

## POLLINATION ECOLOGY, SPECIALIZATION, AND GENETIC ISOLATION IN SYMPATRIC BEE-POLLINATED *SALVIA* (LAMIACEAE)

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**Premise of research.** Previous pollination ecology studies of *Salvia* have shown that there is low specialization for certain subgroups of bees and that pollinator number varies with species and locality. We studied 12 *Salvia* species (three clades with different corolla morphologies and staminal lever mechanisms) that in part co-occur and coflower to examine prezygotic isolation mechanisms and the degree of specialization versus generalization in pollination ecology.

**Methodology.** Pollinators were identified using field observations, photos, and related literature across three sites in Turkey. Video documentation of the visitation rate and the site of pollen placement on the pollinator body, morphometric measurements between flowers and pollinators, flowering time, flower color, handling time, and stigma contact ratio were analyzed. Plant-pollinator networks were constructed.

**Pivotal results.** Mechanical, phenological, and ethological isolation occurs among sympatric *Salvia* species. A morphological fit is evident between flower tube length and the proboscis length of the main pollinators. Pollinator networks indicate that most species are ecological generalists and that only a few are specialists.

**Conclusions.** The 12 *Salvia* species, though phenotypically and functionally specialized by their zygomorphic, bilabiate flowers and bee-pollination syndrome, differ in the degree of their ecological specialization. Most of the sympatric *Salvia* species tend to be ecologically generalized, with two or more main pollinators and a few additional secondary pollinators, while a few *Salvia* species are clearly specialized. Some floral traits (e.g., flower color, morphology, size, corolla tube length and width of the corolla tube entrance, type and size of the staminal lever mechanism, small numbers of flowers in the inflorescence), a short flowering time, and a small population size appear to be correlated with the degree of ecological specialization.

**Keywords:** bee pollination, Lamiaceae, morphometric fitting, pollinator networks, *Salvia*, specialization, sympatric, Turkey.

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### Introduction

The mutualistic interaction between flowers and their animal pollinators is commonly argued to be a key feature in driving both floral diversification and speciation within angiosperms (Fenster et al. 2004; Soltis et al. 2005; Forest et al. 2014; Van der Niet et al. 2014; Breitkopf et al. 2015). Delimiting the role of specialization versus generalization in plant-pollinator relationships is a fundamental research agenda in plant evolutionary biology (Johnson and Steiner 2000; Armbruster 2014, 2017).

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When discussing specialization versus generalization from the plants' perspective, pollination biologists distinguish between phenotypic, functional, and ecological specialization (Fenster et al. 2004; Ollerton et al. 2007; Armbruster 2017). Phenotypic specialization is based on the characters displayed by the flower, for example, floral shape, size, symmetry, color, nectar, and scent. Functional specialization is based on the animal groups acting as pollinators (e.g., bees, butterflies, birds, bats) and the specific adaptations they have to ensure effective pollen transfer. Ecological specialization, finally, is based on the number of different pollinator species per plant species. Commonly, the term generalization is used for plants pollinated by a large number of pollinator species, while the term specialization is used for plants pollinated by relatively few pollinator species or functional groups (Waser et al. 1996; Johnson and Steiner 2000; Armbruster 2017). Pollinator network analysis is now employed to

quantify ecological specialization versus generalization for plants and pollinators (Carstensen et al. 2018).

An emerging model system for exploring specialized versus generalized pollination is the genus *Salvia* L. (sages) in the mint family (Lamiaceae). *Salvia* has a worldwide distribution of nearly 1000 species, displays high vegetative and floral diversity (size, color, shape, staminal lever mechanism, anther connective, and stylar morphology), possesses specific pollination mechanisms, and has adapted in both the Old World and the New World to two main pollinator guilds: bees (including bee flies) and birds (and one instance of hawk moth pollination; Claßen-Bockhoff et al. 2003, 2004; Wester and Claßen-Bockhoff 2006a, 2011; Celep et al. 2014; Reith and Zona 2016; Kriebel et al. 2019, 2020).

In *Salvia*, the pollination process is mostly mediated by a staminal lever mechanism in which each connective of the two anthers is elongated, together forming two lever arms that are movable around the filament tips. The upper connective arm typically bears one fertile theca, with two pollen sacs usually hidden below the upper lip of the corolla. The lower connective arm is placed in the middle of the flower entrance, thus restricting access to nectar. A pollinator searching for nectar pushes this barrier back, thereby lowering the upper connective arm downward, and has pollen grains loaded on its back. When the pollinator visits a second flower, the pollen load is transferred to the stigma (Claßen-Bockhoff et al. 2003, 2004). The staminal lever in *Salvia* has been considered a key innovation (Claßen-Bockhoff et al. 2004) and, along with its associated floral traits, has been considered to play a major role in the adaptive radiation and speciation of *Salvia* because of its function in the process of pollen transfer, individual fitness, and ecological specialization (Claßen-Bockhoff et al. 2004; Wester and Claßen-Bockhoff 2007; Kriebel et al. 2020). A diverse array of staminal lever types has been reported in different clades within *Salvia* (Claßen-Bockhoff et al. 2003; Walker and Sytsma 2007; Zhang et al. 2011; Huang et al. 2015; Claßen-Bockhoff 2017). Because of the different types and sizes of the staminal levers and the diversity of floral morphologies among potentially co-occurring species, pollen grains are loaded on different parts of pollinators' bodies, causing mechanical isolation and facilitating sympatry (Claßen-Bockhoff et al. 2004). On the other hand, the staminal lever mechanism is not functional in some species, and the lower lever arm is highly reduced in some clades, as in the Old World *S. verticillata* group (Claßen-Bockhoff et al. 2003, 2017; Drew et al. 2017; Will and Claßen-Bockhoff 2017).

Although there are pollination ecological studies of *Salvia* (Wester and Claßen-Bockhoff 2006a, 2006b, 2007, 2011; Ott et al. 2016), only a few of them include Old World species (Claßen-Bockhoff et al. 2004; Celep et al. 2014; Şenol et al. 2017). The biogeographic history of *Salvia* indicates that the genus originated in southwest Asia and the Mediterranean region (Will and Claßen-Bockhoff 2017; Kriebel et al. 2019). Turkey is the main center of floral (corolla size, shape, color, and three different staminal lever types) and ecological diversity in the Old World, with over 100 species and 53% endemism (Celep et al. 2015; Celep and Dirmenci 2017).

Our previous study of *S. virgata* showed that 19 bee and fly species pollinate the species (Celep et al. 2014). Thus, despite its phenotypic and functional specialization, the species does not depend on a few pollinators but instead attracts a wide range

of pollinators present at a given locality and time. By extending the study to three populations with 12 *Salvia* species that are placed in three separate clades (subg. *Salvia*, subg. *Sclarea*, and the *S. verticillata* clade) with different stamen types and that in part co-occur and coflower, we can address several key questions: (1) What are the isolation mechanisms among the co-occurring *Salvia* species? (2) Do the study species differ in the degree of their ecological specialization for pollinators? (3) What floral traits control the degree of ecological specialization?

