Plant Ecology and Evolution 154 (1): 49–55, 2021
https://doi.org/10.5091/plecevo.2021.1676

RESEARCH ARTICLE

Reproductive and pollination biology of the Critically Endangered endemic *Campanula vardariana* in Western Anatolia (Turkey)

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Background and aims – *Campanula vardariana* (Campanulaceae) is a critically endangered endemic chasmophyte with a single population situated in the west of Turkey. Very little is known about the reproductive biology of *C. vardariana* and more information is needed to develop a sound conservation strategy for this endemic species.
Material and methods – Floral traits such as flower morphology, nectar, and sugar concentration, as well as pollen viability and stigma receptivity were measured in different floral phases. We observed insect visitations to the flowers and identified pollinators. Additionally, we investigated the effect of cross and self-pollination on fruit and seed production.
Key results – The flowers of *C. vardariana* are protandrous. The length of the styles, which were 8.74 mm during the pollen loading phase, reached 11.35 mm during the pollen presentation phase. The visitor observations made on the *C. vardariana* flowers revealed 11 visitor species from 5 families: 5 Halictidae, 3 Apidae, and one species each from Megachilidae, Colletidae, and Bombyliidae. *Lasioglossum* spp. touched the anthers and stigma using several parts of their bodies and were significant pollinators of *C. vardariana*. Under natural conditions, the mean number of seeds per fruit was around 60 after cross pollination, while no fruits were formed when pollinators were excluded.
Conclusion – *Campanula vardariana* is entirely dependent on pollinators for its reproductive success, and bees, especially Halictidae and to a lesser extent Apidae, play an important role. *Campanula vardariana* is restricted to cracks in calcareous rocks and its population is threatened by goat overgrazing and mining activities (quarry formation). Since seed production is abundant in this population, anthropogenic activities currently form the biggest threat to its existence.

Keywords – *Campanula vardariana*; conservation; endemic; pollination; reproductive biology.

INTRODUCTION

Plants exhibit remarkable differences in their reproductive systems and floral traits to attract pollinators and to achieve successful pollination (Leins & Erbar 2010; Vranken et al. 2014). Many endangered plant species especially suffer from reproductive failure. It is essential to obtain information about the reproductive biology of plant species to understand the reason for this failure (Duan & Liu 2007; Wang et al. 2017) and to design conservation management plans for rare plant species (Gargano et al. 2009; Walsh et al. 2019). For plants pollinated by insects, their ability to attract pollinators is very important (Conner & Rush 1996; Bauer et al. 2017) and secondary sexual characteristics have been developed to increase plant reproductive success, with their effects on the behaviour of insect visitors (Harder & Barrett 1992). The plant pollination process provides important indicators for designing conservation and sustainability strategies for plant populations (Rodríguez-Oseguera et al. 2013; Chen et al. 2018). Comprehensive information about the interactions with pollinators in addition to their pollination systems is required for the conservation of threatened plant species and

Plant Ecology and Evolution is published by Meise Botanic Garden and Royal Botanical Society of Belgium
ISSN: 2032-3913 (print) – 2032-3921 (online)

to develop appropriate conservation strategies (Martinell et al. 2010; Ren et al. 2015). For the conservation management of threatened plant species, it is necessary to understand the ecological and genetic aspects of the plants, as well as their life cycle characteristics (Godefroid et al. 2011; Blambert et al. 2016; Van Rossum et al. 2017).

The genus *Campanula* L. is represented by roughly 420 species in the subtropical and temperate regions of the northern hemisphere (Lammers 2007) and around 150 species in the Mediterranean region (Cronquist 1988; Heywood 1998). In Turkey, the *Campanula* genus is represented by 138 taxa and the endemism rate is approximately 53% (Güner 2000; Yıldırım 2018). *Campanula* flowers are protandrous with a secondary pollen presentation mechanism (Leins & Erbar 1990; Nyman 1992a).

Campanula vardariana, a chasmophytic endemic species in rocky limestone habitats, has a very limited distribution in the west of Turkey (Söke, Aydın) (Damboldt 1978). This species has been classified as "Critically Endangered" (CR) (Ekim et al. 2000). Information on the life cycle of *C. vardariana*, especially the pollination biology and reproductive success of this species, is scarce and incomplete.

