibiting great diversity in flower morphology, nectar composition, and dominant pollinators, allows for the possibility to study some of the above. We studied the concentration and composition of amino acids in the nectar of 38 *Fritillaria* species attracting different groups of pollen vectors (bees, flies, passerines, and hummingbirds). The flowers of fritillaries produced nectar with a varying composition and concentration of amino acids. These differences were mostly associated with the pollinator type. The nectar of passerine bird-pollinated species was rich in amino acids, whereas humming bird-pollinated produced low amino acid nectar. Contrary to previous reports nectar of the insect-pollinated species did not contain a higher amount of proline. Two non-protein amino acids, sarcosine and norvaline, were detected in the floral nectar for the first time.

Nectar is the most crucial floral reward for animal pollinators[1]. Primarily, it is a sugar solution composed of sucrose, fructose and glucose, in varying proportions. Nectar may also contain other sugars, for example mannose, maltose, and melezitose, as well as trace quantities of many other chemical compounds, including amino acids (AAs)[2–5].

While nectar sugars generally represent the nectar's energetic value[1,2], and their proportion seems to be conserved within a species[2,4,6], the biological functions of nectar AAs may vary[2,7,8]. The concentration of nectar AAs is traditionally perceived as an adaptation to various pollinator types. For example, plants pollinated by bees and hummingbirds contain a lower AA concentration, whereas butterfly or passerine bird-pollinated flowers produce nectar that is rich in AAs[9,10]. Floral nectar, for example for insects, serves as a dietary source of essential amino acids (EAAs), which are crucial for growth, somatic maintenance, and reproduction[11,12]. A recent analysis of functional flower trait diversity showed that among various floral characteristics, the concentration of nectar AAs is one of the most important traits shaping plant-pollinator interactions[13]. This may be due to the fact that as an essential source of nitrogen for mutualists nectar AAs[4] also contribute to the taste of the nectar and thus stimulate the chemosensory receptors of insects[2]. Furthermore, compounds such as proline are sources of short-term energy bursts and can be important in the first phases of insect flight[14,15]. Others, such as GABA ($\gamma$-aminobutyric acid), taurine and $\beta$-alanine, appear to influence insect behaviour by limiting excessive states of excitation during stressful conditions[16], or induce higer level of locomotion[17]. Also nonessential amino acids (NEAAs) may play an important role, incorporated from the adult diet into the eggs of butterflies may improve reproductive success of butterflies under suboptimal larval conditions[11].

The role played by nectar AAs extends beyond plant-pollinator interactions. For example, GABA, a non-protein AA (NPAA), may contribute to the protection of nectar from invasion by pathogenic organisms[8].

[1]Botanic Garden, Faculty of Biology, University of Warsaw, Warsaw, Poland. [2]Department of Plant Biochemistry and Toxicology, Institute of Biology, Faculty of Biology and Chemistry, University of Bialystok, Bialystok, Poland. [3]Feature Forest, Trzy Lipy 3, 80-172, Gdańsk, Poland. *email: k.roguz@biol.uw.edu.pl; mzych@biol.uw.edu.pl

1

Moreover, in contrast to the idea of the species-specific constancy of nectar AAs[9], recent studies show a considerable variability of AA composition within a species or among closely related taxa[18–22]. The above arguments demonstrate that floral nectar is much more than a simple food reward for animals. It is under pollinator-mediated selection and should rather be regarded as a complicated multifunctional interface between plants, their mutualists, and antagonists[3,5,8].

Unfortunately, non-sugar constituents of nectar are relatively understudied. This is partly because they constitute a small fraction of nectar, as well as exhibit methodological difficulties[8]. Moreover, little is known about phylogenetic constraints on nectar production. To the best of our knowledge only a handful of studies addressed the issue of the nectar AA profile of closely related taxa[20,21]. To that end, we undertook a broad systematic survey of nectar AAs for 38 species of the monocotyledonous genus *Fritillaria* L. (Liliaceae). It comprised of 100–140 species scattered in the Northern Hemisphere, with a substantial representation concentrated in the Mediterranean region, especially in Turkey, Greece, Iran, and Western North America[23].

Some members of the *Fritillaria* genus have been previously surveyed for nectar diversity[24–26]. These studies, however, only examined nectar sugars in selected members of the genus. Based on sugar nectar profiles, Rix and Rast[24] concluded that most *Fritillaria* species are putatively pollinated by bees and wasps. Indeed, floral visitors to *Fritillaria* flowers include Hymenoptera (mostly various species of bees and wasps), as well as many other taxonomic groups of insects, for example, Diptera, Lepidoptera, and Coleoptera[27–31]. Asiatic *F. imperialis* and some North American species (*F. gentneri* and *F. recurva*) appear to be predominantly bird-pollinated[32–35]. This, however, does not necessarily reflect the complete spectrum of pollination systems, since extensive studies of pollination biology have only been carried out on two *Fritillaria* species; namely *F. imperialis* and *F. meleagris*[23,31,32]. Furthermore, little information is available on the non-sugar nectar constituents of *Fritillaria*. Specifically, data on the AA profile is restricted to a single species, namely the *F. graeca*[36]. Our aim, therefore, was to explore the nectar diversity of a large sample of the *Fritillaria* species, representing various infrageneric taxa, as well as their geographic regions and pollination systems.

## Results

The nectar of all the studied species contained AAs. Thirty AA compounds or groups of AAs compounds in varying proportions were found in the floral nectar of studied fritillaries (Table 1). On average 23 different AAs were present in a single nectar sample.

In general, all the samples contained one to three dominant AAs, which altogether constituted at least 10% of the AAs per sample. A further two to six different AAs jointly constituted a fraction of 5–10%. The most abundant fraction, regarding the number of AAs, included 14–27 AAs and contributed <5% towards the total. We detected a single dominant AA in the nectars of *F. acmopetala*, *F. eduardii*, *F. meleagris*, namely proline (76%) and glutamine (89% and 66%), respectively for each species. In these cases, the fraction in the range of 5–10% was not detected (Supplementary materials: Table 1).

Glutamine was the most abundant AA in the majority of the studied samples (the mean value for all the samples was $4679 \pm 14348$ pmol/µL), it had the highest nectar share of all the studied species – on average $22.5\% \pm 16.6$ in all the studied samples. The EAAs needed by honeybees (methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, valine) were present in the nectar of all the studied species. The percentage of EAAs in the studied species varied between 2% and 51%, with a mean value of 14%. In case of insect-pollinated species, the range lay between 5–51% (mean 16%), for hummingbird-pollinated taxa 6–19% (mean 10%), while in passerine-pollinated 2–6% (mean 4%). Valine was the most common EAA present in 36 samples, followed by threonine (present in seven samples), phenylalanine (present in six samples), leucine (present in three samples), and methionine (present in one sample). The NEAAs (alanine, asparagine, glutamic acid, glutamine, glycine, proline, serine) were also present in all species. The percentage of NEAAs in the studied species varied between 24% and 67%, with a mean value of 59%. In case of insect-pollinated species, the range lay between 2–92% (mean 55%), for hummingbird-pollinated taxa 46–76% (mean 67%), while in passerine-pollinated 82–96% (mean 88%). The NEAAs were present in almost all samples, with the exception of glycine and proline, which were absent in the nectar of five studied species. (Supplementary Materials: Table 1).

BABA (β-Aminobutyric acid) was the rarest AA (the mean value for all the samples was $14.1 \pm 63$ pmol/µL), found in only nine species, and always present in very low concentrations (with a mean percentage value below 1% of the total concentration).

The species with the highest mean concentration of AAs was *F. eduardii* (62058 pmol/µL). The concentration of its AAs was more than 282 times higher than in *F. pallidiflora* (220.4 pmol/µL), the species with the lowest AAs concentration.

Both non-protein (NPAAs) and protein AAs (PAAs) were detected in all samples. The mean proportion of PAAs and NPAAs for all the studied samples was 21:4, and in 50 out of 53 samples the concentration of PAAs was higher. In *F. eduardii* and *F. imperialis* the PAAs usually constituted more than 99% of the total AAs. For *F. crassifolia* and *F. thunbergii*, the quantity of NPAAs was slightly higher than 50%. O-serine + histidine was the most common NPAAs among all the studied species. Two NPAAs, sarcosine and norvaline, were detected in the floral nectar for the first time.

We recorded significant differences between the concentration and proportion of AAs in the same species (the Wilcoxon signed-rank, $p < 0.05$) for most of the studied taxa. The differences were not statistically significant ($p > 0.05$) in *F. recurva*, and *F. acmopetala* (samples from various specimens). Therefore, in the case of these species in further analysis i.e. among species variability the mean values were used.

