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The contribution of pollination interactions to the assemblage of dry grassland communities

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Abstract

Temperate semi-natural dry grasslands are known for the high biodiversity they host. Several studies attempted to pinpoint principles to explain the assembly rules of local communities and disentangle the coexistence mechanisms that ensure the persistence of a high species richness. In this study we examined the influence of pollination interactions on the assemblage of dry grassland communities and in the maintenance of the biodiversity they host. The issue has been addressed from many different perspectives.

We found that similarly to habitat filtering and interspecific interactions for abiotic resources, in dry grassland communities interactions for pollination contribute to influence plant species assemblage. We found entomophilous species flowering synchrony to be a key characteristic, which may favour the long lasting maintenance of rare species populations within the community. Furthermore, plants phenological synchronization was significantly associated with the sharing of pollinator guilds. Plant species exhibited different strategies to reduce competition for pollinators and pollen loss, which included specialization on different pollinators, and temporal and spatial segregation. Aggregation of synchronous pollinator-sharing species occurred among plants diverging in the anther position. Specialist plants flowered during the peak of the flowering season and were pollinated by generalist pollinators, while generalist plants flowered during the start and the end of the flowering season, and were pollinated by specialist pollinators. Thus, pollination interactions resulted in an asymmetric structure. However, while the asymmetric structure was observed to be maintained through the overall flowering season, the degree of selectiveness of the pollination network was not constant and increased in time, highlighting some critical moments on which conservation efforts should focus. Furthermore, we found conservation of dry grassland biodiversity to be improved by the co-occurrence of different dry grassland communities at the landscape scale, which influenced the structure of pollination networks, by favoring the maintenance of a higher richness of pollinators.

The assessment of pollination interaction in dry grassland communities helped us to reveal that pollinator availability was less limiting than expected for food-deceptive orchid species, which were found to be highly generalized for pollinators.

Lastly, beside plant-plant interactions for pollination we found that also an indirect pollination filtering might occur in dry grassland of different altitude, further confirming the contribution of pollination related processes to dry grasslands assemblage and maintenance.

Introduction and study framework

Biological extinctions are altering key processes pivotal to the productivity and sustainability of Earth's ecosystems. Evidence exists that current species extinction rates are higher than the pre-human background rate (May, 2010), with hundreds of anthropogenic extinctions documented in prehistoric and historic times (Stuart et al., 2004; Pereira et al., 2010). Further, species loss will accelerate changes in ecosystem processes (Hooper et al., 2012), but it is unclear how these effects occur in comparison to the direct effects of other forms of environmental change (e.g. land use changes, elevated CO₂, nutrient pollution, etc.) that are both driving diversity loss and altering ecosystem function. Biodiversity loss in the 21st century could rank among the major drivers of ecosystem change, and species loss is expected to reduce biomass production with effects on plant production comparable in magnitude to those of ultraviolet radiation and climate warming (Hooper et al., 2012). Understanding the side-effect of biodiversity loss on ecosystem functioning and services is thus currently a major aim of ecology (Loreau et al., 2001; Fontaine et al., 2006; Reiss et al., 2009).

Experimental evidence of diversity effects on the functioning of terrestrial ecosystems is mainly available for plants that, as primary producers, play a central role in the flow of energy within ecosystems. Overall, results show that declining plant diversity may damage such ecosystem properties as plant biomass, primary production, nutrient cycling and decomposition. Theoretical models that consider a single trophic level usually predict consistent patterns (e.g., Tilman et al., 1997).

However, several works suggested that changes in biodiversity may affect ecosystem processes through trophic interactions among species. Naeem et al. (2000) found that in aquatic microcosms, diversity at the level of producers (such as photosynthetic algae) and decomposers (bacteria) influenced ecosystem productivity. Duffy et al. (2003) showed that increasing grazer diversity in a seagrass system reduced both algal biomass and total community diversity. Finally, Montoya et al. (2003) suggested that changes in food-web structure and complexity could affect higher-level ecosystem services. However, even though consensus is mounting about the interdependence between biodiversity and ecosystem functioning (Sachs et al., 2009; Hooper et al., 2012), there are still contrasting views. For example, Schwartz et al. (2000) suggested that only few species are needed to sustain most processes and functions. Accordingly, increased attention was paid to processes involving relatively dominant species (Schwartz et al., 2000; Lawler et al., 2001; Cardinale et al., 2006). Recently, however, several authors have emphasized the role of the whole diversity, comprising subordinate species, in assuring ecosystem stability and functioning across space and time (Allan et al., 2011). All these findings concur to identify the understanding of how trophic interactions affect the relationship between biodiversity and ecosystem functioning as an important current challenge.