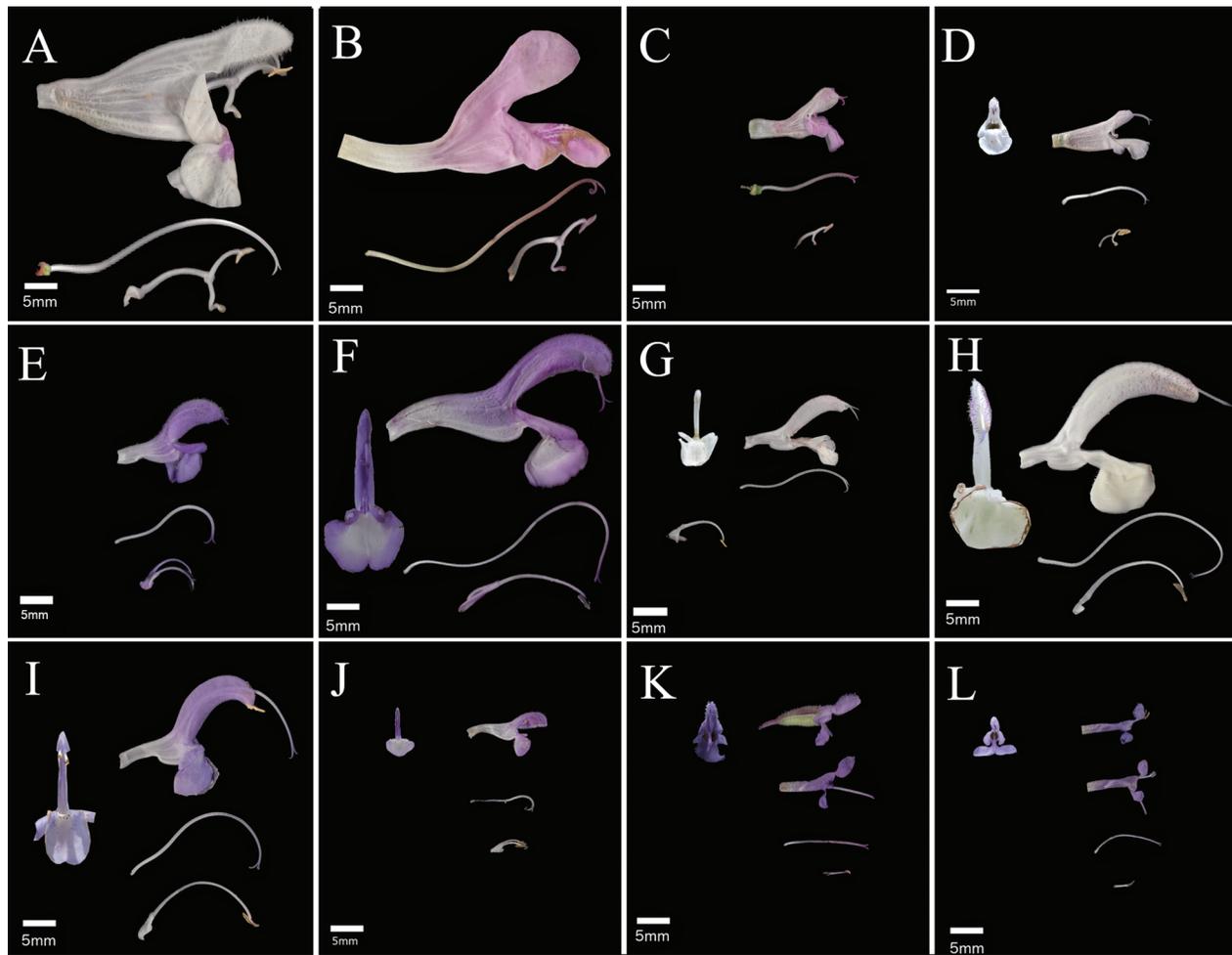
## Material and Methods

### Study Areas

Pollination studies were done from April to August between 2010 and 2014, with additional field observations done in both 2015 and 2016 in Ankara (study area 1) and in the Nevşehir-Cappadocia region (in two populations, study areas 2 and 3) in Central Anatolia, Turkey. The distance between the first study area and the other two is about 300 km. The distance between the second and the third is approximately 10 km. Study area 1 is a protected steppe region at an altitude of 920–980 m, lat. 39° 52'56.20"N, long. 32°46'34.37"E (fig. A1A, A1B; figs. A1, A2 are available online), on the Middle East Technical University (METU) campus, Ankara. Study area 2 is also a protected steppe region at an altitude of 1138–1140 m, lat. 38°40'40.84"N, long. 34°53'23.35"E (fig. A1C), between Ürgüp and Avanos, above Devrent Valley, Nevşehir. Although there are some planted trees (*Pinus nigra* J.F. Arnold and *Cedrus libani* A. Rich) and natural shrubs in the first study area, the vegetation in the first and the second study areas is mainly open steppe and is dominated by herbaceous members of Asteraceae, Fabaceae, Boraginaceae, Poaceae, and Lamiaceae. Study area 3 is in a protected valley called Zemi Valley with woody and bushy vegetation at an altitude of 1330–1360 m, lat. 38°36'38.19"N, long. 34°48'38.87"E, between Uçhisar and Göreme, Nevşehir (fig. A1D). The vegetation in the third study area is dominated by old fruit trees, old vineyards, old planted *Populus alba*, and herbaceous plants.

### Studied *Salvia* Species

Twelve *Salvia* species were studied in three study areas. Photos of flowers and the primary pollinators for each species are depicted in figures 1 and 2. In study area 1 (METU, Ankara), we studied 10 sympatric *Salvia* species: *S. absconditiflora* Greuter & Burdet, *S. tchihatcheffii* (Fisch. & C.A. Mey.) Boiss., *S. virgata* Jacq., *S. hypargeia* Fisch. & C.A. Mey., *S. aethiopsis* L., *S. candidissima* Vahl, *S. cyanescens* Boiss. & Balansa, *S. viridis* L., *S. verticillata* subsp. *amasiaca* (Freyn & Bornm.) Bornm., and *S. russellii* Benth. In study area 2 (Devrent, Nevşehir), we studied three sympatric *Salvia* species: *S. blepharochlaena* Hedge & Hub.-Mor., *S. absconditiflora*, and *S. hypargeia*. In study area 3 (Zemi Valley, Nevşehir), only *S. recognita* Fisch. & C.A. Mey. was studied. According to Will and Claßen-Bockhoff (2017), these species are placed in *Salvia* clade I (subclades I-C and I-D and the *S. verticillata* group). Using the most recent molecular phylogenetic tree and classification of Drew et al. (2017), the study species are placed in three separate clades: subg. *Salvia* (corresponding to subclade I-D), subg. *Sclarea* (corresponding to subclade I-C), and the *S. verticillata* clade (now placed in subg.