For this reason, in order to establish a conservation strategy for this endemic species, our research was aimed at determining (1) *C. vardariana* floral traits, such as flower morphology, nectar and sugar concentration, (2) flower visitation rates and pollinator identity, (3) pollen viability in addition to stigma receptivity in different floral phases, and (4) the effect of cross and self-pollination on fruit and seed production.

MATERIAL AND METHODS

Study site and species description

Campanula vardariana Bocquet is a perennial plant which is a critically endangered endemic with a single population in the west of Turkey (Alçitepe & Yildiz 2010; Subaşı 2014). Flowering time is between May and June. It grows in a habitat of calcareous rocks at an elevation of 1–100 m. The study was conducted in the only known population (Söke, Aydın) located in the Aegean region of Turkey (37°49′21.26″N, 27°29′3.12″E) between May 2012 and September 2014. The mean annual temperature in this region is 17.7°C (min: 11.9°C, max: 24.5°C), and the annual mean rainfall is around 664 mm (Turkish State Meteorological Service 2021). Dominant species in the habitat are *Sarcopoterium spinosum* (L.) Spach, *Plantago lagopus* L., *Trifolium campestre* Schreb., *Bromus intermedius* Guss., *Medicago minima* L., *Micromeria myrtifolia* Boiss. & Hohen., and *Filago vulgaris* Lam.

Floral biology

As in other *Campanula* species, the flowers of *C. vardariana* are protandrous. Before anthesis, the introrsely opening anthers release pollen onto the middle and upper part of the style (pollen loading stage). When the flower opens, the pollen is already fully loaded onto the stylar hairs with a closed stigma (pollen presentation stage). A total of 30 flowers from 15 plants in the pollen presentation stage were fixed in Formalin Acetic Alcohol (FAA: acetic acid 5%, formaldehyde 5%, and ethanol 90%). In the laboratory, the diameter and length of the corolla tube and pistil length were measured using digital callipers and a digital microscope (Dino-Lite AM313). Stigma lobe length, anther and style length, stigma diameter, and stigma-anther distance were measured in mature flowers (in the pollen presentation stage), and flower buds (in the pollen loading stage) in 15 randomly selected individuals. Voucher specimens of *C. vardariana* were deposited in the EGE Herbarium at the Department of Botany, Ege University (EGE–41681).

Nectar volume was measured between the hours of 09:00–10:00, 12:00–13:00, and 16:00–17:00 using micropipettes in 30 open flowers taken from randomly selected individuals. Sugar concentration in these samples was determined using a handheld refractometer, 0–50% Brix (Bellingham and Stanley model 45-81, Tunbridge Wells, UK). The total sugar content was calculated using the exponential regression formula proposed by Galetto & Bernardello (2005).

For the statistical analysis, the floral morphometric data of the floral traits were compared between phases using Student's t-tests. Nectar parameter means of the three different situations were compared with a one-way ANOVA. All statistical tests were carried out using Paleontological Statistics (PAST) v.4.03 (Hammer et al. 2001).

Pollen viability was tested in flowers in both phases of flower development by applying 1% TTC (1,2,3-triphenyl tetrazolium chloride) to 20 flowers and 20 flower buds (Mulugeta et al. 1994). After the pollen preparations were stained and incubated at 30°C, they were examined under the light microscope at a magnification of 160–400× using a light microscope (Leica: 10×18 ocular, 4×/0.10 objective). Viable pollen was dyed in red and light red, while non-viable pollen grains were not dyed. The viable and non-viable pollen grains were counted from ten randomly assigned fields of view at a magnification of 10× until a total of 100 grains was examined, and this was repeated five times. Control experiments were performed using heat-killed pollen (80°C for 2 h; Dafni & Firmage 2000).

To determine stigma receptivity, Peroxidase Test Paper (Perex Tesmo KO, Macherey-Nagel) (Dafni & Motte-Maués 1998) was used on stigmas from 20 mature flowers in the pollen presentation stage, and 20 flower buds in the pollen loading stage. This test indicates the main receptive area on the stigma surface, which turns dark blue or purple when in contact with the solution.