Plant–pollinator interactions play a central role in the reproduction of the plants and the life histories of the insects, having a marked influence on ecological community dynamics and diversity. Furthermore, animal-mediated pollination is one of the essential critical ecosystem services provided to humankind (Costanza et al., 1997; Kearns et al., 1998), with 75% of crops grown worldwide, benefiting at least to some degree from animal-mediated pollination (Martin, 2015). Thus, the comprehension of such functional relationships between species of two different trophic levels, which reciprocally influence each other, is of great importance for the conservation of their populations and the maintenance of the ecosystem services that pollination provides. Pollination systems are currently under threat of anthropogenic extinction due to habitat fragmentation, land use change, modern agricultural practices, use of chemicals such as insecticides and herbicides, and invasions of non-native plants and animals (Kearns et al., 1998). Under this scenario, increasing concerns have been raised regarding the "pollination crisis"; in fact, the potential decline of pollinators, both in terms of species richness and abundance, might have critical implications for both wild flowers and crops. To improve conservation of wild pollinators, several actions have been implemented worldwide, from the sowing of wildflower strips (Feltham et al., 2015) to the banning of insecticides.

However, conservation of natural or semi-natural habitats such as wildflower-rich grasslands have been suggested as one of the most effective measure in pollinator species conservation (Orford et al., 2016). Being included in oligo- to meso-trophic semi-natural communities, European temperate dry grasslands are an example of great interest: at small spatial scales they are among the most diverse plant communities of the world, mirroring intricate coexistence mechanisms (Wilson et al., 2012). Moreover, they support a significant proportion of rare plant and pollinator species and characterize a typical traditional agro-environmental landscape where mowing, grazing or fire were the dominant land use types (Wellstein et al. 2014). In Europe dry grassland communities are included in the habitat 6210(*) "Semi-natural dry grasslands and shrubland facies on calcareous substrates (Festuco-Brometalia)" by the 92/43/EEC Directive, and are considered 'habitat of priority importance' if they host populations of orchid species (EU Commission, 2013). However, dry grasslands are facing a significant decrease both in quality and surface due to changes in land use and farming practices throughout Europe (Stoate et al., 2001; Poschlod et al., 2005), and they are currently listed as Vulnerable in the European Red List of Habitat (Janssen et al., 2016). In the light of these considerations, the general aim of this research project was to assess the influence of pollination interactions on the assemblage of dry grassland communities and on the maintenance of the biodiversity they host. The issue has been addressed from many different perspectives.

In the 1st chapter "Does flowering synchrony contribute to the sustainment of dry grassland biodiversity?" we tested whether in dry grassland communities there is a non-random flowering pattern and if the pattern influences species richness, and the richness of rare and common species. Our findings suggested that flowering synchrony might be a key characteristic which contributes to shape dry grassland composition by favouring the long lasting maintenance of rare species populations within the community. Given the high degree of flowering synchrony, pollinators can be regarded as a limiting resource for which entomophilous plant species might interact to assure pollination, an event pivotal for their reproduction and population maintenance.

In the 2nd chapter "New insights into plants coexistence in species-rich communities: the pollination interaction perspective" we explored if spatially aggregated co-flowering species exhibit suites of reproductive traits capable to minimize competition for pollination and pollen loss due to heterospecific pollen transfer, thereby assuring a steady co-existence of a high number of animal-pollinated species. Our study provided evidence that similarly to habitat filtering and interspecific interactions for abiotic resources, in dry grasslands interactions for pollination filter plant species by reducing competition for pollinators and pollen loss through specialization on different pollinators, or temporal and spatial segregation. Aggregation of synchronous pollinator-sharing species occurred among plants diverging in the anther position.

In the 3rd chapter "The resilience of pollination interactions: importance of temporal phases" we examined if a temporal approach can help to reveal critical moments during the flowering season, when the pollination network may be less resilient to perturbations, and if pollination interactions evaluated at species, guild and network level show different patterns when assessed through time. Our results pointed out that when taking into account the temporal dimension of interactions, in dry grassland community differences could be detected at different levels of organization (i.e., species, guild and network). If, at the species level, no relationship was disclosed between partner diversity and time, when assessing the temporal trend of partner diversity separately for the guild of plants and pollinators we could observe an asymmetric structure of interactions. Pollination interactions showed to be asymmetric throughout the flowering season, however evenness of interactions and network selectiveness showed a significant positive relationship with time, revealing a poorer network of interactions during the end of the flowering season. The temporal analysis of pollination interactions revealed a stronger risk of secondary extinctions during the end of the flowering season, due to a lower degree of redundancy and thus of resilience of the overall network.