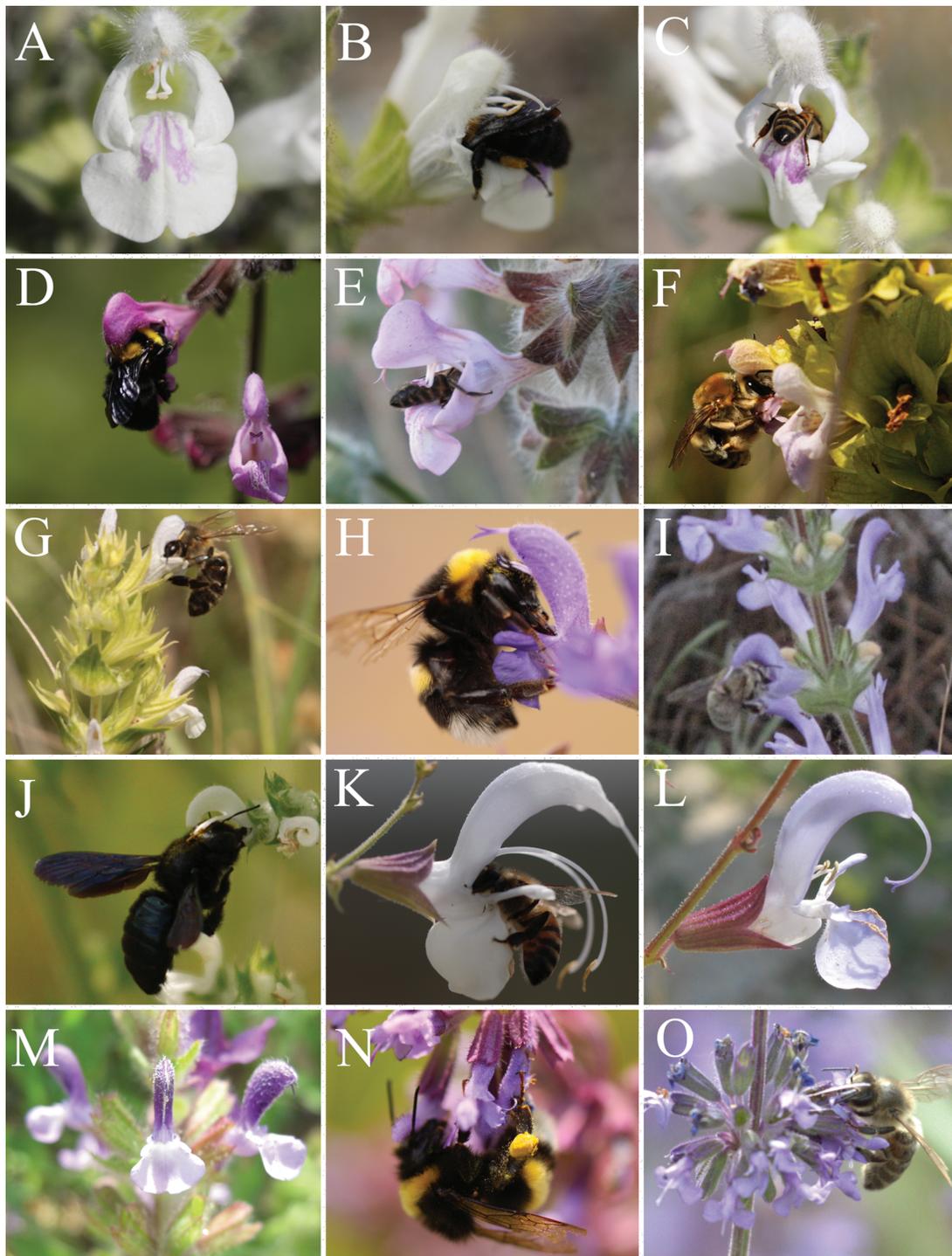
**Fig. 1** Flower front, flower side view, style, and stamen of the studied species. A, *Salvia blepharochlaena* (for frontal view, see fig. 2A). B, *Salvia recognita* (for frontal view, see fig. 2D). C, *Salvia absconditiflora* (for frontal view, see fig. 2F). D, *Salvia tchibatcheffii*. E, *Salvia virgata*. F, *Salvia hypargeia*. G, *Salvia aethiopsis*. H, *Salvia candidissima*. I, *Salvia cyanescens*. J, *Salvia viridis*. K, *Salvia verticillata* subsp. *amasiaca*. L, *Salvia russellii*.

“*Heterosphace*”; Kriebel et al. 2019). Here we use the clade names in Drew et al. (2017).

*Salvia blepharochlaena*, *S. recognita*, *S. absconditiflora*, and *S. tchibatcheffii* belong to the subg. *Salvia* clade. In this clade, flowers have a more or less straight upper lip and a three-lobed lower lip with one big middle lobe and two small to large revolute lateral lobes. The corolla tube is gradually wider toward the throat and is straight and annulate (hairy ring at the lower part of the corolla tube; figs. 1A–1D, 2A–2G). The corolla tube entrance is considerably wider (particularly in large-flowered *S. blepharochlaena* and *S. recognita*) than that of the species placed in the *Sclarea* or *S. verticillata* clades. The upper lever arm is slightly longer than the lower one or subequal to it. The anther connective tissue is more or less the same length as the filament, and the lower lever arm bears some fertile pollen grains that are positioned close to the corolla tube entrance (fig. 1A–1D; stamen type A, according to Hedge [1982]). The style is exerted from the upper lip of the corolla and is slightly curved because of

the straight upper lip of the corolla. Hedge (1974) considered these species to be basal/ancestral *Salvia* species, although they appear to be nested well within subg. *Salvia* on the basis of molecular data (Kriebel et al. 2019).

*Salvia virgata*, *S. hypargeia*, *S. aethiopsis*, *S. candidissima*, *S. cyanescens*, and *S. viridis* belong to the subg. *Sclarea* clade. Flowers in this clade have semifalcate to strongly falcate upper lips (to better touch and adhere anthers to the insect body) and three-lobed lower lips with one large middle lobe. The latter acts as the landing platform for pollinators, with the two prominent lateral lobes functioning as footholds. The corolla tube is gradually wider toward the throat and is either invaginated or not, or the tube is ventricose and is either squamulate or not. The corolla tube entrance is very narrow, and the lower staminal lever arm is sterile and spoon shaped and completely blocks the corolla tube entrance (figs. 1E–1J, 2H–2M). The upper lever arm is considerably longer than the lower one, and the anther connective tissue is also considerably longer than the filament (stamen



**Fig. 2** A–G, Species from the subg. *Salvia* clade. A–C, *Salvia blepharochlaena*. A, Front view. B, Pollination by *Bombus argillaceus*; the head and thorax are inserted into the corolla tube, and the lever mechanism and stigma touch its thorax. C, *Apis mellifera* entering the corolla tube without triggering the lever mechanism. D, E, *Salvia recognita*. D, Pollination by *B. argillaceus*; the head and thorax are inserted into the corolla tube, and the lever mechanism and stigma touch its thorax. Front view. E, *Apis mellifera* entering the corolla tube without triggering the lever mechanism. F, *Salvia absconditiflora* pollination by *Amegilla quadrifasciata*; lever mechanism touches the posterior head and anterior thorax. G, *Salvia tchihatcheffii* pollinated by *A. mellifera*. H–M, Species from the subg. *Sclarea* clade. H, *Salvia virgata* pollinated by *Bombus terrestris*; lever mechanism touches the head of the pollinator. I, *Salvia hypargeia* pollinated by *Anthophora aestivalis*. J, *Salvia aethiopsis* pollinated by *Xylocopa* cf. *iris*; lever mechanism touches the thorax of the pollinator. K, *Salvia candidissima* visited by *A. mellifera*; lever mechanism does not touch the pollinator body. L, Female flowers of *S. cyanescens*, stamens distorted and style elongated and curved inward. M, *Salvia viridis* flowers. N, O, Species from the *S. verticillata* clade. N, *Salvia verticillata* subsp. *amasiaca* pollinated by the head of *B. terrestris*; stigmas of the other flowers touch the ventral part of the pollinator. O, *Salvia russellii* pollinated by *A. mellifera*; stigmas of the other flowers touch the head of the pollinator.

type B; figs. 1E–1J, A2A). The style is exerted from the upper lip of the corolla and is highly curved because of the semifalcate to falcate shape of the corolla upper lip.