Flower visitation and pollinator identification

These observations were performed in 15 randomly assigned individuals for 3 days between the hours of 09:00–10:00, 12:00–13:00, and 16:00–17:00, when the weather conditions were appropriate (sunny and only slightly windy). The species of insect visitors were identified and the number of each visiting species was recorded. At least one individual of each visiting species was captured as a voucher and for further identification. Floral visitors in the widest sense were recorded, with all animals touching a flower were counted as visitors. A visitor was considered as legitimate if it made

Table 1 – Floral morphology of *Campanula vardariana*.

Floral trait	n	Range (mm)	Mean ± sd (mm)
Corolla diameter	30	14–25	19.2 ± 5.06
Corolla length	30	17–32	26.86 ± 7.10
Pistil length	30	15–32	19.2 ± 8.32

Table 2 – Morphological measurements (mean ± standard deviation) of pistil and stamen in flowers of *Campanula vardariana* at the pollen loading and presentation stages.

Stage	Number of individuals (n)	Anther length (mm)	Style length mm)	Stigma diameter (mm)
Pollen loading	15	6.53 ± 0.81	8.74 ± 1.39	1.65 ± 0.70
Pollen presentation	15	6.79 ± 0.90	11.35 ± 2.12	2.49 ± 0.71
Student t		0.85134	4.0691	3.3608
p value		0.3976	0.0007	0.0019

Table 3 – Nectar parameters in *Campanula vardariana* flowers (n = 30) measured during the day in the pollen presentation stage.

Time of day	Nectar volume (µL)		Sugar concentration (%)		Sucrose (mg/µL)	
	Mean ± standard deviation	Range	Mean ± standard deviation	Range	Mean ± standard deviation	Range
09:00–10:00	1.98 ± 0.92	0.83–4.16	39.35 ± 9.71	12–55	0.46 ± 0.13	0.12–0.69
12:00–13:00	2.30 ± 0.95	0.83–4.00	42.83 ± 7.39	29–58	0.51 ± 0.10	0.32–0.74
16:00–17:00	1.65 ± 0.64	1.00–3.00	35.97 ± 18.15	4–58	0.43 ± 0.23	0.04–0.74
F	0.0076		1.917		291	
p value	0.9307		0.1533		< 0.001	

contact with the stigma of the flower during its visit, whereas those which did not achieve contact were considered as non-legitimate visitors. Additionally, for each visitor, the arrival and departure times were recorded. The identification of the floral visitors was performed at Hacettepe University, Faculty of Science, Department of Biology and Zoology. The voucher specimens were deposited in the museum of Ege University, Faculty of Science, Department of Biology.

Reproductive success

To determine fruit set, 100 flowers (two flowers per individual) were randomly selected from a total of 50 plants in the population and labelled. All mature fruits were collected 45 days after anthesis and the number of seeds per fruit was determined. To determine the ovule number, the ovaries of 20 flowers taken from 20 individuals were collected and the number of ovules per flower was counted using a light microscope. Mean seed set was then calculated by dividing mean seed production by mean ovule number. To determine if seeds can be produced by autonomous self-pollination, 100 flower buds on 50 individuals were isolated using pollination bags to prevent flower visitation. After approximately 1 month, the isolated flowers were brought to the laboratory, where fruit set and number of seeds per fruit were determined.

RESULTS

Floral biology

Corolla length ranged between 17 and 32 mm (table 1), while the pistil was a few mm shorter than the corolla. The corolla diameter was between 14 and 25 mm.

Both the anthers and style were longer in the pollen presentation stage compared to the pollen loading stage of the flower buds (table 2). The mean stigma diameter in the pollen presentation stage was larger than in the pollen loading stage. The style length differed significantly between the pollen loading stage and the pollen presentation stage (t-test, $t = 4.0691$, $p = 0.0007$).

Nectar parameters were measured in the morning, around midday, and at the end of the afternoon (table 3). The highest sugar concentration, nectar volume, and sugar content were measured around midday (table 3). The sucrose (mg/µL) content around midday was significantly higher than in the morning and at the end of the afternoon ($F = 291$, $p < 0.001$).