In the 4th chapter "Co-occurring grassland communities: the functional role of exclusive and shared species in the pollination network organization" we evaluated the effects of co-occurring dry grassland communities (pioneer vs. late-successional) on the patterns of pollination interactions at the landscape scale. In fact, grasslands importance for the provision of pollination services in agro-environments has led several studies to attempt to find which condition mostly contributes to the maintenance of plant and pollinator species richness. Different grassland communities were found to support different plant and pollinator species, however the biodiversity resulting from the co-occurrence of different grassland communities at the landscape scale has to be clarified. Our results highlighted that the co-occurrence of different grassland communities plays a key role in the maintenance of pollination network stability at the landscape scale and in the sustainment of species requiring their co-occurrence to survive.

Since orchid species presence in dry grasslands is especially important from both the ecological and policy point of view, in the 5th chapter "Are food-deceptive orchid species really functionally specialized for pollinators?" we explored if food-deceptive orchid species typical of dry grassland communities are pollinator limited. In fact, food-deceptive orchid species have traditionally been considered pollination specialized to bees or butterflies. However, it is unclear to which concept of specialization this assumption is related; whether to that of phenotypic specialization or of functional specialization. In contradiction to expectations derived from the phenotypic point of view, food-deceptive orchid species were found to be highly functionally generalized for pollinators, and no differences in the pollinator guild spectra could be revealed among orchid groups.

In the 6th chapter "Altitudinal patterns of floral morphologies in dry calcareous grasslands" we verified if in dry grassland communities there is a non random distribution of different blossom types along an altitudinal gradient, which may exert a selective pressure on both plants and insects, as well as on their mutualistic relationships. Our study revealed that altitude might affect species assemblage in dry grassland communities not only by selecting plant species according to their tolerance to different environmental conditions, but also according to their floral morphology, evoking the possibility of an indirect pollination filtering to occur.

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Does flowering synchrony contribute to the sustainment of dry grassland biodiversity?



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ABSTRACT

Phenological relationships among entomophilous species for pollination may play an important role in structuring natural plant communities.

The main aim of this work was to test whether in dry grassland communities there is a non-random flowering pattern and if the pattern influences the species richness, and the richness of subordinate and common species.

Field sampling was carried out in temperate dry grasslands in NE Italy. Species composition and the flowering phenology were monitored in 45 2 m × 2 m plots randomly placed over dry grasslands.

To quantify the degree to which insect-pollinated species overlap in their flowering time we developed a “co-flowering index” (CF-index). The significance of the observed flowering pattern was tested using a null model.

A positive correlation was found between the synchronic flowering and the number of subordinate species. Subordinate species showed shorter flowering length than the common species and a mostly specialized pollination system.

Our findings suggest that flowering synchrony might be a key characteristic which may contribute to shape dry grassland composition by favouring the long lasting maintenance of rare species populations within the community.

The comprehension of such functional relationships between species of different trophic levels is of great importance for the conservation of dry grasslands and the maintenance of the ecosystem services that pollination provides.

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1. Introduction

Biotic interactions have long been investigated as a possible mechanism governing and maintaining species richness within a community (Brooker et al., 2008; Bonanomi et al., 2011; Hallett et al., 2014). Among the wide variety of biotic interactions, phenological relationships among entomophilous species for pollination have been hypothesized as playing an important role in structuring natural plant communities (Feldman et al., 2004; Moeller, 2004; Ghazoul, 2006; Callaway, 2007; Brooker et al., 2008). Polli-

nators are assumed to exert comparable selection pressures, thus generating correlations among floral traits (e.g., color, long and narrow corolla tubes, or particular nectar quantities and concentrations) (Armbruster et al., 1999, 2000; Fenster et al., 2004). Although generalist pollination systems are frequent on a global scale, also specialization is common and is assumed to have been integral to angiosperm diversification (Johnson and Steiner, 2000). Most angiosperms produce recognizable suites of convergent floral traits and reward sources that recur in flowers of different evolutionary origin but that share similar pollinators (Rodríguez-Gironés and Santamaría, 2004; Thomson and Wilson, 2008; Willmer, 2011; Sonkoly et al., 2016). In this way, plant-plant relationships may have been modified in order to ensure and/or enhance the visitation rate, eventually influencing community structure and dynamics. Especially, the sharing of the same temporal niche (synchronous flowering periods) by different plant species increases the abundance of floral resources. This in turn could enhance the possibility