A comparison of closely related taxa, namely *F. recurva* and *F. affinis*, and their cross *F. gentneri*, also revealed significant differences ($p < 0.005$) in their concentration of AAs. There were also differences in the composition of AAs between parent plants and the hybrid species (*F. affinis* and *F. recurva* vs. *F. gentneri*).

| | ASP | GLU | ASN | SER | GLN | OSER+HIS | GLY | THR | CIT | ARG | BALA | ALA | TAU | GABA | BABA | TYR | AABA | CY2 | VAL | MET | NVA | TRP | PHE | ILE | ORN | LEU | LYS | HYP | SAR | PRO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Subgenus Fritillaria** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F. acmopetala | 56 | 96 | 55 | 113 | 348 | 13 | 152 | 135 | 187 | 20 | 17 | 97 | 15 | 19 | 0 | 40 | 6 | 5 | 312 | 0 | 4 | 4 | 39 | 148 | 16 | 39 | 5 | 6 | 56 | 6359 |
| F.amana | 132 | 399 | 77 | 140 | 95 | 180 | 264 | 244 | 19 | 24 | 10 | 282 | 0 | 22 | 77 | 0 | 52 | 9 | 188 | 34 | 0 | 11 | 325 | 75 | 95 | 0 | 19 | 3 | 18 | 7 |
| F.carica | 45 | 126 | 29 | 117 | 21 | 9 | 58 | 30 | 0 | 12 | 0 | 109 | 0 | 0 | 0 | 17 | 0 | 0 | 38 | 0 | 0 | 42 | 20 | 23 | 11 | 25 | 7 | 5 | 95 | 48 |
| F.crassifolia | 27 | 67 | 7 | 28 | 20 | 9 | 9 | 0 | 0 | 17 | 0 | 34 | 0 | 0 | 0 | 12 | 14 | 0 | 20 | 0 | 0 | 0 | 7 | 10 | 10 | 0 | 7 | 1 | 273 | 0 |
| F.drenovskii | 43 | 59 | 8 | 43 | 150 | 3 | 195 | 9 | 12 | 17 | 0 | 65 | 21 | 11 | 0 | 13 | 2 | 0 | 13 | 0 | 0 | 0 | 26 | 0 | 0 | 19 | 0 | 6 | 61 | 56 |
| F.ehrhartii | 60 | 553 | 76 | 17 | 348 | 9 | 0 | 46 | 0 | 61 | 0 | 57 | 0 | 25 | 0 | 14 | 30 | 0 | 49 | 0 | 0 | 43 | 32 | 18 | 18 | 18 | 0 | 33 | 435 | 84 |
| F.elwesii | 369 | 585 | 4512 | 550 | 2646 | 54 | 121 | 389 | 50 | 63 | 42 | 383 | 13 | 14 | 0 | 220 | 19 | 7 | 615 | 0 | 0 | 27 | 548 | 174 | 16 | 85 | 16 | 5 | 28 | 2419 |
| F.gracilis | 283 | 1770 | 238 | 101 | 3412 | 46 | 30 | 117 | 44 | 14 | 24 | 101 | 0 | 34 | 0 | 21 | 0 | 0 | 315 | 76 | 0 | 0 | 158 | 214 | 36 | 0 | 0 | 31 | 60 | 210 |
| F.graeca | 20 | 71 | 24 | 35 | 228 | 13 | 24 | 16 | 6 | 10 | 0 | 29 | 0 | 17 | 0 | 8 | 3 | 4 | 25 | 0 | 4 | 0 | 11 | 13 | 9 | 0 | 0 | 3 | 124 | 0 |
| F.kotschyana | 155 | 2888 | 94 | 278 | 2371 | 577 | 40 | 410 | 0 | 0 | 30 | 246 | 0 | 693 | 36 | 12 | 5 | 0 | 488 | 14 | 239 | 22 | 202 | 0 | 63 | 0 | 12 | 0 | 0 | 20 |
| F. lusitanica | 25 | 99 | 10 | 26 | 56 | 4 | 0 | 13 | 14 | 7 | 0 | 25 | 0 | 2 | 0 | 5 | 0 | 3 | 18 | 6 | 0 | 6 | 6 | 9 | 7 | 8 | 0 | 15 | 136 | |
| F.meleagris | 216 | 472 | 137 | 374 | 6581 | 46 | 0 | 0 | 30 | 88 | 0 | 479 | 63 | 77 | 0 | 48 | 0 | 35 | 358 | 63 | 0 | 31 | 165 | 298 | 0 | 155 | 11 | 16 | 82 | 173 |
| F. meleagroides | 210 | 728 | 35 | 281 | 133 | 17 | 643 | 61 | 32 | 37 | 4 | 348 | 61 | 77 | 0 | 30 | 0 | 30 | 129 | 246 | 10 | 31 | 26 | 43 | 4 | 41 | 4 | 11 | 86 | 140 |
| F.michai lovskyi | 159 | 223 | 26 | 170 | 370 | 14 | 885 | 44 | 30 | 32 | 2 | 252 | 99 | 29 | 0 | 25 | 1 | 28 | 217 | 213 | 3 | 9 | 39 | 156 | 3 | 105 | 14 | 16 | 477 | 185 |
| F.pallidiflora | 27 | 37 | 36 | 37 | 112 | 5 | 134 | 18 | 6 | 21 | 5 | 58 | 15 | 40 | 13 | 8 | 5 | 13 | 17 | 3 | 11 | 0 | 10 | 7 | 7 | 10 | 6 | 5 | 40 | 30 |
| F.pontica | 107 | 486 | 282 | 566 | 2040 | 1362 | 497 | 521 | 56 | 36 | 49 | 417 | 52 | 26 | 0 | 33 | 0 | 17 | 440 | 24 | 11 | 0 | 117 | 232 | 26 | 64 | 5 | 10 | 187 | 103 |
| F.pyrenaica | 56 | 51 | 23 | 146 | 145 | 16 | 96 | 31 | 0 | 53 | 0 | 83 | 0 | 175 | 0 | 0 | 0 | 39 | 952 | 0 | 36 | 60 | 0 | 17 | 25 | 0 | 127 | 16 | 102 | 48 |
| F.stribrnyi | 248 | 410 | 30 | 75 | 822 | 8 | 26 | 80 | 0 | 11 | 0 | 70 | 0 | 10 | 0 | 30 | 0 | 0 | 182 | 0 | 0 | 22 | 84 | 92 | 0 | 43 | 4 | 7 | 93 | 17 |
| F.thessala | 41 | 83 | 89 | 385 | 964 | 1916 | 78 | 0 | 36 | 18 | 27 | 112 | 0 | 26 | 436 | 0 | 0 | 11 | 333 | 21 | 0 | 29 | 27 | 278 | 48 | 0 | 0 | 5 | 132 | 87 |
| F.tubiformis | 222 | 852 | 552 | 1573 | 9621 | 98 | 182 | 595 | 6 | 44 | 96 | 656 | 0 | 123 | 0 | 43 | 21 | 0 | 1063 | 24 | 23 | 36 | 312 | 380 | 0 | 248 | 17 | 5 | 32 | 163 |
| F.ussuriensis | 79 | 844 | 146 | 17 | 788 | 515 | 0 | 0 | 13 | 3 | 5 | 28 | 0 | 26 | 0 | 15 | 0 | 0 | 550 | 0 | 0 | 15 | 104 | 211 | 0 | 38 | 2 | 19 | 35 | 12 |
| F. uvavulpis | 126 | 1561 | 47 | 313 | 34 | 51 | 256 | 37 | 0 | 9 | 0 | 509 | 0 | 63 | 23 | 0 | 12 | 117 | 364 | 14 | 12 | 150 | 77 | 0 | 147 | 0 | 20 | 22 | 54 | 37 |
| F. verticillata | 16 | 8 | 0 | 36 | 48 | 2 | 14 | 7 | 0 | 6 | 0 | 24 | 0 | 0 | 6 | 0 | 0 | 0 | 2 | 10 | 3 | 3 | 7 | 6 | 4 | 7 | 0 | 2 | 422 | 116 |
| F.whittallii | 46 | 154 | 35 | 49 | 641 | 34 | 15 | 32 | 0 | 0 | 46 | 103 | 0 | 0 | 0 | 20 | 0 | 5 | 72 | 0 | 0 | 22 | 52 | 1 | 18 | 4 | 6 | 1 | 82 | 11 |
| **Subgenus Japonica** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F.amabilis | 146 | 278 | 1112 | 138 | 1145 | 37 | 16 | 123 | 8 | 28 | 27 | 69 | 32 | 14 | 0 | 73 | 115 | 0 | 304 | 0 | 11 | 19 | 102 | 112 | 0 | 23 | 0 | 1 | 5 | 748 |
| **Subgenus Korolkowia** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F.sewerzowii | 87 | 166 | 45 | 100 | 824 | 6 | 465 | 24 | 24 | 31 | 17 | 172 | 53 | 43 | 32 | 24 | 0 | 22 | 43 | 5 | 41 | 0 | 22 | 18 | 15 | 54 | 0 | 8 | 61 | 196 |
| **Subgenus Liliorhiza** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F.affinis | 299 | 402 | 32 | 337 | 473 | 23 | 1817 | 103 | 41 | 67 | 0 | 560 | 106 | 42 | 0 | 38 | 16 | 39 | 70 | 0 | 0 | 0 | 53 | 37 | 27 | 106 | 0 | 70 | 625 | 336 |
| F. camt schatcensis | 298 | 322 | 18 | 378 | 437 | 17 | 1804 | 40 | 34 | 50 | 0 | 511 | 106 | 26 | 0 | 34 | 0 | 37 | 53 | 0 | 0 | 0 | 29 | 27 | 0 | 86 | 0 | 32 | 860 | 336 |
| F. eastwoodiae | 49 | 105 | 17 | 53 | 79 | 23 | 42 | 49 | 7 | 8 | 2 | 54 | 0 | 0 | 0 | 16 | 6 | 2 | 44 | 6 | 0 | 7 | 49 | 17 | 0 | 23 | 0 | 1 | 18 | |
| F.gentneri | 39 | 100 | 19 | 42 | 209 | 2 | 196 | 15 | 6 | 10 | 0 | 69 | 20 | 16 | 0 | 9 | 1 | 8 | 20 | 0 | 4 | 3 | 11 | 8 | 2 | 12 | 0 | 6 | 39 | 59 |
| F.recurva | 39 | 59 | 4 | 23 | 88 | 0 | 189 | 1 | 4 | 7 | 0 | 65 | 16 | 8 | 0 | 5 | 1 | 6 | 11 | 0 | 0 | 0 | 7 | 4 | 2 | 12 | 0 | 4 | 17 | 39 |
| **Subgenus Petilium** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F. eduardii | 179 | 368 | 114 | 1370 | 62058 | 47 | 348 | 403 | 18 | 13 | 44 | 2789 | 18 | 12 | 9 | 125 | 21 | 4 | 393 | 61 | 0 | 19 | 504 | 242 | 119 | 369 | 30 | 5 | 21 | 244 |
| F.imperialis | 211 | 1851 | 118 | 334 | 6261 | 16 | 272 | 164 | 52 | 18 | 45 | 1493 | 28 | 42 | 0 | 23 | 0 | 0 | 254 | 0 | 18 | 45 | 70 | 107 | 9 | 81 | 0 | 6 | 56 | 227 |
| F.raddeana | 54 | 196 | 43 | 103 | 719 | 14 | 0 | 70 | 55 | 26 | 0 | 160 | 0 | 9 | 0 | 25 | 0 | 10 | 75 | 0 | 0 | 22 | 40 | 37 | 0 | 44 | 0 | 0 | 50 | 103 |
| **Subgenus Rhinopetalum** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F. stenanthera | 76 | 194 | 0 | 83 | 65 | 25 | 0 | 70 | 16 | 19 | 0 | 215 | 0 | 0 | 0 | 20 | 28 | 11 | 60 | 0 | 0 | 27 | 33 | 51 | 62 | 31 | 0 | 11 | 753 | 119 |
| **Subgenus Theresia** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F.persica | 16 | 326 | 25 | 32 | 136 | 5 | 25 | 10 | 0 | 0 | 0 | 143 | 0 | 0 | 0 | 8 | 0 | 6 | 15 | 0 | 0 | 12 | 20 | 7 | 9 | 0 | 6 | 1 | 70 | 0 |
| **Other species** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| F.grandiflora | 189 | 790 | 1370 | 1937 | 7842 | 3966 | 444 | 1152 | 55 | 121 | 201 | 4347 | 36 | 132 | 0 | 141 | 37 | 82 | 2390 | 206 | 51 | 275 | 987 | 948 | 0 | 392 | 226 | 105 | 12 | 275 |