*Salvia verticillata* subsp. *amasiaca* and *S. russellii* belong to the *S. verticillata* clade. This small clade comprises four species and five taxa (*S. verticillata* subsp. *verticillata*, *S. verticillata* subsp. *amasiaca*, *S. russellii*, *S. napifolia*, and *S. judaica*) that can be easily recognized by their densely crowded flowers in verticillasters (12–40 or more flowers per verticillaster) with many small (6–12 mm in length) violet-blue, lilac, or, very rarely, whitish flowers. These species lack the typical *Salvia* lever mechanism because of the reduction of their lower lever arms and their inarticulate filament (stamen type C; fig. 1K, 1L). Instead of using a lever mechanism, bee pollinators must push back the movable upper lip to gain access to the nectar (Hildebrand 1865; Correns 1891; Claßen-Bockhoff et al. 2004).

Ten of the 12 studied *Salvia* species are hermaphroditic and protandrous with an acropetal flowering sequence. After the male phase of the flowers, the style elongates, lowers down, and occupies the position previously held by the anthers. Therefore, the successful stigma contact ratio can increase or decrease depending on the early or late flowering phases (Claßen-Bockhoff 2017). *Salvia candidissima* and *S. cyanescens* are gynodioecious, a sexual system with both hermaphroditic and female individuals occurring within a population (Darwin 1877; Cardoso et al. 2018). In gynodioecious populations, female flowers have distorted stamens with upper lever arms highly reduced and no pollen-producing anthers (fig. 2L).

#### Pollinator Observations

To estimate the visitation rate of pollinators, we observed insect visitors (table A1; tables A1–A3, A5 are available online) for more than 220 h (about 100 h in study area 1, about 60 h in study area 2, and about 60 h in study area 3), covering the entire flowering period of the *Salvia* species, including all daylight hours and weather conditions. An insect was classified as a pollinator only when it came into contact with both the pollen sacs and the stigmatic surface. Observations were documented with photographs and videos using Canon 80D, 70D, G10, and 5D digital cameras as well as a Sanyo TH1 video camera. We filmed the behavior of visitors on both the flowers and the inflorescences. If the studied *Salvia* population was small and there were few visitors, we tried to film the visitor until it left the population. On the other hand, if the studied *Salvia* population was large, with many visitors and flowers at the same time, we tried to film the individual visitor as long as possible in the inflorescence and in the population. We included previous video records and observations made for *S. virgata* and *S. verticillata* subsp. *amasiaca* (Celep et al. 2014). On the basis of 135 videos and field observations, the handling times of the main bee visitors were calculated (table A2), and the same information for fly visitors was obtained from Celep et al. (2014). To determine the handling time, we tracked the movements of 185 bees using video records in slow-motion mode. Because of pollinator scarcity and fast pollinator movements from flower to flower, only a limited number of video records were obtained for *S. blepharochlaena*, *S. absconditiflora*, *S. hypargeia*, *S. candidissima*, and *S. cyanescens*. However, detailed observations done by eye, extensive notes, and photographic documentation dur-

ing field studies allowed for the determination of pollinator behavior, pollen loading areas on pollinator bodies, visiting sequences to flowers, and handling times for each of these species.

The videos and field observation notes were also used to reconstruct the percentage of legitimate manipulations of the lever mechanism (defined as pollen placement) and the percentage of stigma contact of the main pollinator species (table A2). These percentages were obtained based on the number of legitimate visits relative to all visits by that pollinator to each *Salvia* species. Stigma contact is defined as when the pollinator touches the stigmatic surface with the pollen-bearing part of its body so that pollen can be transferred to the stigma. The site of pollen placement on an insect's body was easily determined by the yellow or white color of the pollen grains and by the increase in the yellow or white spots on the insect's body after repeated flower visitation. Because of the high year-to-year flux in pollinator numbers and their visitation frequency, pollinator visiting frequency was classified as high (+++, observed continuously at a plant in almost every visit in the flowering period), medium (++, number of observations per flowering period, between 10 and 50 times), or low (+, number of observations per flowering period, <10 times). More than 180 captured bees and flies that were first observed as flower visitors were examined under a dissecting microscope to verify the site of pollen placement and to obtain morphometric measurements of the pollinator. Pollen grains were removed from the insect's body and compared with pollen grains from the studied species using a light microscope. To ensure that pollen grains belonged to these species, the size, shape, and aperture type of the pollen grains were compared with the results of a recent palynological study (Özler et al. 2011, 2013). Insects were identified by F. Dikmen. Voucher specimens of the plants and insects were deposited at the Department of Biological Sciences, METU.

#### Morphometric Measurements

To examine morphological matching between flowers and floral visitors, 11 floral (table A3) and nine insect morphological traits (table A4, deposited in the Dryad Digital Repository, <https://doi.org/10.5061/dryad.fttdz08qr>; Celep et al. 2020; Wester and Claßen-Bockhoff 2006b; Celep et al. 2014) were measured using digital calipers and a Leica DM1000 light microscope (fig. A2). We measured at least 20 haphazardly selected flowers from different individuals of each *Salvia* species and 1–12 individuals of each pollinator species. The nectar stand, or the distance from the floral tube entrance to the nectar source, was determined in at least 20 flowers that were collected haphazardly and dissected. The average value of these measurements was subtracted from the floral tube length and was defined as the length pollinators have to bridge to gain nectar. To determine the length of the mouthparts of the bee species, the maxilla was measured but not the tongue length (for technical reasons). We estimated the tongue (glossa) length of the main pollinators by using data taken from specimens with fully exerted mouth parts ( $n = 20$ –30 per species) from the Senckenberg Museum, Frankfurt am Main, Germany (E. Tweraser and R. Claßen-Bockhoff, unpublished data).

Additional floral morphometric measurements were made as some insect visitors can penetrate into the corolla tube of some larger-flowered *Salvia* with their head and thorax parts. For

example, *S. blepharochlaena* and *S. recognita* have extremely large corolla tube entrances because of their corolla morphology and size; their lower staminal lever arms do not fully close the corolla tube entrances and thus allow large *Bombus argillaceus* and *Anthophora* species to penetrate farther into the tube. Similarly, *Apis mellifera* can partly enter its head into the corolla tube of *S. absconditiflora*. Therefore, morphological comparisons between the nectar stand distance and the maximum length accessible to the pollinator (fig. A2G) were done for some insects: we separately calculated glossa length; head plus glossa length; and the sum of head, thorax, and glossa length (table A4). In addition, the main floral (i.e., flower size, color, stamen type, flower number per individual) and vegetative characters (i.e., growth form, stem height), flowering time, and population size information are given in table A5.