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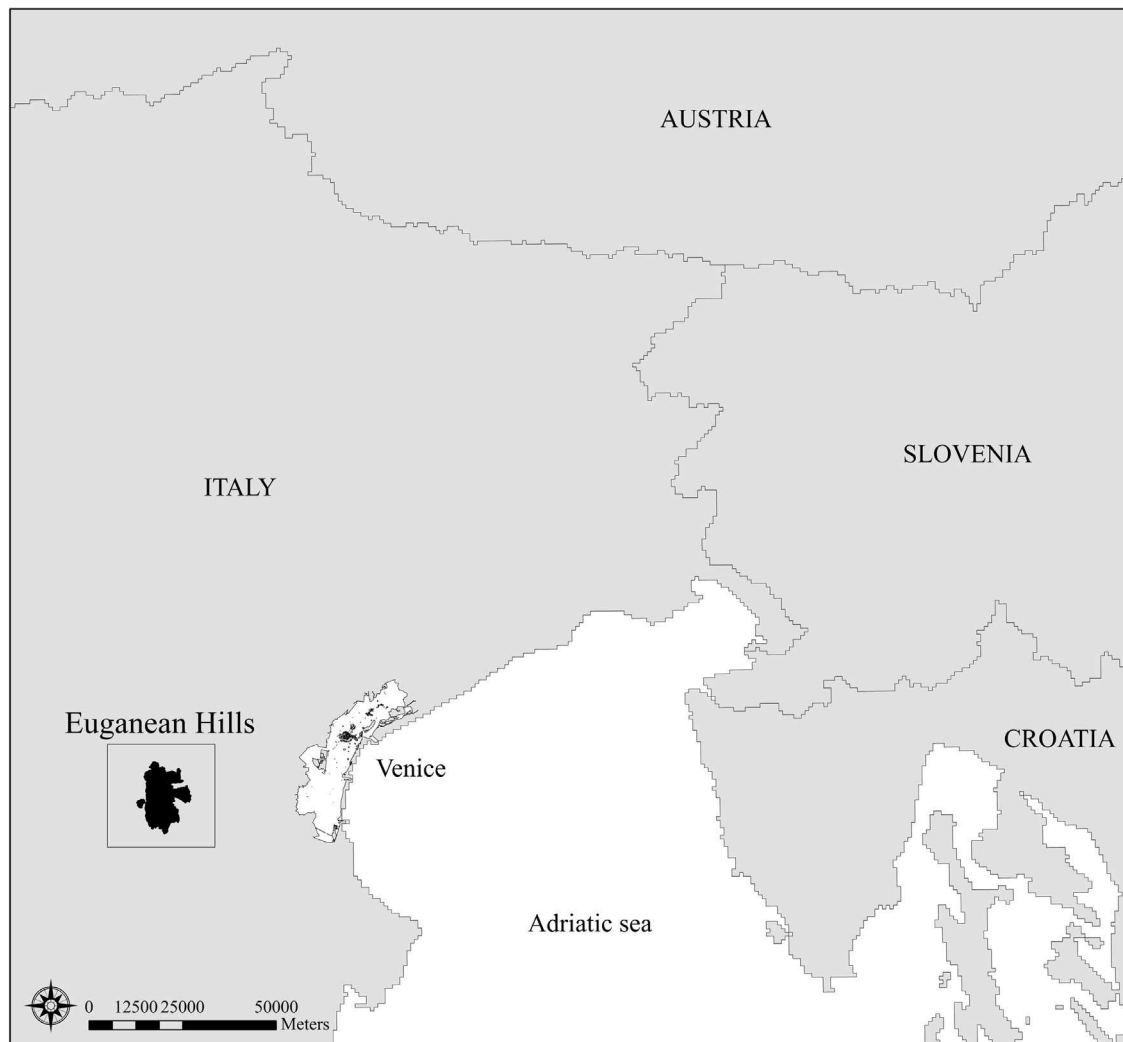


Fig. 1. Study area. Euganean Hills are enclosed within the black square.

of pollinator sharing and ensure the survival of rare and extremely selective entomophilous species (Lavery, 1992; Johnson et al., 2003; Ghazoul, 2006).