**Table 1.** Composition of amino acids (pmol/µL) in subgenera *Fritillaria, Japonica, Korolkowia, Liliorhiza, Petilium, Rhinopetalum, Theresia* and other species; ASP – Asparagine, GLU - Glutamic acid, ASN – Asparagine, SER – Serine, GLN – Glutamine, OSER + HIS – O-Serine + Histidine, GLY – Glycine, THR – Threonine, CIT – Citrulline, ARG – Arginine, BALA – β-Alanine, ALA – Alanine, TAU – Taurine, GABA - Gamma-Aminobutyric Acid, BABA - β-Aminobutyric acid, TYR – Tyrosine, AABA - α-Aminobutyric acid, CY2 – Cystine, VAL – Valine, MET – Methionine, NVA – Norvaline, TRP – Tryptophan, PHE – Phenylalanine, ILE – Isoleucine, ORN – Ornithine, LEU – Leucine, LYS – Lysine, HYP – Hydroxyproline, SAR – Sarcosine, PHE – Phenylalanine.
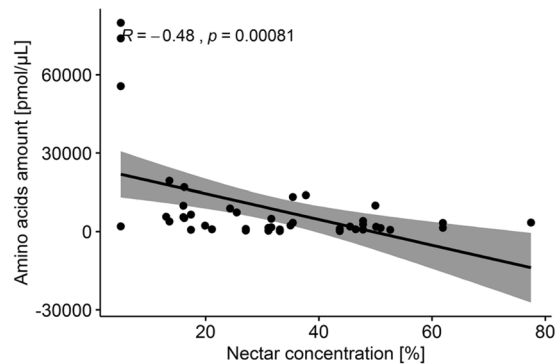
**Figure 1.** The correlation between the mean concentration of amino acids and nectar concentration (F-statistic: 2.2664, r = −0.48. p = 0.00081), an outlier points are for *F. eduardii*.

Pearson's correlation revealed negative correlation between sugar and AAs concentration (p < 0.00) and lack of correlation between nectar volume and AAs concentration (Fig. 1). PGLS analysis did not show correlation between nectar properties (sugar concentration and volume, values presented in Table 2) and the total concentration of AAs (P > 0.5).

The first two principal components of the AAs concentration, with the main pollinator and subgenera as explanatory variables, explained 69.2% and 60% (Fig. 2AB) of the total variance, respectively. A PERMANOVA was performed on the AAs composition. Pollinators, subgenera and main sugar were used as categorical variables to complement the graphics evaluation derived by PCA. They were highly significant (p < 0.05) between studied sections, with the exception of the main sugar type (p = 0.099) (Table 3).

Random forest analysis revealed a strong influence of phylogenetic affinity on the composition and concentration of AAs, resulting in 20 out of 25 samples correctly assigned to proper subgenus (with a class error value of 32.26%). The main pollinators type resulted in 23 out of 31 samples correctly assigned to proper subgenus (with a class error value of 25.8%). The variation in the profile of AAs was not explained by origin and the main sugar component of nectar (these saw class errors, respectively of 58% and 51.9%).

The phylogenetic signal was not present ($\lambda \ll 1$).

## Discussion

The mean number of different AAs found in *Fritillaria* nectar ([23]23), corresponds well with a similar analysis performed on other taxa[22,36–38]. All samples of fritillaries analyzed here contained NPAAs, while Baker and Baker[10] reported NPAAs to be present in only 36% of the samples, which took part in their extensive study. Such a discrepancy may be related to the applied techniques (the dansylation-UV fluorescence method vs. HPLC), alternatively it might be due to species-specific differences. Two NPAAs, sarcosine and norvaline, were detected in the floral nectar for the first time. A minimum of two and a maximum of nine NPAAs were found in the nectar of fritillaries. This resembles the results obtained for other species from other genera and families studied previously[22,36–39]. The mean concentration of AAs identified in the *Fritillaria* nectar was 8633 ± 16776 pmol/µL and proved to be relatively high when compared to species from other genera and families cited above.

Our study revealed that the total concentration and composition of AAs varies widely within and between the *Fritillaria* species. While a variation in the total concentration of AAs has been previously reported[9,22,39], the variability in composition is rather unexpected, especially in the case of specimens derived from the same location. There is only one study on supergeneralistic species *Angelica sylvestris* L. showing differences in nectar AAs composition within a species. In this case, however, samples were collected along an ~700-km transect, and the differences in AAs composition could be a result of "adaptive wandering" rather than related to pollination ecotypes adapted to local pollinator assemblages[22]. The mean value of the correlation between AAs composition from nectar samples of the same species, showing infraspecific variability, was r = 0.6 (88% of significant correlations), and was lower compared to the results presented by Gardener and Gillman[39]: namely r = 0.88 (all 544 obtained correlations were statistically significant). The low value of this factor, obtained in our study, may indicate that the species-specific AA composition is not fixed, and might be modulated by environmental factors rather than being determined by genetics alone[18,40].

Our results are in contrast to the work of Lohaus and Schwerdtfeger[41] who hypothesised that the avoidance of excessively high nitrogen loss by plants producing large nectar volumes might be achieved by a reduction in the AAs concentration. Both *Petilium* species, *F. imperialis* and *F. eduardii*, produce the highest nectar volumes of all the studied *Fritillaria* species[26], which also has a high AA concentration.

According to Baker and Baker[42], in case of hybridization the hybrid nectar is an 'additive', which means it contains each of the AAs of the parents, but does not contain any AAs that are not present in the parents' nectar. The nectar components of *F. gentneri* (a cross between *F. recurva* and *F. affinis*) may be regarded as an 'additive' only to some extent. There are two novel AAs not present in either of the parents' nectar. Moreover, few AAs present in both parental nectars are absent in the nectar of some hybrid flowers. The differences might be related to the fact that Baker and Baker's[42] plants were F1 hybrids, while *F. gentneri* is probably a well-established hybrid capable of sexual reproduction[34]. Detected differences might thus indicate an adaptation to pollinators, since *F. recurva* and *F. gentneri* are visited (and probably pollinated) by hummingbirds[34], whereas *F. affinis* is bee-pollinated.