During the pollen loading stage, all pollen grains in the anthers were viable. In the pollen presentation stage, mean pollen viability was 66.6 ± 7.1%. Stigmas in the pollen loading phase and stigmas in the presentation phase that were not curled were found to be non-receptive, while stigmas that

Table 4 – Flower visitors of *Campanula vardariana*, the number of visits recorded, the percentage of the total number of visits, the duration of a single flower visit, and the resource collected (P = pollen, N = nectar). The total number of visits observed is 551.

Family	Flower visitors	Number of visits	% of total	Time spent per flower visit(s)	Resource collected
Apidae	*Apis mellifera* Linnaeus, 1758	10	1.81	3	P/N
Apidae	*Ceratina* sp.	10	1.81	10.2	P
Apidae	*Bombus terrestris* Linnaeus, 1758	20	3.63	18	P/N
Halictidae	*Lasioglossum morio* Fabricius,1793	143	25.95	1.62	P/N
Halictidae	*Lasioglossum discum* Smith, 1853	24	4.36	15	P/N
Halictidae	*Lasioglossum obscuratum* Morawitz, 1876	58	10.53	17.43	P/N
Halictidae	*Lasioglossum glabriusculum* Morawitz, 1872	117	21.23	7.29	P/N
Halictidae	*Lasioglossum setulellum* Strand, 1909	30	5.44	7.26	P/N
Megachilidae	*Osmia* sp.	18	3.27	11.66	P/N
Colletidae	*Hylaeus* sp.	13	2.36	8.92	P/N
Bombyliidae	Bombyliinae	108	19.60	7.12	P/N

Table 5 – Reproductive success after different pollination treatments in *Campanula vardariana*.

Treatment	n fruit set	Fruit set (%)	n seed set	Number of seeds per fruit	Seed set (%)
Open pollination	100	83.48	50	603.6 ± 122.9	63.93
Autonomous self-pollination	100	0	–	–	–

were completely curled during the pollen presentation phase were viable.

Flower visitation and pollinator identification

The pollinator observations on the flowers of *C. vardariana* revealed 11 visitors from 5 families: 5 taxa from Halictidae, 3 from Apidae, and 1 taxon from each of the three families Megachilidae, Colletidae, and Bombyliidae (table 4). The three most frequently visiting taxa were *Lasioglossum morio* (25.95%), *Lasioglossum glabriusculum* (21.23%), and Bombyliinae (19.60%), respectively. The taxa with the longest visiting time were *Lasioglossum morio* and *Apis mellifera*. Two-thirds of the total number of the flower visits in *C. vardariana* was performed by *Lasioglossum* spp. (table 4, fig. 1).

In addition, the members of the Coleoptera (Curculionidae and Cantharidae), Formicidae, and Thomicidae that visited in order to sleep/rest or feed on flower parts were non-legitimate visitors. The Apidae (*Apis mellifera*, *Bombus terrestris*, and *Ceratina* sp.), which represented 7.25% of the visits, are considered as legitimate visitors in that they not only touched the floral reproductive parts but also moved between inflorescences. The time spent of their visits ranged from 3 to 18 s per flower, and among them, the longest visit per flower was made by *Bombus terrestris*. *Osmia* (Megachilidae) and *Hylaeus* (Colletidae) were rare visitors (5.6%). The time spent during their visits ranged from 8.92 to 11.66 s per flower.

Reproductive success

The mean number of ovules per flower was 944.16 ± 245.44 (n = 50). In the open pollination treatment, 83.48% of the flowers set fruit, and almost 64% of the ovules set seed, producing around 600 seeds per fruit on average (table 5). No fruit formation was observed when pollinators were excluded.

DISCUSSION

Our floral morphology observations revealed a clear temporal separation in the maturation of male and female structures. Shetler (1979) reported that *Campanula* flowers were protandric, pollen grains were loaded before the enlargement of the stigmatic lobes, and that pollen grains were often dispersed during the budding phase or opening of the buds. Denisow et al. (2014) reported a similar situation for *Campanula bononiensis* L. Pollen grains are rapidly released when anthers open during the budding stage and all the pollen grains released are presented on the styles when the corolla opens. Pollen presentation in *C. bononiensis* flowers is extended by about 2 days per flower due to the secondary pollen presentation.