The phenological synchronization of flowering and fruiting is supposed to affect inter-specific patterns at several scales, from population to community and landscape (Frankie et al., 1974; Ollerton and Lack, 1992, 1998; Bronstein, 1995; Vilela et al., 2014; Borchert et al., 2005). For example, asynchronous ripening of fruits among certain species in a community was interpreted as a mechanism for avoiding competition for seed dispersers (Wheelwright, 1985). Conversely, flowering synchrony of individuals in a given population has been proved to influence both the quantity and genetic quality of their offspring, by affecting the number of potential mates and the foraging efficiency of pollinators (Schemske, 1977). At a different scale of observation, Dante et al. (2013) revealed how relationships among co-flowering entomophilous species drive species assemblage and distribution within an old-field plant community, highlighting the importance of pollination interactions in influencing ecosystem structure and functioning.

During the last decade, there has been a consensus in ecological and environmental sciences about the interdependence between species richness and ecosystem functioning (Sachs et al., 2009; Hooper et al., 2005), although with contrasting views. Schwartz et al. (2000) proposed that this relationship generally follows an asymptotic trend, suggesting that only few species are needed to

sustain most processes and functions. Accordingly, increased attention was paid to processes involving relatively dominant species (Schwartz et al., 2000; Lawler et al., 2001; Cardinale et al., 2006). Recently, however, several authors have emphasized the role of the whole diversity, comprising less common species, in assuring ecosystem stability and functioning across time (Allan et al., 2011; Mouillot et al., 2013).

According to Tilman (2004), the abundance of a species is proportional to the amount of the habitat that has the environmental conditions that match the species requirements. Thus, less common species are those better adapted to less common environmental conditions in a habitat and are likely to possess functional traits distinct from those of common species. Indeed, rare or subordinate species have been recognized as increasing both species richness (Wellstein et al., 2014) and the functional diversity of communities (Richardson et al., 2012); they are also expected to support ecosystem functioning under future environmental conditions (Mouillot et al., 2013). At the same time, in the majority of ecological communities, rare species are expected to be at higher risk of extinction than dominant ones (Purvis et al., 2000). Therefore, the identification of patterns related to the maintenance of species diversity and, in particular, of the rare or subordinate species in ecological communities should be mandatory when investigating ecosystem functioning (Allan et al., 2011; Mouillot et al., 2013).

European temperate calcareous dry grasslands provide a particularly suitable example to this specific issue. Their biological diversity is high and includes a variety of rare and endangered species from different taxonomic groups (Van Helsdingen et al., 1996; Szymank et al., 1998). They are among the most diverse plant communities of the world at small spatial scales, mirroring intricate coexistence mechanisms (Wellstein et al., 2014; Dengler et al., 2014). A high species richness is also found for butterflies and other invertebrates (Bobbink and Willems, 1988; Van Swaay, 2002). In Europe, semi-natural dry grassland make up the majority of High Nature Value farmland and harbor the majority of EU farmland biodiversity (Collins, 2012; Habel et al., 2013). Given their importance they are listed in Annex I of the EU Habitat Directive (CE 43/92) as priority habitats, i.e. “natural habitat types in danger of disappearance”.

In light of these considerations, this work’s main aim was to analyze flowering synchrony among entomophilous species in temperate dry grassland communities and to elucidate the relationship between the flowering pattern and the structure of the community, in terms of rare and common species.

Specifically, we addressed the following questions (i) is there a non-random flowering pattern among entomophilous species in dry grassland communities? (ii) Does the flowering pattern influence the total species richness and the relative richness of subordinate and common species at community level? And (iii) do subordinate and common plant species show different patterns in the flowering time length and in the pollination system (i.e. generalized vs. specialized).

2. Material and methods

2.1. Study area and site selection

Field sampling was carried out in the Euganean Hills (NE Italy), a group of volcanic hills extending for 15096 ha (Fig. 1; pictures in Supplementary material). The area is a historically cultivated landscape with a mosaic of arable fields, semi-natural grasslands and deciduous forests. The site has been under protection since 1989 and was recently included in the Natura 2000 network as SCI/SPA. Bioclimatic classification, performed according to the Worldwide Bioclimatic Classification System (<http://www.globalbioclimatics.org/>), shows a Temperate-Oceanic type.