| | OR | SUB | V | % | MS | FRU | GLU | SUC | POL | N/NF/NP | SOU | PIC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *F. acmopetala* Boiss. | EUR | FRI | 35 | 35 | FRU | 101 | 30 | 1 | BEE | 2/1/2 | BG | 1 |
| *F. affinis* (Schult. & Schult. f.) Sealy | AM | LIL | 15.4 | 13 | FRU | 222 | 144 | 14 | BEE | 1/1/1 | BG | 2 |
| *F. amabilis* Koidzumi | EUR | JAP | | | FRU | 216 | 118 | 158 | BEE | 1/1/1 | BG | 3 |
| *F. amana* (Rix) R. Wallis & R.B. Wallis | EUR | FRI | 8 | 48 | FRU | 145 | 11 | 364 | BEE | 1/1/1 | BG | 4 |
| *F. camtschatcensis* L. | AM/AS | LIL | NA | NA | GLU | 24 | 16 | 0.6 | FLY | 1/3/1 | BG | 5 |
| *F. carica* Rix | EUR | FRI | 14 | 21 | FRU:GLU | 163 | 153 | 25 | BEE | 1/1/1 | BG | 6 |
| *F. crassifolia* Boiss. & A. Huet | EUR | FRI | | | SUC | 29 | 33 | 55 | BEE | 1/1/1 | BG | 7 |
| *F. drenovskii* Degen & Stoj. | EUR | FRI | 36 | 27 | GLU | 164 | 189 | 1 | BEE | 2/1/2 | PK | 8 |
| *F. eastwoodiae* R.M. Macfarlane. | AM | LIL | 34 | 16 | FRU | 98 | 48 | 5 | BEE | 1/1/1 | CE | |
| *F. eduardii* A. Regel ex Regel | AS | PET | 57 | 5 | FRU | 15 | 4 | | PAS | 3/1/3 | BG | |
| *F. ehrhartii* Boiss. & Orph | EUR | FRI | 9 | 50 | NA | NA | NA | NA | BEE | 1/1/1 | BG | 9 |
| *F. elwesii* Boiss. | EUR | FRI | 51 | 38 | FRU | 103 | 39 | 93 | BEE | 1/1/1 | BG | 10 |
| *F. gentneri* Gilkey Madroño | AM | LIL | 54 | 31 | FRU | 267 | 155 | 104 | HUM | 2/1/2 | BG | 11 |
| *F. gracilis* Smiley | EUR | FRI | 54 | 26 | SUC | 36 | 3 | 124 | BEE | 1/1/1 | BG | |
| *F. graeca* Boiss. & Sprun. | EU | FRI | 10 | 53 | FRU | 98 | 86 | 33 | FLY | 1/1/1 | BG | 12 |
| *F. grandiflora* Grossh. | EUR | OTH | NA | | NA | NA | NA | NA | BEE | 1/1/1 | BG | |
| *F. imperialis* L. | AS | PET | 205 | 14 | FRU:GLU | 33 | 34 | 1 | PAS | 3/1/3 | PK | 12 |
| *F. kotschyana* Herbert | EUR | FRI | 55 | 24 | FRU | 97 | 37 | | BEE | 1/1/1 | BG | 13 |
| *F. liliacea* Lindl. | AM | LIL | 34 | 48 | NA | NA | NA | NA | BEE | 2/1/1 | CE | 14 |
| *F. lusitanica* Wikstr. | EUR | FRI | 51 | 9 | SUC | 42 | 5 | 109 | BEE | 1/1/1 | LH | 15 |
| *F. meleagris* L. | EUR | FRI | NA | 50 | FRU:GLU | NA | NA | NA | BEE | 1/1/1 | BG | 16 |
| *F. meleagroides* Patrin ex Schult. f | EUR | FRI | 39 | 32 | FRU | 146 | 34 | 64 | BEE | 2/1/2 | PK | 17 |
| *F. michailovskyi* Fomin | EUR | FRI | 4 | 17 | FRU | 76 | 31 | 55 | BEE | 2/1/2 | BG | 18 |
| *F. pallidiflora* Schrenk | AS | FRI | 26 | 44 | GLU | 147 | 158 | 72 | BEE | 3/1/3 | BG | 19 |
| *F. persica* L. | EUR | THE | 4 | 47 | GLU | 215 | 570 | 41 | BEE | 1/1/1 | BG | 20 |
| *F. pontica* Wahlenb. | EU | FRI | 6 | 16 | FRU | 54 | 28 | 9 | BEE | 2/1/2 | BG | 21 |
| *F. pyrenaica* L. | EU | FRI | 52 | 20 | SUC | 74 | 29 | 98 | FLY | 1/1/1 | BG | 22 |
| *F. raddeana* Regel. | AS | PET | 9 | 50 | SUC | 99 | 69 | 119 | BEE | 1/1/1 | BG | 23 |
| *F. recurva* Benth. | AM | LIL | 49 | 33 | FRU | 95 | 54 | 15 | HUM | 3/1/3 | BG | |
| *F. sewerzowii* Regel. | EUR | KOR | 25 | 62 | FRU:GLU | 140 | 140 | | BEE | 2/1/2 | BG | 24 |
| *F. stenanthera* (Regel) Regel | AS | THE | 1 | 46 | GLU | 70 | 139 | 10 | BEE | 1/1/1 | BG | 25 |
| *F. stribrnyi* Velen. | EU | FRI | 7 | 35 | NA | NA | NA | NA | BEE | 1/1/1 | BG | 26 |
| *F. thessala* (Boiss.) Kamari | EUR | FRI | 14 | 16 | SUC | 40 | 5 | 89 | BEE | 1/1/1 | BG | 27 |
| *F. tubiformis* Gren. & Godr. | EUR | FRI | 1 | 46 | SUC | 57 | 34 | 72 | BEE | 1/1/1 | BG | |
| *F. ussuriensis* Maxim. | EUR | FRI | 3 | 78 | SUC | 87 | 7 | 283 | FLY | 1/1/1 | BG | 28 |
| *F. uva vulpis* Rix | EUR | FRI | 12 | 48 | GLU | 90 | 99 | 53 | BEE | 2/1/2 | BG | 30 |
| *F. verticillata* Willd. | AS | FRI | 1 | 17 | GLU | 374 | 407 | 1 | BEE | 1/1/1 | BG | 31 |
| *F. whittallii* Baker | EUR | FRI | 14 | 51 | FRU | 186 | 52 | 167 | BEE | 1/1/1 | BG | 32 |

**Table 2.** Overview of all examined *Fritillaria* species showing OR – origin of the plants (EUR – Europe, AM – North America, AS – Asia), SUB – subgenera (JAP – *Japonica*, FRI – *Fritillaria*, KOR – *Korolkovia*, LIL – *Liliorhiza*, THE – *Theresia*); some of their main features (v – mean nectar volume produced in one flower, % - mean nectar concentration, MS – main nectar sugar type, FRU, GLU, SUC – amount of different sugar types (mg/ml) following information in Roguz *et al*. 2018), POL – main pollinator type derived from literature data, N/NF/NP – number of samples/number of flowers used for one sample/number of plants used to obtain nectar samples, SOU – source of samples, PIC – corresponding picture.

Furthermore, the lack of 'additiveness' and the appearance of 'new' AAs means that AAs profiles are not predictive of *Fritillaria* phylogeny, and contrary to Baker and Baker[42] environmental influence is more significant than genetic control. This aspect of nectar biology, however, deserves further attention.

Baker and Baker's[42] study discovered that AAs are universally present and follow a species-specific pattern. Similarly to carbohydrates, nectar AAs can play an important role in the attraction of pollinators[6,10,20,43,44], as they constitute an important nitrogen source for several animal groups[20,45]. The adaptive significance of floral nectar (and its AAs composition) is reflected by its ability to attract potential pollinating agents[20,46,47]. However, pollinators searching for nectar would usually probe multiple flowers[19] and the nectar composition variability between individuals of one species may be perceived as random noise[19,48]. Therefore, even in the case of pollinators' preference for a particular nectar composition, the selective pressure on a favoured floral nectar type would be hampered by the high variation within co-flowering individuals[19].

Nevertheless, the influence of potential pollinators should not be neglected as their flower choice seems to be a key element in the debate concerning the ecological role of AAs in floral nectar[49]. Results from previous studies
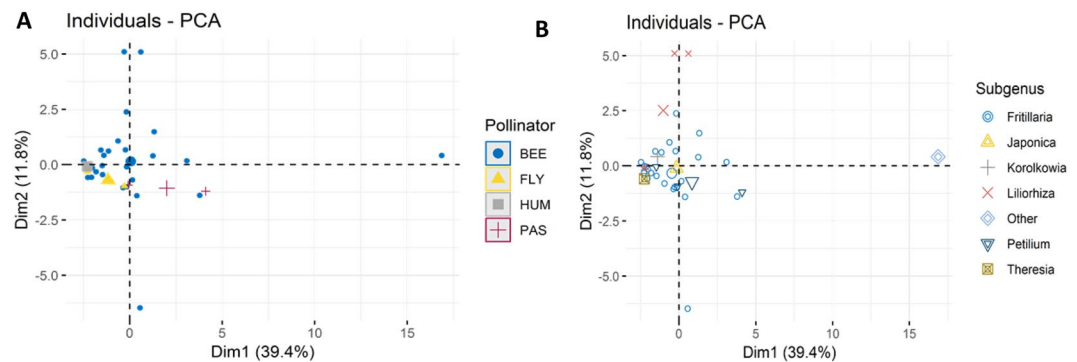
**Figure 2.** Scatterplot of PCA, (**A**) – data grouped by pollinator, (**B**) – data grouped by subgenus identity.

| PERMANOVA | Df | F | $R^2$ | *p* |
|---|---|---|---|---|
| **AAs** | | | | |
| Pollinators | 5 | 2.85 | 0.23 | 0.001*** |
| Subgenera | 6 | 1.98 | 0.21 | 0.001*** |
| Main sugar | 3 | 1.4 | 0.09 | 0.099 |
| Origin | 4 | 1.47 | 0.11 | 0.06 |

**Table 3.** Results of the PERMANOVA: Degrees of freedom (Df). pseudo-F(F). $R^2$ and p value.

demonstrate the prevailing importance of direct reward measurements, indicating that AAs concentration is one of the most important traits to shape plant-insect interaction[13]. One of the studies of AAs composition – of floral nectars in a phryganic community – revealed that flower-visiting insects were the most decisive players in shaping nectar chemical composition[36]. This might be related to the fact that insects are a group of pollinators that strongly rely on a flowers' food source, which results in a strong coevolution of nectar and insect-pollinators[6,39]. This seems to be the case in *Fritillaria* pollinated by different groups of animals. Our studies revealed substantial differences in the nectar composition of fritillaries, even among closely related species. This suggests an adaptation to potential pollinators. These differences relate not only to AA compounds but also to carbohydrates[26].