Variability was observed in the amount of nectar measured at different times during the day. In flowers of *C. vardariana* in the pollen presentation phase, the highest sugar concentration, volume, and content were measured between 12:00 and 13:00, which corresponds with the findings for *C. patula* L. (Denisow et al. 2018). The mean sugar concentration for *C. vardariana* is similar compared to other *Campanula* species. Strzałkowska-Abramek et al. (2018) showed that mean nectar sugar concentration ranged from 40.1% (*C. glomerata* L.) to 56.7% (*C. trachelium* L.). Carlson & Harms (2006) classified protandrous Campanulaceae as female-biased nectar producers. Male-biased or female-biased nectar production has been documented in several protandrous species and is thought to be driven by sexual selection or avoidance of inbreeding (Carlson 2007).

The degree of protandry is highly variable among species (Nyman 1992a, 1992b) and a considerably shorter male phase is usually observed in selfing compared to that of the xenogamous species (Nyman 1993). Fruit formation does not occur in *C. vardariana* flowers that are shielded from pollinators, and the presence of protandry confirms that seed production is pollinator-dependent.

In *C. vardariana*, the stigmas were not functional during the pollen loading phase and completely curled stigmas were only functional during the pollen presentation phase. However, the percentage of viable pollen grains decreased in this phase. This is undoubtedly a mechanism that prevents self-pollination and encourages outcrossing.

A study on *C. microdonta* Koidz. and *C. punctata* Lam. reported that especially members of the Megachilidae species