Statistical Analyses

Floral and pollinator morphometric calculations (minimum, maximum, mean ± SD, box plots) to show morphometric fit and possible legitimate visitation between flowers and pollinators were done with Microsoft Excel 2016. For example, large flowers, which tend to ecological specialization, were able to pollinate only with large bees, which must have a large body size to contact pollen sacs and long glossae to reach nectar in the corolla tube because of the long upper staminal lever arms of the flowers. A *t*-test with two independent means (*P* < 0.05) was used for analyzing differences in flower size between two populations of *S. absconditiflora* (in the appendix, available online) with Minitab (ver. 13.1, Minitab, State College, PA).

Pollinator network analysis was done in the R package bipartite (Dormann et al. 2008) using RStudio (ver. 1.2.5019). In the pollinator network analysis, flower visitors and their visiting frequencies were shown for each studied taxon on the basis of flower color, studied clades, and pollinator functional group. Pollinator network analysis has been shown to be a useful tool to show links between flowers and their pollinators visually and to identify ecological specialization versus generalization (Cars-

tensen et al. 2018). *Salvia* species with a single primary pollinator in terms of visitation would be classified as ecological specialists, whereas species with several to many pollinators, each with a variety of visitation numbers, would be classified as ecological generalists. The data for pollinator network analysis were collected from all study sites between 2010 and 2014. However, the data were collected for *S. blepharochlaena* from study site 2 and for *S. recognita* from study site 3. For the other 10 species, the data for pollinator network analysis were mainly obtained from study site 1. *Salvia absconditiflora* and *S. hypargeia* grow in both study area 1, with a big population, and study area 2, with a small population. Although there were different visitor species in each study site, the pollinator network analyses were done with data combined from both study sites.

Results

The flowering times of the 12 *Salvia* species from the three study sites are provided in figure 3. Detailed information about the population sizes of each *Salvia* species and pollinator diversity at the three study sites is provided in the appendix. In addition, specifics of the pollination process (pollinators, visitation numbers, pollinator behavior, nectar robbing) for each *Salvia* species from different subclades with different stamen types are also provided in the appendix.

Pollinator Network Analysis: Clades, Flower Color, Pollinator Features

Variation in the number of pollinator species per individual species of *Salvia* was clearly shown in the pollinator network analysis (table A1; fig. 4). A total of 39 bee and three fly species visited the *Salvia* species across all three populations. For example, *S. blepharochlaena* is a pollinator specialist that mainly relies on the large *Bombus argillaceus* for pollination. On the other hand, *S. virgata* is a pollinator generalist that uses 23 different pollinators.

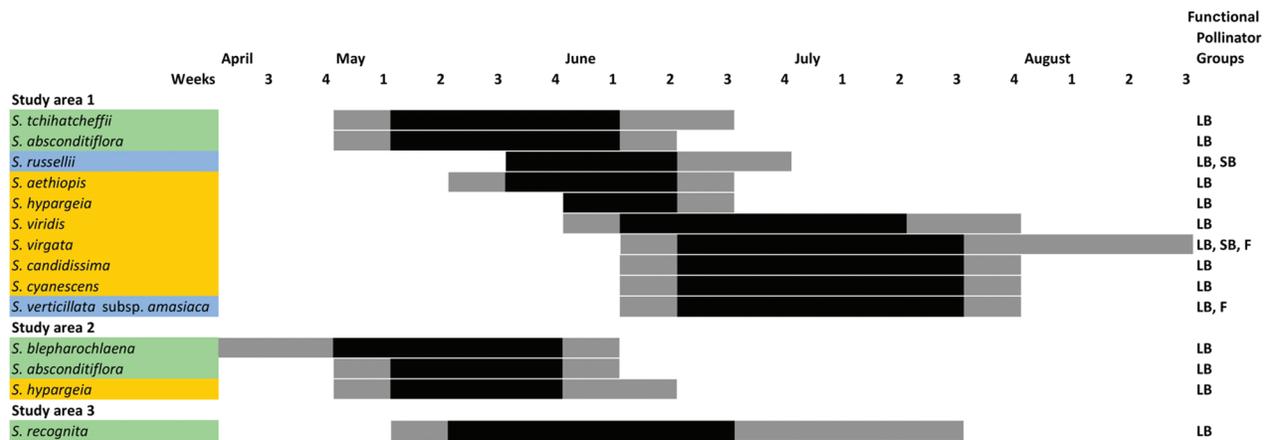
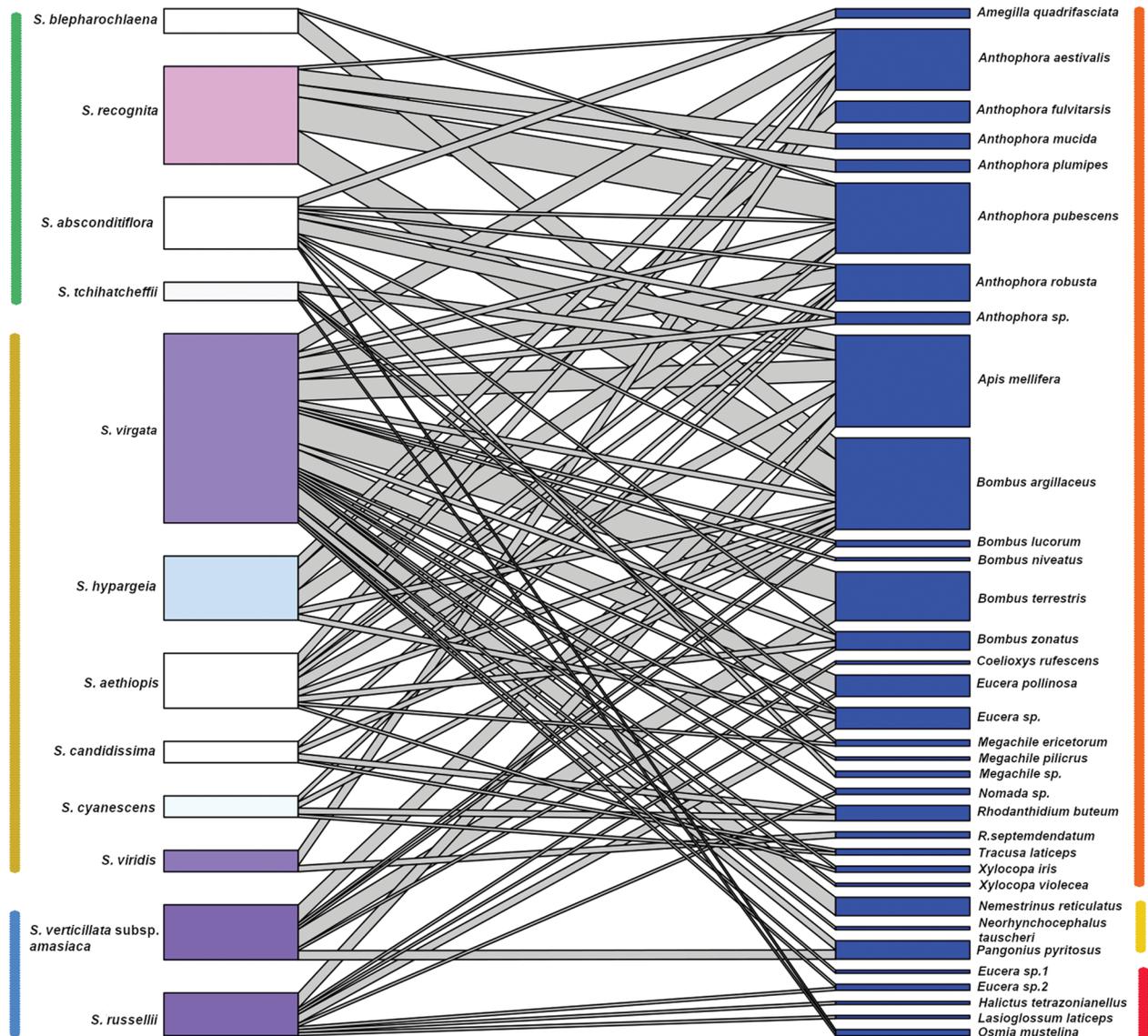