Although data concerning pollination in *Fritillaria* is scarce, nectar properties and nectary location indicate that bees are most likely the commonest pollinator of *Fritillaria* flowers[26]. Therefore, we expected the nectar of fritillaries to be rich in proline, which is oxidized in the insects' flight muscle, especially during the first phase of flight[15]. Moreover, proline is a type of AA, which accumulates at a high concentration in nectars of many angiosperm species[2,4,7,50,51], regardless of their phylogenetic distances[8,51]. Since insects have the ability to taste proline and favour proline-rich nectar, they are likely to impose selective pressure on plant species producing proline-containing nectars[14]. Surprisingly, in the case of fritillaries proline was the most abundant AA in only a single insect-pollinated species, namely *F. acmopetala*, and the share of this AA in the nectar of other *Fritillaria* was relatively low. If present, the quantity of proline in the nectar of insect-pollinated species was variable and ranged from 7 to 6359 pmol/μL. Such low concentration of proline may result from the fact that proline is metabolically more expensive than other nectar compounds[14]. Phylogenetic constraints may also play a role for some subgenera, as some closely related species have a similar concentration of proline. These include *F. verticillata* and *F. pallidiflora*. Phenylalanine, which is also one of the EAAs, generates a strong phagostimulatory effect on bees[36], and was also present in small concentration in the nectar of the bee-pollinated *Fritillaria* species (with a mean ratio of 2%). From the three most abundant AAs in *Fritillaria* nectar i.e., glutamine, glutamic acid, and glycine, only the role of glycine is known. This AA has a strong influence on honey bee-learning behaviour[52]. As for the other two AAs, these seem to be consistent and sizable nectar components of certain species[18,39]. However, the evaluation of their role in plant-pollinator interaction must await further study. We still lack evidence, but the *Fritillaria* reward composition may be related to the pollinator's life cycle. Fritillaries are spring–flowering plants. They flower almost immediately after the snow melts and are thought to be Queen bee pollinated[23]. The AAs requirements of *Fritillaria* pollinators could thus be very specific, for instance, for reproduction.

We also lack studies on the metabolism and ecological role of NPAAs. However, an 'ecophysiological' picture has emerged[8]. Proline is utilized during the first phase of insect flight, while nectar sugars propel long-distance flights, and taurine, GABA and β-alanine increase the efficiency of flight muscles. Moreover, proline and GABA increase the insect's appetite for nectar due to phagostimulatory activity[7]. Surprisingly, β-alanine is not present in *Fritillaria* nectar, while taurine is present only rarely, and GABA is found in low concentration. We found two NPAAs, sarcosine and norvaline, for the first time in floral nectar, but its influence on pollinators is not known.

Some studies have revealed a trade-off between carbohydrate quantity and AAs abundance. This carbohydrates-to-AAs ratio could play a functional role as, for example, it has been demonstrated that honey bees would rather acquire essential AAs than sugars[4,53]. Our study showed a similar trend for *Fritillaria*, but the results were statistically non-significant. Moreover, not all EAAs were detected in the nectar of melittophilous species. However, since pollen seems to represent an additional source of nitrogen and AAs, bees do not appear to solely

rely on nectar to supply these substances[21]. In this case the relative abundance of the different AAs (including the essential ones), could play an important role in providing potential pollinators with specific taste-information used in the field for food resource selection[54].

Similarly to butterfly-pollinated flowers, a high level of AAs was also described in flowers pollinated by carrion flies[3,10,45]. Flesh flies have been known to select nectar containing a mixture of AAs[55]. Six EAAs (i.e. valine, leucine, isoleucine, methionine, phenylalanine, tryptophan) elicited a feeding response by stimulating flies' chemoreceptors[56]. Flies were reported to visit flowers of *F. camtschatcensis*, however, contrary to previous results obtained by Baker and Baker[10] for other fly-pollinated species, the relative concentration of AAs in *F. camtschatcensis* nectar was considerably lower than the mean value. Moreover, the six AAs that caused a feeding response in flies were also hardly present in the nectar of this species.

The phylogeny[57] and field observation of the *Fritillaria* pollination system suggests that there have been at least two transitions from entomophily to passerine or hummingbird pollination[25,26,32,33,58]. These transitions involve several floral modifications, and are accompanied by changes in nectar volume and concentration, as well as sugar composition[26]. Previous studies of Baker and Baker[10] indicate, that AAs content may also change due to a pollinator shift. *Fritillaria imperialis*[32,33], and the closely related *F. eduardii*, both likely pollinated by passerine birds[26], were indeed reported to have a distinct AAs concentration and composition. The total concentration of AAs in the nectar of these two species was higher than the mean value, which is in accordance with several other observations of passerine pollinated species[2,10,21,45]. Such a high AAs concentration in bird-pollinated flowers may also have a repellent character, since a hymenoptera dominated pollinator community avoids high AAs concentration in floral nectar[13].

Similarly to the passerine-pollinated *Erythrina* species[2], glutamine, in bird-pollinated *Fritillaria*, occurred in much higher concentrations. Although EAAs were found to be commonly present in passerine-pollinated species[2], they are virtually absent in the nectar of both *F. imperialis* and *F. eduardii*. NEAAs are the main drivers of the variable concentration in fritillaries. Interestingly, the third member of the subgenus *Petilium*, *F. raddeana*, produced nectar with a lower concentration of AAs and higher percentage of both EAAs and PAAs. All the above-mentioned facts seem to indicate that the non-sugar components of nectar may play an important role in the plant-pollinator interactions[2,59]. A high AAs concentration with low nectar sugar concentration may play the role of a phenotypic filter, deterring illegitimate pollinators and antagonists, since various insects have shown a distaste for a high concentration of AAs. This could be masked by a higher concentration of carbohydrates[4].

In the case of *F. gentneri* and *F. recurva*, which are both hummingbird-pollinated species, the total AAs concentration was 11 times lower than the mean value counted for all studied species. This dichotomy of AAs concentration in ornithophilous species, with a high concentration in passerine-pollinated species and lower concentrations in hummingbird-pollinated species, has already been described in other taxa[2,10,21,45], as well as experimental studies[60]. Low AAs concentration in hummingbird-pollinated flowers may also have a repellent effect, aiding avoidance of competition with bees favouring higher AAs concentrations[3,10,45]. On the other hand, the shortage of AAs in a bird's diet could be overcome via additional food sources, e.g. insects[61]. Similarly to subgenus *Petilium*, we found differences in AAs concentration and composition in the nectar of closely related species in the subgenus *Liliorhiza*. *Fritillaria affinis*, *F. eastwoodiae*, *F. liliacea* produced nectar with a higher AAs concentration. Furthermore, a higher concentration of bee-preferred proline was present in species of this subgenus presumed to be insect-pollinated, which also indicates a strong influence of pollinators.

The influence of colonizing microorganisms on AAs, and their effect on plant-pollinator interaction, is also an important factor[51,62,63]. However, due to difficulties in obtaining nectar samples we could not study this aspect of *Fritillaria*. Nevertheless, we assume that pending flowers of most *Fritillaria* species are a kind of protection from microorganisms transported with the air, and therefore can reduce the number of nectar-inhabiting microorganisms. However, further research is needed before drawing any strong conclusions in this regard. Other interesting aspects for future AAs studies include the impact of non-standard, psychoactive AAs on pollinators[7,64]. Moreover, since the AAs composition in nectar correlates with AAs composition in phloem sap[41], the next step in studying nectar variability would be to examine to what extent the differences between and within the species are correlated with the composition of phloem sap versus influenced by a pollinator.

AAs concertation and composition in *Fritillaria* may be influenced by several factors. While phylogeny plays a role, as several closely related species have a similar AAs composition and concentration, the prevailing evidence is that AA composition and concentration can be variable even within a single species, as well as between closely related taxa. This is in agreement with the results of Gijbels *et al.*[19] and Lanza *et al.*[18], who found differences in the concentration and composition of AAs at the species level. Glutamine, glutamic acid, and glycine were the most abundant AAs in the nectar of the *Fritillaria* species analyzed in this study, but further analysis is needed to assess the potential role of these AAs. However, our results contradict other studies, which suggest that proline is the most abundant AA in floral nectar[9,36,65].

The nectar traits studied can be subject to selection, which is imposed by potential pollinators, a finding confirmed by other researchers[36,66,67]. Changes in AAs concentration and composition might play an important role in attracting new floral visitors in case of a pollinator shift. Our analysis revealed high concentration of AAs in passerine bird-pollinated species, and very low AAs concentration in hummingbird-pollinated species. These tendencies were not reflected in closely related species from the same subgenus.