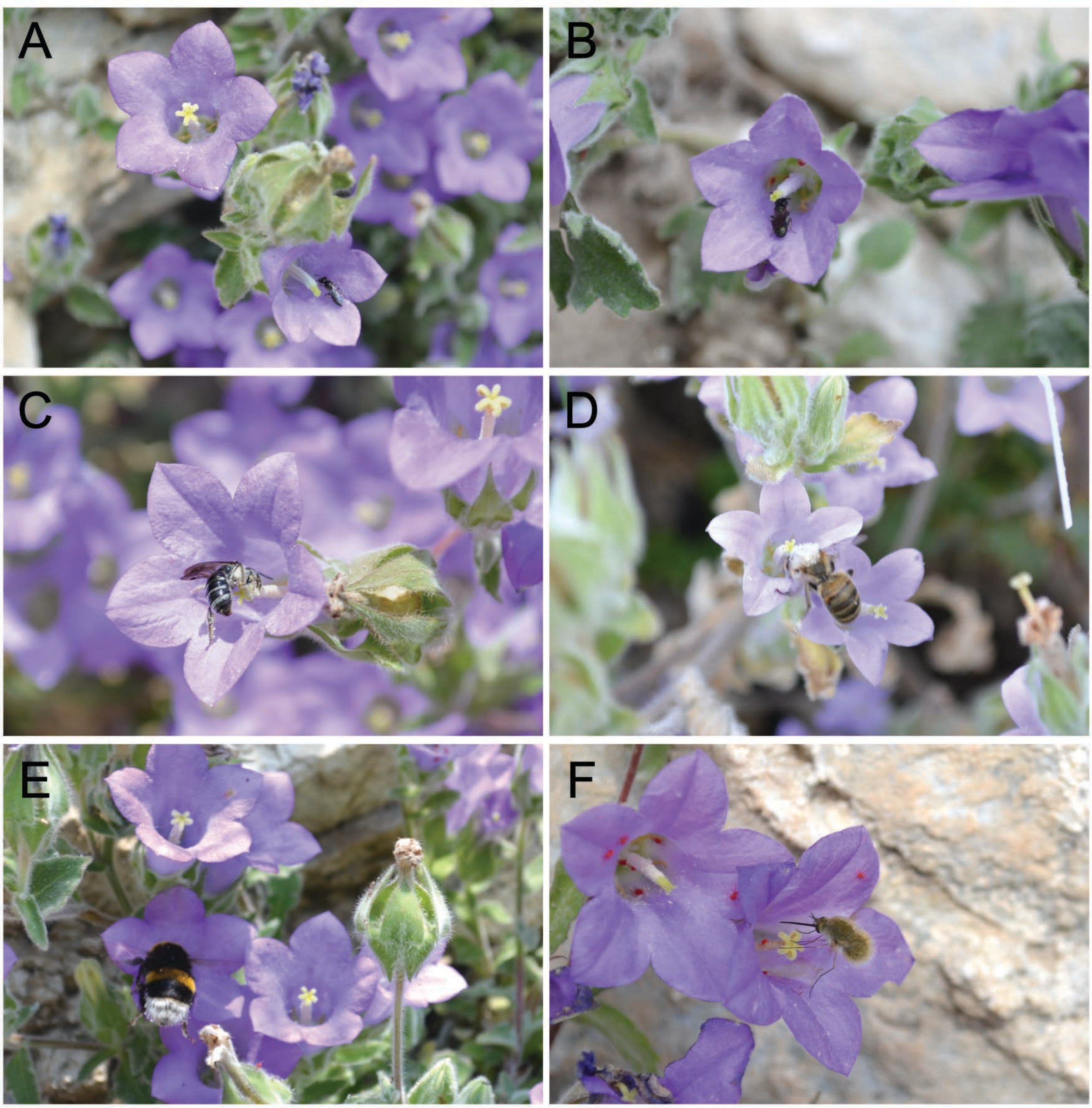

Figure 1 – Pollinators of *Campanula vardariana*. **A**. *Lasioglossum* (*Evylaeus*) *morio* (Fabricius, 1793). **B**. *Lasioglossum* (*Evylaeus*) *glabriusculum* (Morawitz, 1872). **C**. *Lasioglossum discum* (Smith, 1853). **D**. *Apis mellifera* Linnaeus, 1758. **E**. *Bombus terrestris* (Linnaeus, 1758). **F**. *Bombylius* sp. Photographs by Ümit Subaşı.

visited larger flowers, whereas responses of bumble bees and halictid bees did not show particular trends in flower size (Inoue et al. 1995). However, Inoue (1990) reported that although members of Megachilidae were locally important pollinators of *Campanula*, Halictidae and *Bombus* bees were much more effective. Denisow et al. (2014) found that both nectar and pollen in *C. bononiensis* flowers attracted not only Apoidea but also various other insects. Our results showed that 67.5% of the visitors of *C. vardariana* flowers were visited by *Lasioglossum* spp. (Halictidae). Koski et al. (2018) reported that *C. americana* flowers were frequently visited by small bees (including Halictidae and Apidae).

As expected for Campanulaceae (D'Antraccoli et al. 2019), no fruits were obtained in our experiments for the bagged flowers, indicating the absence of apomixis and autonomous self-pollination. The effective reproduction of *C. vardariana* is therefore entirely dependent on pollinator visits. *Lasioglossum* spp. were able to reach the anthers and stigma using several parts of their bodies and are therefore considered as important pollinators for *C. vardariana*.

In conclusion, our study contributed important information that can help to prevent the extinction of *C. vardariana* and determine appropriate conservation strategies. The Halictidae and with Apidae species play an important role in the pollination of *C. vardariana* and their activities ensure a high rate of seed production.

Campanula vardariana is a local endemic chasmophyte from Söke (Aydın, Turkey), limited to cracks in calcareous rocks. Goat overgrazing and mining activities (quarries) as well as habitat loss pose a threat to its single population. Bees are considered to be the most important taxon of pollinators (Kearns et al. 1998) and they are also faced with these anthropogenic threats. The population seems genetically healthy, since seed production is not compromised, so with good conservation efforts to halt the destruction of this population, its long-term future could be ensured.

ACKNOWLEDGEMENTS

The present study was supported by the Ege University Rector Scientific Research Project Office (Project No: 2012-FEN-019), the Scientific and Technological Research Council of Turkey (TUBITAK Project No: 112T598), and the Science, Technology Application and Research Center of Ege University (EBILTEM Project No: 2013/BİL/017). We would like to extend our sincere thanks to Dr Fatih Dikmen, İstanbul University, Science Faculty, Biology and Zoology Department for his valuable contributions to the identification of pollinators.

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Communicating editor: Renate Wesselingh.

Submission date: 14 Sep. 2019

Acceptance date: 18 Dec. 2020

Publication date: 23 Mar. 2021