Fig. 3 Flowering times of the studied species at the three study sites. Gray areas show early or late flowering times, indicating relatively few flowers in the populations. Black areas show the main peak of the flowering time. Green-highlighted species belong to the subg. *Salvia* clade, yellow-highlighted species belong to the subg. *Sclarea* clade, and blue-highlighted species belong to the *S. verticillata* clade. F = fly; LB = large bee; SB = small bee.



**Fig. 4** Pollinator network between *Salvia* species and their pollinators. Bars on the left display *Salvia* species, their flower colors, and clades (green: subg. *Salvia*, yellow: subg. *Sclarea*, blue: *S. verticillata* clade). Bars on the right display the pollinator names and their functional groups (orange: large bee pollinators, yellow: fly pollinators, red: small bees, mostly visitors). Bar width increases with the relative number of interactions between flowers and pollinators. Line thickness represents the number of insects visiting each *Salvia* species (see table A4).

Four species from the subg. *Salvia* clade were pollinated by 14 bee species (from seven genera). Six species from the subg. *Sclarea* clade were pollinated by 24 bee species (from nine genera) and three fly species (three genera). Two species from the *S. verticillata* clade were pollinated by 15 bee species (from seven genera) and one fly species (fig. 4; tables A1, A2).

Across all studied species of *Salvia*, the number of pollinators varied tremendously with floral color (fig. 4). White flowers are found in *S. blepharochlaena*, *S. absconditiflora*, *S. tchihatcheffii*, *S. aethiopsis*, and *S. candidissima*. The number of pollinators of white flowers varied from three (one common and two rare pollinator species) for *S. blepharochlaena* to eight for *S. aethiopsis*. Pink flowers are found only in *S. recognita*, which had four species of pollinators. Purplish-violet flowers occur in *S. virgata*, *S.*

*hypargeia*, *S. cyanescens*, *S. viridis*, *S. verticillata* subsp. *amasiaca*, and *S. russellii*. The pollinators of purplish-violet flowers exhibited the greatest number and variation: from two pollinators for *S. viridis* to 23 (20 bee and three fly species) for *S. virgata*.

All bee pollinators are classified as large (body size, >10 mm; Danfort et al. 2006; Vargas et al. 2017) except for six species (fig. 4): *Osmia mustelina* (9.2–9.6 mm), *Osmia versicolor* (6.5 mm), *Lasioglossum laticeps* (4.9–5.8 mm), *Halictus tetrazonianellus* (7.7 mm), *Eucera* sp. 1 (9.6 mm), and *Eucera* sp. 2 (9.2 mm). All small-size bees were observed visiting their respective flowers only a few times. The first four of the small-size bees also have short glossae/tongues (see Danfort et al. 2006). Therefore, all the main pollinators visiting these studied *Salvia* species

are classified as large-size and long-tongued bees. The glossa length is >10 mm in *B. argillaceus*, *Bombus zonatus*, *Anthophora mucida*, *A. pubescens*, *A. plumipes*, and *A. fulvitaris* (tables A1, A2).

*Anthophora*, with 10 species, was the most species-rich genus of pollinators visiting *Salvia*, followed by *Eucera* (seven species) and *Bombus* (five species). Pollinators visiting the most species of *Salvia* across the three study sites included *A. pubescens* (six species) and *B. argillaceus* (five species). *Apis mellifera* pollinated six species of *Salvia*, but it also robbed nectar from an additional four species (fig. 4; table A1).

#### Morphological Matching between Floral Reward and Pollinator Mouthparts

*Salvia blepharochlaena* and *S. recognita* have very long corolla tubes and very wide corolla tube entrances (table A3). Therefore, their main pollinators can insert not only their mouthparts but also their head, thorax, and some part of the abdomen (maximum accessible length) to access the nectar (fig. 2; table A4). Thus, not only glossa length but also body size is important for proper pollination (release of the staminal lever mechanism and stigma contact), and they assure mutual benefit between the pollinator and *S. blepharochlaena* and *S. recognita* (figs. 2, 5; tables A3, A4).

The remaining species in subg. *Salvia* (*S. absconditiflora* and *S. tchihatcheffii*), subg. *Sclarea* (*S. virgata*, *S. hypargeia*, *S. aethiopsis*, *S. candidissima*, *S. cyanescens*, and *S. viridis*), and the *S. verticillata* clade (*S. verticillata* subsp. *amasica* and *S. russellii*) have short corolla tubes and/or very narrow corolla tube entrances. Therefore, pollinators can insert only their mouthparts (glossae) into the corolla tubes to access the nectar. Thus, glossa length is a limiting factor for proper pollination in these species. Our detailed morphometric measurements from the flowers and pollinators show that the main pollinators' glossae are long enough to reach the nectar in the corolla tube (figs. 2, 5; table A4). However, the rare visitor *L. laticeps* cannot reach the nectar cover area because of the very short length of its glossa. It is an illegitimate visitor but does collect pollen grains from *S. tchihatcheffii*, *S. aethiopsis*, and *S. russellii*. However, because of the specific flower construction of *S. russellii* and its lack of a staminal lever mechanism, *L. laticeps* may contribute somewhat to its reproductive success. Similarly, *O. versicolor* cannot reach the nectar but can collect pollen grains from *S. tchihatcheffii*.

Across all studied species, the smallest flowers were found in *S. verticillata* subsp. *amasica* (7.5–10.1 mm,  $8.6 \pm 0.8$ ,  $n = 30$ ) and *S. russellii* (8.1–10.7 mm,  $9.4 \pm 0.7$ ,  $n = 30$ ; table A3). All visitors to *S. verticillata* had glossae that were longer than the distances between flower entrances and nectar stands and were effective pollinators.