## Material and Methods

**Taxon sampling.** Nectar samples used for this study were obtained from the *Fritillaria* species cultivated at the University of Warsaw Botanic Garden (hereafter BG) and in the private collections of Colin Everett (Somerton, Somerset, UK; hereafter CE), Laurence Hill (Richmond, Surrey, UK; hereafter LH) and Paweł Kalinowski (Szczeglacin, Korczew, Poland; hereafter PK) (Fig. 3). Most of the *Fritillaria* species are rare in
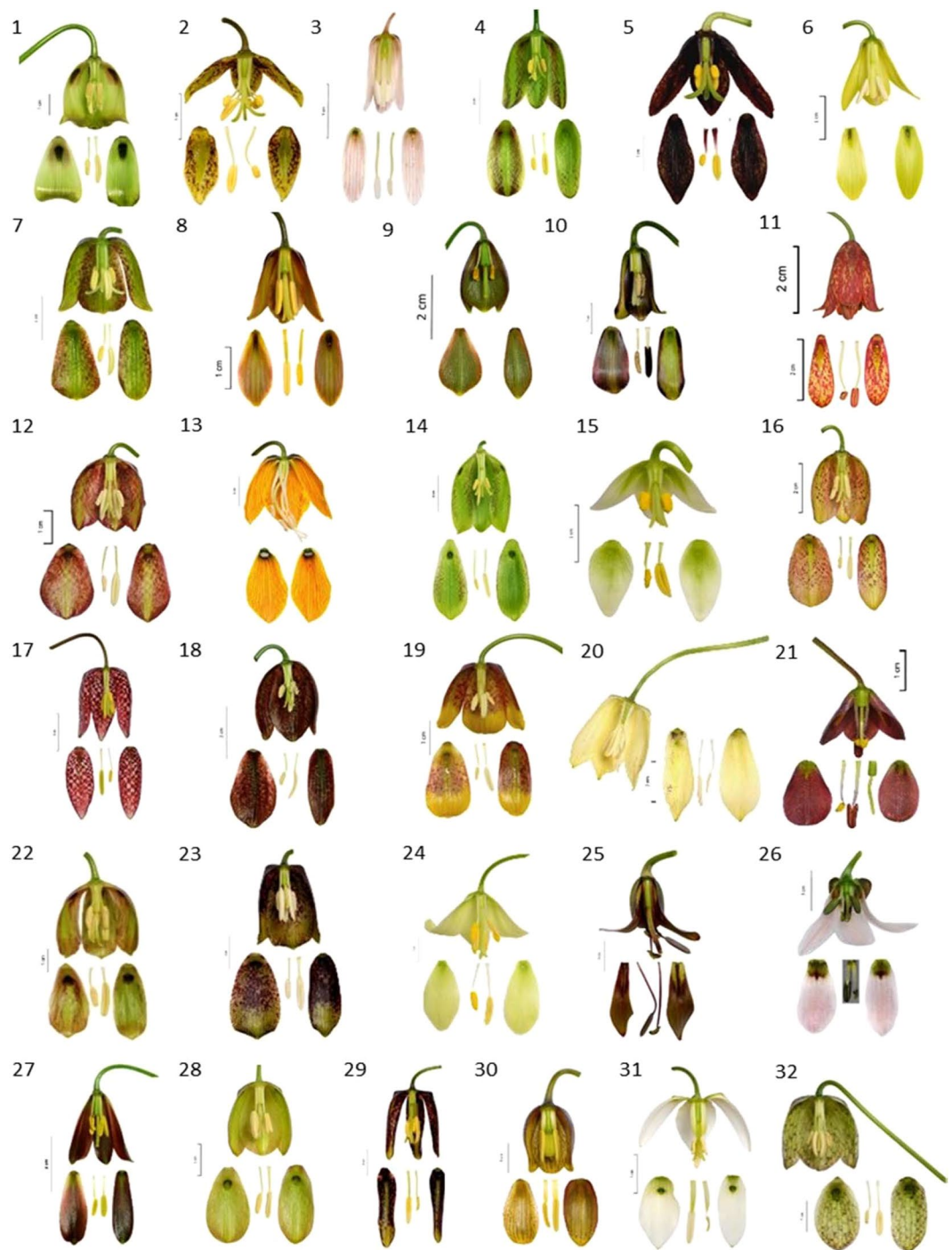
**Figure 3.** Flowers and nectaries of selected *Fritillaria* species in full anthesis (species' names in Table 2).

cultivation, and the number of specimens used in the study varied due to the availability of flowers or their nectar (the accession numbers for species used in this study are listed in Table 2). Flowers in the collections of BG and PK were first selected at the bud stage (flowers still closed) and bagged with nylon mesh (net size 0.5 mm) to prevent visits by insects. In all cases the nectar was sampled at the anthesis stage before the anthers had dehisced. In case of LH's and CE's collections, nectar sampling was performed on unbagged flowers exposed to animal visitors (possible contamination of floral nectar with pollen grains during insects visits). Due to possible influence of potential flower visitors in the case of unbagged flowers, these species were excluded (*F. liliacea, F. eastwoodiae, F. lusitanica*) from analyzis.

All the available nectar was sampled with microcapillary pipettes from the nectaries of all six tepals and treated as one sample per flower. In the case of *F. camtschatcensis*, where nectar volumes were very small, samples were taken under a Nikon SMZ 1000 stereomicroscope (Nikon Corp., Japan). For species producing small

quantities of nectar several flowers of the same specimen were used to collect it. Only the *F. liliacea* samples taken from different flowers of a single specimen were considered to be separate samples. During sampling we tried to avoid possible pollen, phloem sap or other plant tissue contamination, since it could affect the obtained results by releasing additional AAs[68–70]. All the nectar samples were collected around noon (between 11am and 1 pm). The collected nectar was frozen at −20 °C in 1.5 ml Eppendorf tubes prior to analysis.

**Nectar AA composition analysis.**     The composition of the nectar's AAs was analyzed using high-performance liquid chromatography (HPLC). The samples were frozen (−20 °C) until determination. After thawing the samples to an ambient temperature the nectar was diluted to a volume of 20 μL (10 μL of nectar was mixed with 10 μL of distilled water). The sample was filtered through a spin column with a 0.4 μm pore size membrane filter (A&A Biotechnology, Poland) before injection by centrifugation for 2 min at 9000 g (relative centrifugal force). The supernatant was loaded into the insert and analyzed by a HPLC. The samples were analyzed using an Agilent Technologies 1260 Infinity series system consisting of a 1260 Infinity Agilent Quaternary pump G1311B, a 1260 Infinity Diode Array Detector (DAD) G1315D, a 1260 Infinity Fluorescence Detector (FLD) G1321B, a 1260 Infinity ALS G1329B Automated Sample Injector, a 1290 Infinity Autosampler Thermostat G1330B and a thermostatted column oven 1290 Infinity TCC G1316C. The system was controlled by Agilent OpenLab ChemStation software. The analysis of AAs in 10 μL aliquots of nectar collected from flowers was performed by gradient HPLC using an Agilent Zorbax Eclipse Plus C18 (4.6 × 150 mm, 5 μm) column with a guard, i.e. Agilent Zorbax Eclipse Plus C18 (4.6 × 12.5 mm, 5 μm). The extracts, containing primary and secondary AAs were pre-column derivatized with *o*-phtalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) reagent. An injector program was used for the derivatization. Following derivatization, a mixture of each sample was injected into a pre-equilibrated column operated at 40 °C. The primary (OPA-derivatized) AAs were monitored at 388 nm by DAD while the secondary (FMOC-derivatized) AAs were monitored by FLD, at an excitation wavelength of 266 nm and an emission wavelength of 305 nm. Mobile phase A was 40 mM $NaH_2PO_4$ (pH 7.8 adjusted using 10 M NaOH solution), while mobile phase B was acetonitrile:methanol:water (45:45:10. v/v/v). The following gradient profile was seen: 0–5 min: 0% B t- 10% B; 5- 25 min: 10% B - 40.5% B; 25–30 min: 40.5% B - 63% B; 30–35 min: 63% B - 82% B; 35–37 min: 82% B - 100 B; 37–39 min: 100% B; 39–40 min: 100% B- 0% B; 40 43 min: 0% B. A flow rate of 1 mL/min was used. The total analysis time was 43.0 min.

**Data analysis.**     The total concentration of AAs was determined for each species. If several samples were obtained the results were used in the analyzis as separate records. The percentage of EAAs, non-protein amino acids (NPAAs), and protein amino acids (PAAs) was calculated. The Wilcoxon signed-rank test was applied to determine significant differences between the AAs composition in samples from the same or very closely related species. The correlations between nectar properties (concentration of sugars and volume) were calculated using the Pearson's product moment correlation coefficient. Additionally, the phylogenetic generalized least squares (PGLS) correlation was calculated, since this approach accounts for independent variables[71].

A principal component analysis (PCA) was performed and plotted against subgenera and the main pollinator, to visualize the differences in the concentration of AAs. The data on pollinators (see Table 1) was gained from the available literature[27,29–34,72–74], personal field observations (*F. michailovskyi, F. raddeana, F. carica, F. pontica, F. sewerzowii, F. ussuriensis, F. uva-vulpis F. persica, F. acmopetala, F. pallidiflora*), or based on the morphological adaptations of *Fritillaria* flowers. In the latter case, predictions of the pollinators syndrome were made from the following flower characteristics: corolla shape, size, colour, position on a stem, and nectar sugar composition and concentration[26].

A permutational multivariate analysis of variance (PERMANOVA) was conducted to identify the relative importance of all the sugar concentrations, pollinators, subgenera, and origin of the species. The Adonis routine was used for this purpose (it offers a multivariate analysis of variance using distance matrices based on the permutation test). 'Random forest' analysis was used to test whether nectar AAs differed between subgenera and the pollinators type and origin[75]. This machine-learning algorithm allows the assignment of nectar samples to pre-defined groups of subgenera and a pollinator type and origin. Random forest returned a confusion matrix showing the number of correctly assigned samples for each request.

The maximum likelihood (ML) tree was inferred with the use of the matK sequences obtained from GenBank. The fast bootstrap method implemented by IQ-TREE 1.6.8 was used for ML bootstrap analysis[76]. Lengths of tree branches were obtained from a ML tree of the *Fritillaria* genus. The phylogenetic correlation λ was calculated to assess the phylogenetic signal in the analyzed data. Pagel's λ is the transformation of the phylogeny, ensuring the best fit of studied trait data to a Brownian Motion model[77,78].

All the statistical analysis was performed using[79] (version 3.3.5. www.r-project.org.).

## Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## References
1. Willmer, P. *Pollination and floral ecology*. (Princeton University Press, 2011).
2. Nicolson, S. W. & Thornburg, R. W. In *Nectaries and nectar* (eds Nicolson, S. W. & Nepi, M.) 215–264 (Springer, 2007).
3. Heil, M. Nectar: generation, regulation and ecological functions. *Trends Plant Sci* **16**, 191–200, https://doi.org/10.1016/j.tplants.2011.01.003 (2011).

4. Roy, R., Schmitt, A. J., Thomas, J. B. & Carter, C. J. Review: Nectar biology: From molecules to ecosystems. *Plant Science.* **262**, 148–164, https://doi.org/10.1016/j.plantsci.2017.04.012 (2017).

5. Parachnowitsch, A. L., Manson, J. S. & Sletvold, N. Evolutionary ecology of nectar. *Annals of Botany* **123**, 247–261, https://doi.org/10.1093/aob/mcy132 (2018).

6. Baker, H. G. & Baker, I. In *Hadbook of experimental pollination ecology* Vol. 117–141 (eds Jones, C. & Little, R.) (Van Nostrand Reinhold, 1983).

7. Nepi, M. Beyond nectar sweetness: The hidden ecological role of non-protein amino acids in nectar. *Journal of Ecology* **102**, 108–115, https://doi.org/10.1111/1365-2745.12170 (2014).

8. Nepi, M. *et al.* Amino acids and protein profile in floral nectar: Much more than a simple reward. *Flora: Morphology, Distribution, Functional Ecology of Plants* **207**, 475–481, https://doi.org/10.1016/j.flora.2012.06.002 (2012).

9. Baker, H. G. & Baker, I. The occurrence and significance of amino acids in floral nectar. *Plant Systematics and Evolution* **151**, 175–186, https://doi.org/10.1007/BF02430273 (1986).

10. Baker, H. G. & Baker, I. In *Biochemical aspects of evolutionary biology* (ed. Nitecki, M.) 131–171 (University of Chicago Press, 1982).

11. Mevi-Schütz, J. & Erhardt, A. Amino acids in nectar enhance butterfly fecundity: A long-awaited link. *The American Naturalist* **165**, 411–419, https://doi.org/10.1086/429150 (2005).

12. Paoli, P. P. *et al.* Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids* **46**, 1449–1458, https://doi.org/10.1007/s00726-014-1706-2 (2014).

13. Fornoff, F. *et al.* Functional flower traits and their diversity drive pollinator visitation. *Oikos* **126**, 1020–1030, https://doi.org/10.1111/oik.03869 (2017).

14. Carter, C., Shafir, S., Yehonatan, L., Palmer, R. G. & Thornburg, R. A novel role for proline in plant floral nectars. *Naturwissenschaften* **93**, 72–79, https://doi.org/10.1007/s00114-005-0062-1 (2006).

15. Teulier, L., Weber, J. M., Crevier, J. & Darveau, C. A. Proline as a fuel for insect flight: enhancing carbohydrate oxidation in hymenopterans. *Proceedings of the Royal Society B: Biological Sciences* **283**, https://doi.org/10.1098/rspb.2016.0333 (2016).

16. Stevenson, P. A. Colocalisation of taurine- with transmitter-immunoreactivities in the nervous system of the migratory locust. *Journal of Comparative Neurology* **404**, 86–96, doi:10.1002/(SICI)1096-9861(19990201)404:1<86::AID-CNE7>3.0.CO;2-8 (1999).

17. Felicioli, A. *et al.* Effects of nonprotein amino acids on survival and locomotion of *Osmia bicornis. Insect Molecular Biology* **27**, 556–563, https://doi.org/10.1111/imb.12496 (2018).

18. Lanza, J., Smith, G. C., Sack, S. & Cash, A. Variation in nectar volume and composition of Impatiens capensis at the individual, plant, and population levels. *Oecologia* **102**, 113–119, https://doi.org/10.1007/BF00333318 (1995).

19. Gijbels, P., Van den Ende, W. & Honnay, O. Landscape scale variation in nectar amino acid and sugar composition in a Lepidoptera pollinated orchid species and its relation with fruit set. *Journal of Ecology* **102**, 136–144, https://doi.org/10.1111/1365-2745.12183 (2014).

20. Antoń, S., Komoń-Janczara, E. & Denisow, B. Floral nectary, nectar production dynamics and chemical composition in five nocturnal *Oenothera species* (Onagraceae) in relation to floral visitors. *Planta* **246**, 1051–1067, https://doi.org/10.1007/s00425-017-2748-y (2017).

21. Tiedge, K. & Lohaus, G. Nectar sugars and amino acids in day- and night-flowering *Nicotiana* species are more strongly shaped by pollinators' preferences than organic acids and inorganic ions. *PLoS ONE* **12**, 1–26, https://doi.org/10.1371/journal.pone.0176865 (2017).

22. Zych, M. *et al.* Spatiotemporal variation in the pollination systems of a supergeneralist plant: is *Angelica sylvestris* (Apiaceae) locally adapted to its most effective pollinators? *Annals of Botany*, https://doi.org/10.1093/aob/mcy140 (2019).

23. Zych, M., Stpiczyńska, M. G. & Roguz, K. Pollination Biology and Breeding System of European Fritillaria meleagris L. (Liliaceae). *Reproductive Biology of Plants*, 147–163, https://doi.org/10.1201/b16535-9 (2014).

24. Rix, E. M. & Rast, D. Nectar sugars and subgeneric classification in *Fritillaria. Biochemical Systematics and Ecology* **2**, 207–209, https://doi.org/10.1016/0305-1978(75)90063-0 (1975).

25. Stpiczyńska, M., Nepi, M. & Zych, M. Secretion and composition of nectar and the structure of perigonal nectaries in *Fritillaria meleagris* L. (Liliaceae). *Plant Systematics and Evolution* **298**, 997–1013, https://doi.org/10.1007/s00606-012-0609-5 (2012).

26. Roguz, K. *et al.* Functional diversity of nectary structure and nectar composition in the genus *Fritillaria* (Liliaceae). *Frontiers in Plant Science* **9**, 1246, https://doi.org/10.3389/fpls.2018.01246 (2018).

27. Hedström, I. Kungsängsliljans pollinationsekologi. *Svensk Bot Tidskr* **77**, 313–325 (1983).

28. Kawano, S., Masuda, J. & Hayashi, K. Life-history monographs of Japanese plants. 10: *Fritillaria koidzumiana* Ohwi (Liliaceae). *Plant Species Biology* **23**, 51–57, https://doi.org/10.1111/j.1442-1984.2008.00208.x (2008).

29. Zox, H. & Gold, W. Ecology of black liliy (*Fritillaria camschatcensis*) a Washington State sensitive species. *Douglasia Fall*, 17–22 (2008).

30. Zych, M. & Stpiczyńska, M. Neither protogynous nor obligatory out-crossed: Pollination biology and breeding system of the European Red List *Fritillaria meleagris* L. (Liliaceae). *Plant Biology* **14**, 285–294, https://doi.org/10.1111/j.1438-8677.2011.00510.x (2012).

31. Zych, M., Goldstein, J. & Roguz, K. The most effective pollinator revisited: pollen dynamics in a spring-flowering herb. 315–322, https://doi.org/10.1007/s11829-013-9246-3 (2013).

32. Búrquez, A. Blue tits, *Parus caeruleus*, as pollinators of the crown imperial, *Fritillaria imperialis*, in Britain. *Oikos* **55** (1989).

33. Peters, W. S., Pirl, M., Gottsberger, G. & Peters, D. S. Pollination of the Crown Imperial *Fritillaria imperialis* by Great Tits *Parus major. Journal of Ornithology* **136**, 207–212 (1995).

34. Pendergrass, K. & Robinson, A. Recovery plan for *Fritillaria gentneri* (Gentner's fritillary) (2005).

35. Cronk, Q. & Ojeda, I. Bird-pollinated flowers in an evolutionary and molecular context. *Journal of Experimental Botany* **59**, 715–727, https://doi.org/10.1093/jxb/ern009 (2008).

36. Petanidou, T., Van Laere, A., N. Ellis, W. & Smets, E. What shapes amino acid and sugar composition in Mediterranean floral nectars? *Oikos* **115**, 155–169, https://doi.org/10.1111/j.2006.0030-1299.14487.x (2006).

37. Gottsberger, G., Arnold, T. & Linskens, H. F. Intraspecific variation in the amino acid content of floral nectar. *Botanica Acta, Journal of the German Botanical Society* **102**(2), 141–144 (1989).

38. Piechowski, D. Reproductive Ecology, Seedling Performance, and Population Structure of Parkia pendula in an Atlantic Forest Fragment in Northeastern Brazil. *Structure* **46**, 203–204 (2007).