### Discussion

Although there are about 1000 *Salvia* species distributed worldwide that have diverse floral traits and two main pollinator guilds (bees, bee flies, and birds), pollination ecology studies of *Salvia* are relatively scarce (Wester and Claßen-Bockhoff 2006a, 2006b, 2007; Celep et al. 2014, 2015; Huang et al. 2015; Ott et al. 2016). The lack of pollination ecology studies is particularly evident in the Irano-Turanian phylogeographic

region, a key area in terms of *Salvia* endemism and evolution (Celep and Dirmenci 2017; Will and Claßen-Bockhoff 2017; Kriebel et al. 2019). Here we present detailed perspectives on pollination ecology in a phylogenetic framework of 12 *Salvia* species that co-occur in Turkey in three study sites; eight of these (*S. blepharochlaena*, *S. recognita*, *S. absconditiflora*, *S. tchihatcheffii*, *S. hypargeia*, *S. candidissima*, *S. cyanescens*, and *S. russellii*) are studied here for the first time. These *Salvia* species belong to three different groups (subg. *Salvia*, subg. *Sclarea*, and the *S. verticillata* clade) with a wide range of flower sizes, colors, and shapes and three different staminal lever mechanisms. The data accumulated on floral biology, pollinators, and geographical area allow an evaluation of a pollination network for *Salvia* for the first time.

#### Prezygotic Isolating Mechanisms

The detailed analyses of pollination across 12 species of *Salvia* (see the appendix for specifics) presented here provide evidence for several isolating mechanisms between co-occurring species.

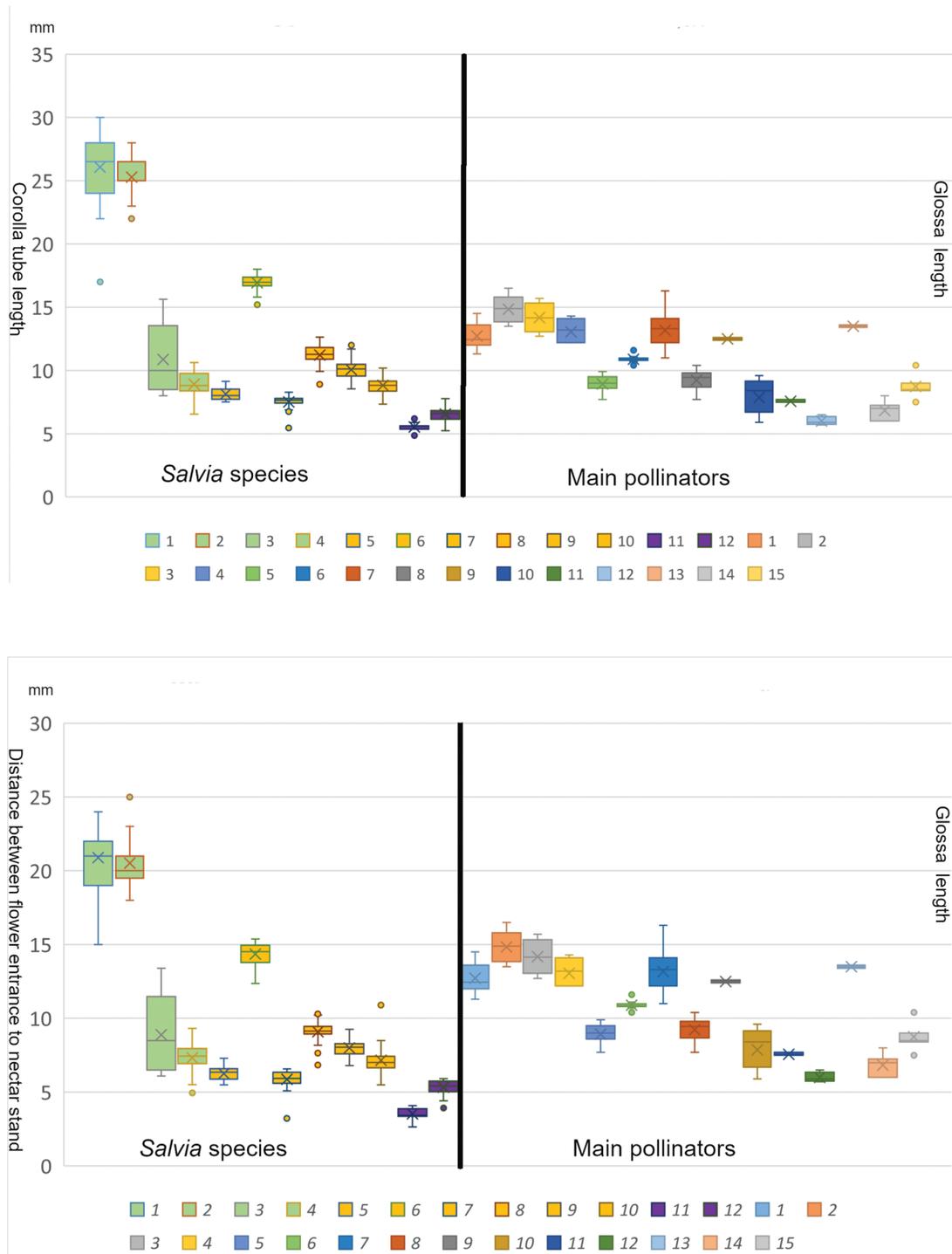
#### Phenological and Behavioral Isolation

Phenological isolation that prevented hybridization despite sharing of the same pollinators was well documented in study site 1. The 10 *Salvia* species flowered between early May and late August (fig. 3), but sets of species partitioned that flowering time into three time periods. For example, *S. aethiopsis* and *S. virgata* extensively shared pollinators, but *S. aethiopsis* flowered in the second time period and *S. virgata* in the third, with minimal overlap. Behavioral preferences by bees for certain species, despite the presence of other apparently suitable species, were documented for *S. blepharochlaena*, *S. recognita*, and *S. hypargeia*. Two closely related and similar-looking species, *S. russellii* and *S. verticillata* subsp. *amasica*, were visited by different pollinator assemblages. Although not tested, these two species appear to have different floral scent profiles, and pollinators may be cued by these features.

#### Mechanical Isolation: Examples from Both Flowers and Pollinators

Probably the best-documented isolating mechanism in this study, as well as in other studies of *Salvia*, is mechanical isolation (different flower sizes, staminal levers, pollinators, and pollen deposition). In a pollination ecology study of six sympatric species, Claßen-Bockhoff et al. (2004) described clear mechanical isolations occurring among the sympatric species. Wei et al. (2017) studied two coflowering sympatric *Salvia* species (*S. liguliloba* Y.Z.Sun and *S. bowleyana* Dunn), both pollinated by *Bombus trifasciatus*. They showed that pollen from anthers of the two species is loaded on different parts of the pollinator, thereby effectively eliminating natural hybridization and increasing effective pollination.

A diversity of forms of mechanical isolation appears to operate extensively among sympatric species of both subg. *Salvia* and subg. *Sclarea* in these study sites. Most species in the subg. *Salvia* clade have larger flowers (particularly, much larger corolla tubes) than species in subg. *Sclarea* and the *S. verticillata* clade do



**Fig. 5** Morphometric fit between corolla tube length and glossa length in the upper panel. Morphometric fit between distance from flower entrance to nectar stand and glossa length in the lower panel. The first 12 box plots show the studied *Salvia* species: *S. blepharochlaena* (1), *S. recognita* (2), *S. absconditiflora* (3), *S. tchibatcheffii* (4), *S. virgata* (5), *S. hypargeia* (6), *S. aethiopis* (7), *S. candidissima* (8), *S. cyanescens* (9), *S. viridis* (10), *S. verticillata* subsp. *amasiaca* (11), and *S. russellii* (12). The following 15 species show the main pollinators: *Anthophora pubescens* (1), *A. mucida* (2), *A. plumipes* (3), *A. fulvitaris* (4), *A. aestivalis* (5), *A. robusta* (6), *Bombus argillaceus* (7), *Bombus terrestris* (8), *Bombus zonatus* (9), *Apis mellifera* (10), *Eucera pollinosa* (11), *Rhodanthidium septemdentatum* (12), *Amegilla quadrifasciata* (13), *Pangonius pyritosus* (14), and *Nemestrinus reticulatus* (15).