39. Gardener, M. C. & Gillman, M. P. Analyzing variability in nectar amino acids: Composition is less variable than concentration. *Journal of Chemical Ecology* **27**, 2545–2558, https://doi.org/10.1023/A:1013687701120 (2001).

40. Gilliam, M. *et al.* Amino acids in the floral nectar of cotton. *Apidologie* **12**, 125–132 (1981).

41. Lohaus, G. & Schwerdtfeger, M. Comparison of sugars, iridoid glycosides and amino acids in nectar and phloem sap of *Maurandya barclayana, Lophospermum erubescens*, and *Brassica napus. PLoS One* **9**, e87689, https://doi.org/10.1371/journal.pone.0087689 (2014).

42. Baker, I. & Baker, H. G. Analyses of amino acids in flower nectars of hybrids and their parents, with phylogenetic implications. *New Phytologist* **76**, 87–98 (1976).

43. Pacini, E., Nepi, M. & Vesprini, J. L. Nectar biodiversity: a short review. *Plant Systematics and Evolution* **238**, 7–21, https://doi.org/10.1007/s00606-002-0277-yNectar (2003).

44. Escalante-Pérez, M. & Heil, M. In *Secretions and Exudates in Biological Systems. Signaling and Communication in Plants* Vol. 12 (eds Vivanco, J. & Baluška, F.) 27–49 (Springer, 2012).
45. Nicolson, S. In *Nectaries and nectar* (eds Nicolson, S. W. & Nepi, M.) 289–342 (Springer, 2007).
46. Freeman, C., William, H. & Worthington, R. Patterns of floral nectar-sugar composition of *Ipomopsis longiflora* (Polemoniaceae) near the contact zone of its subspecies Longiflora and Australis. *American Journal of Botany* **72**, 1662–1667 (1985).
47. Bernardello, G. In *Nectries and Nectar* (eds Nicolson, S., Nepi, M. & Pacini, E.) 19–128 (Springer, 2007).
48. Herrera, C. M., Pérez, R. & Alonso, C. Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. *American Journal of Botany* **93**, 575–581, https://doi.org/10.3732/ajb.93.4.575 (2006).
49. Gardener, M. C., Rowe, R. J. & Gillman, M. P. Tropical Bees (*Trigona hockingsi*) Show No Preference for Nectar with Amino Acids. *Biotropica* **35**, 119–125, https://doi.org/10.1111/j.1744-7429.2003.tb00269.x (2003).
50. García, M. T. A. & Gottsberger, G. Composition of the floral nectar of different subgenera of Argentinian *Passiflora* species. *Plant Systematics and Evolution* **283**, 133–147, https://doi.org/10.1007/s00606-009-0215-3 (2009).
51. Borghi, M. & Fernie, A. R. Floral Metabolism of Sugars and Amino Acids: Implications for Pollinators' Preferences and Seed and Fruit Set. *Plant Physiology* **175**, 1510–1524, https://doi.org/10.1104/pp.17.01164 (2017).
52. Kim, Y. & Smith, B. Effect of an amino acid on feeding preferences and learning behavior in the honey bee, *Apis mellifera*. *Journal Of Insect Physiology* **46**, 793–801, https://doi.org/10.1016/s0022-1910(99)00168-7 (2000).
53. Hendriksma, H. P., Oxman, K. L. & Shafir, S. Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant-pollinator interactions. *Journal of Insect Physiology* **69**, 56–64, https://doi.org/10.1016/j.jinsphys.2014.05.025 (2014).
54. Rodriguez-Pena, N. *et al.* Nitrogen and amino acids in nectar modify food selection of nectarivorous bats. *Journal of Animal Ecology* **82**, 1106–1115, https://doi.org/10.1111/1365-2656.12069 (2013).
55. Rathman, E. S., Lanza, J. & Wilson, J. Feeding preferences of flesh flies (*Sarcophaga bullata*) for sugar-only vs. sugar-amino acid nectars. *The American Midland Naturalist* **124**, 379 (1990).
56. Shiraishi, A. & Kuwabara, M. The effects of amino acids on the labellar hair chemosensory cells of the fly. *Journal of General Physiology,* **56** (1970).
57. Day, P. D. *et al.* Evolutionary relationships in the medicinally important genus *Fritillaria* L. (Liliaceae). *Molecular Phylogenetics and Evolution* **80**, 11–19, https://doi.org/10.1016/j.ympev.2014.07.024 (2014).
58. Ojeda, I. *et al.* Comparative Micromorphology of Petals in Macaronesian *Lotus* (Leguminosae) Reveals a Loss of Papillose Conical Cells during the Evolution of Bird Pollination. *International Journal of Plant Sciences* **173**, 365–374, https://doi.org/10.1086/664713 (2012).
59. Inouye, D. *et al.* The effects of nonsugar nectar constituents on estimates of nectar energy content. *Ecology* **61**, 992–996 (1980).
60. Hainsworth, F. & Wolf, L. Nectar characteristics and food selection by hummingbirds. *Oecologia* **25**, 101–113 (1976).
61. Yanega, G. & Rubega, M. Feeding mechanisms: Hummingbird jaw bends to aid insect capture. *Nature* **428**, 615 (2004).
62. Peay, K. G., Belisle, M. & Fukami, T. Phylogenetic relatedness predicts priority effects in nectar yeast communities. *Proc Biol Sci* **279**, 749–758, https://doi.org/10.1098/rspb.2011.1230 (2012).
63. Lenaerts, M. *et al.* Impact of microbial communities on floral nectar chemistry: potential implications for biological control of pest insects. *Basic and Applied Ecology*, 1–10, https://doi.org/10.1016/j.baae.2015.10.001 (2015).
64. Nepi, M., Grasso, D. A. & Mancuso, S. Nectar in Plant-Insect Mutualistic Relationships: From Food Reward to Partner Manipulation. *Frontiers in Plant Science* **9**, 1063, https://doi.org/10.3389/fpls.2018.01063 (2018).
65. Terrab, A. *et al.* Analysis of amino acids in nectar from Silene colorata Poiret (Caryophyllaceae). *Botanical Journal of the Linnean Society* **155**, 49–56, https://doi.org/10.1111/j.1095-8339.2007.00673.x (2007).
66. Antoń, S., Kamińska, M. & Stpiczyńska, M. Comparative structure of the osmophores in the flower of Stanhopea Graveolens Lindley and Cycnoches Chlorochilon Klotzsch (Orchidaceae). *Acta Agrobotanica* **65**, 11–22, https://doi.org/10.5586/aa.2012.054 (2012).
67. Schmidt-Adam, G., Murray, B. G. & Young, A. G. The relative importance of birds and bees in the pollination of *Metrosideros excelsa* (Myrtaceae). *Austral Ecology* **34**, 490–498, https://doi.org/10.1111/j.1442-9993.2009.01949.x (2009).
68. Raguso, R. A. & Gottsberger, G. An ode to osmophores: Stefan Vogel's seminal contributions to the study of scent. *Flora: Morphology, Distribution, Functional Ecology of Plants* **232**, 150–152, https://doi.org/10.1016/j.flora.2017.02.014 (2017).
69. Gottsberger, G., Arnold, T. & Linskens, H. Variation in floral nectar amino acids with aging of flowers, pollen contamination and flower damage. *Israel J Bot* **39**, 167–176 (1990).
70. Gottsberger, G., Schrauwen, J. & Linskens, H. F. Amino acids and sugars in nectar, and their putative evolutionary significance. *Plant Systematics and Evolution* **145**, 55–77, https://doi.org/10.1007/BF00984031 (1984).
71. Martins, E. & Hansen, T. Phylogenies and the comparative method: A general approach to incorporating phylogenetic information into the analysis of interspecific data. *Am Nat*, 646–667 (1997).
72. White, G. *The natural history & antiquities of Selborne.* (J. M. Dent & Sons Ltd., 1789).
73. Bernhardt, P. *The rose's kiss. A natural history of flowers.*, (Island Press, 1999).
74. Naruhashi, N., Takata, Y. & Negoro, H. Pollinators and dispersing insects of seeds in *Fritillaria koidzumiana* (Liliaceae). *The Journal Of Phytogeography And Taxonomy* **54**, 57–63 (2006).
75. Breiman, L. Random forests. *Machine Learning* **45**, 5–32 (2001).
76. Trifinopoulos, J., Nguyen, L., von Haeseler, A. & Minh, B. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucl Acids Res* **44**, 232–235, https://doi.org/10.1093/nar/gkw256 (2016).
77. Freckleton, R., Harvey, P. & Pagel, M. Phylogenetic analysis and comparative data: A test and review of evidence. *Am Nat* **160**, 712–726 (2002).
78. Pagel, M. & Harvey, P. Comparative methods for examining adaptation depend on evolutionary models. *Folia Primatol (Basel)* **53**, 203–220 (1989).
79. R: A Language and Environment for Statistical Computing (Vienna, 2011).

## Acknowledgements

## Author contributions

K.R. and M.Z. conceived the study; K.R. assembled field data; A.B., M.C., A.G. performed nectar analysis, K.R., A.R. and M.Z. analyzed the data; K.R. and M.Z. wrote the draft version of the paper. All authors contributed to the final version.

## Competing interests

The authors declare that the research was conducted in the absence of any financial/commercial and non-financial interests, which could be construed as a potential conflict of interest.

## Additional information

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41598-019-51170-4.

**Correspondence** and requests for materials should be addressed to K.R. or M.Z.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.