(Will and Claßen-Bockhoff 2017; Kriebel et al. 2020). In subg. *Salvia*, we studied two large-flowered species (*S. blepharochlaena* and *S. recognita*), one medium-size species (*S. absconditiflora*), and one small- to medium-size species (*S. tchibatcheffii*). Here the size of the corolla tube effectively filters the set of bees as potential pollinators for each species because of the long and varying distance between the corolla entrance and the nectar stand—a common mechanical isolating feature seen in *Salvia* (fig. 5; tables A3, A4).

Species of the subg. *Sclarea* clade exhibited some of the most precise mechanical isolating mechanisms seen in this study. The pollination ecology and floral morphology of six species were evaluated. Their flowers are ideally suited to bee pollination (Hedge 1974; Westerkamp and Claßen-Bockhoff 2007) with small- to medium-size corollas (between 10 and 30 mm long), semifalcate to falcate upper lips, short corolla tubes, and narrow and fully closed corolla tube entrances with spoon-shaped lower lever arms (fig. 2I–2M). However, two floral features (corolla tube length and staminal lever construction) determine whether there is pollination success or pollinator exclusion, and species in subg. *Sclarea* have evolved a diversity of these two floral traits. The first, because of the very narrow and fully closed corolla tube entrance, is that the glossa length of pollinators should be equal to or longer than the distance between the corolla tube entrance and the nectar source (fig. 5). The second is that the pollinator's body should be longer than the upper staminal connective arm to allow for contact with both the pollen sacs and the stigma tip.

A clear example of this type of mechanical isolation is seen in three sympatric species from subg. *Sclarea*. *Salvia hypargeia* has the longest corolla (26–30 mm) and corolla tube (15–18 mm) and was pollinated by large *Anthophora aestivalis*, *A. fulvitaris*, *A. pubescens*, and *Bombus argillaceus*. On the other hand, *S. candidissima* and *S. cyanescens* have slightly shorter corollas (24–29 mm in *S. candidissima*; 20–27 mm in *S. cyanescens*) and corolla tubes (9–13 mm in *S. candidissima*; 8.5–12 mm in *S. cyanescens*). These latter species were also pollinated by *B. argillaceus*. However, the length of the upper staminal lever arm differs between *S. hypargeia* (12.2 mm), *S. candidissima* (17.4 mm), and *S. cyanescens* (13.8 mm; tables A3, A4). Therefore, when *B. argillaceus* visited all of these species in one study site, pollen grains were loaded on the thorax by *S. hypargeia* but on the abdomen by *S. candidissima* and *S. cyanescens* (table A2). Thus, slight differences in corolla and staminal morphologies can lead to mechanical isolation (Claßen-Bockhoff et al. 2004; Celep et al. 2014), here between *S. hypargeia* and related sympatric species. Not surprisingly, considering the shared pollinators and the similar placement of pollen on the abdomen, we observed some hybrid specimens of *S. candidissima* and *S. cyanescens* in the study sites, as was also previously reported (Hedge 1982).

A second example of this type of mechanical isolation involves *S. candidissima* and *S. cyanescens* relative to their smaller-flowered relatives *S. virgata* and *S. aethiopsis*. When the common pollinator *Apis mellifera* visited the larger-flowered *S. candidissima* and *S. cyanescens*, it did trigger the staminal lever mechanism. However, because of the smaller body size of the pollinator (10.8–11.5 mm; table A4) and the long upper staminal lever arms of the flowers (13.0–22.6 mm in *S. candidissima*; 8.0–18.0 mm in *S. cyanescens*; table A3), pollen sacs often did not contact the pollinator's back. On the other hand, *A. mellifera*

was quite successful in pollinating *S. virgata* and *S. aethiopsis* because of their shorter upper staminal lever arms (5.9–9.3 mm in *S. virgata*; 4.8–14.6 mm in *S. aethiopsis*). Thus, larger flowers (>20 mm) in subg. *Sclarea* effectively filter out some pollinators (e.g., *A. mellifera*) because of differential lengthening of the staminal lever mechanism relative to the pollinator body size.

#### Generalization versus Specialization in the Pollination Ecology of *Salvia*

In conclusion, our study shows that these 12 *Salvia* species, though phenotypically and functionally specialized because their zygomorphic and strongly bilabiate flowers display a bee-pollination syndrome, differ in their degree of specialization in pollination ecology. Our studies show that most of the sympatric *Salvia* species tend to be ecologically generalized, with two or more main pollinators and a few additional secondary pollinators (table A1), while a few *Salvia* species are clearly specialized. A diversity of pollinators was available at each study site, but species of *Salvia* varied in terms of how many pollinators they actually utilized. This is readily apparent when the pollinator network is examined (fig. 4). Specialization involves not only a smaller number of pollinator species but also more precise and effective pollen transfer mechanisms. *Salvia blepharochlaena* is a prime example of a pollinator specialist in that it almost exclusively relies on the large *B. argillaceus* for pollination. This specialization is probably driven by a suite of characteristics: *S. blepharochlaena* possesses a flower that is very white and a large corolla with a constricted corolla tube entrance, displays a short flowering time with a small number of flowers in a short inflorescence, and has a small population size. Conversely, *S. virgata* is the classic pollinator generalist, using 23 different pollinators, each to a different degree (see also Celep et al. 2014). This generalization is likewise probably driven by its own suite of characteristics: *S. virgata* has medium-size blue-violet flowers, a large number of flowers in a tall inflorescence, and the longest flowering season among the *Salvia* studied. Importantly, this generalization occurs in *S. virgata* despite the phenotypic and functional specialization of its staminal lever mechanism and nearly closed corolla tube entrance.

This study is the most comprehensive pollination ecological study of *Salvia* based on the number of species studied so far. This study is also the first to examine generalization and specialization in the context of plant-pollinator networks of *Salvia*. Such studies are important not only for the species-specific information they provide (Carstensen et al. 2018) but also because they are crucial as tools to assess the effects of invasive species, resilience to human-mediated landscapes or climatic changes, and conservation efforts for threatened and endangered species (Ballantyne et al. 2015). *Salvia* offers ample opportunities to explore these issues further using pollination ecology and pollinator-plant network analyses, as it has diversified around the world and in the context of quite different pollinators (Will and Claßen-Bockhoff 2017; Kriebel et al. 2019). As both floral evolution and phylogenetic relationships across *Salvia* are coming into focus (Kriebel et al. 2019, 2020), we have the opportunity to design and implement pollination ecology studies in different biomes and to use both trait (including floral color spectrum and scent) and phylogenetic history as contributors

to pollinator network structure more explicitly in *Salvia* (Chamberlain et al. 2014).

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