The study was conducted on semi-natural, oligo-to mesotrophic, *Bromus erectus*-dominated dry grasslands. They establish on poorly developed, shallow and skeletal, calcareous soils characterized by low water retention capacity and high leaching rates (Bini, 2001). The community structure is mainly determined by hemicryptophytes and, subordinately, chamaephytes. The floristic composition of the Euganean dry grasslands reflects their particular geographic position, in a transitional connection area among the Mediterranean, the Alpine and the South-Eastern European phytogeographical regions (Villani et al., 2016). The vegetation is dominated by few, highly covering, anemophilous species (e.g. *B. erectus*, *Artemisia alba*, *Catapodium rigidum*, *Koeleria pyramidata*, *Carex hallerana*, *Bothriochloa ischaemum*) and numerous, scarcely covering, entomophilous species, the most common of which include *Bupleurum gussonei*, *Scabiosa triandra*, *Fumana procumbens*, *Helianthemum nummularium* ssp. *obscurum*, *Convolvulus cantabrica* and *Globularia bisnagarica*. Based on recent syntaxonomic revision (Terzi, 2015), Euganean dry grasslands can be included in the *Festuco-Brometea* Br.-Bl. & Tx. ex Klika & Hadač 1944 class and the SE-European-Illyrian order *Scorzoneretalia villosae* Kovačević 1959 (= *Scorzonero-Chrysopogonetalia*), represented by several diagnostic species, with high frequency values (*Chrysopogon gryllus*,

Dorycnium pentaphyllum ssp. *herbaceum*, *Eryngium amethystinum*, *Salvia pratensis* ssp. *bertolonii*, *Sanguisorba minor*, *S. triandra*, etc.). Given the high percentage of Mediterranean and Illyrian taxa and the phytogeographical and ecological context, the community can be referred to the alliance *Saturejion subspicatae* Tomić-Stanković 1970.

Originally, sampled grasslands had been managed by low-intensive mowing practices. Since the foundation of the Park and the SPA/SCI management plan, they have been subject to yearly mowing and grazed every three years.

2.2. Data collection

We selected 15 dry grassland areas extending from 0.27 ha to 6.95 ha (ranging from 68 m a.s.l. to 284 m a.s.l.). Altogether 45 plots of 2 m × 2 m (4 m²) were selected using a stratified random sampling design. For each plot, species composition was recorded and flowering phenology of all entomophilous species was monitored every ten days (1st April–26th September in 2015) for a total of eighteen monitoring intervals. Flowering was considered started when the first flower was observed to be open (Pleasants, 1980) and ended when individual plants no longer possessed any flower with anthers (Dante et al., 2013). All species surveyed in the plots flowered within the monitoring period and no differences in flowering time were observed among individuals of the same species occurring in different plots. Species nomenclature was standardized following Conti et al. (2005).

Plant traits have been widely used to detect general ecological patterns irrespective of species identity (Lavorel and Garnier, 2002; Díaz et al., 2004). When applied to floral characters (e.g. position of the nectar in the flower, flower shape, floral symmetry, flower openness and accessibility of floral rewards), floral traits combinations allow the identification of functional groups of flowers and corresponding functional groups of flower visitors. This, in turns, allows to define the type of pollination system and determine plant species degree of specialization. Following Ellis and Ellis-Adam’s classification (in Corbet, 2006), all the entomophilous species recorded were grouped on the basis of the accessibility of nectar. Three categories of entomophilous plant species were recognized: (a) allophilous species, which have fully exposed nectar; (b) hemiphilous species, with partly concealed nectar, and (c) euphilous species, that have deeply concealed nectar. Allophilous and hemiphilous species can be visited by a high number of small, short tongued insect species, and some of medium size and tongued length (Corbet, 2006; Willmer, 2011); thus, their pollinator spectrum is wide and the pollination system can be regarded as generalized. Euphilous species, on the contrary are mainly visited by large, long tongued insect. Given the restricted spectrum of effective pollinators, their pollination system can be considered as specialized (Corbet, 2006; Willmer, 2011). On this basis, entomophilous species were grouped according to the pollination system, as “generalists” (allophilous and hemiphilous) and “specialists” (euphilous).

2.3. Data analysis

2.3.1. Flowering synchrony

Several quantitative methods have been proposed to measure flowering synchrony (hereafter FS) (Sargent and Ackerly, 2008). Most of them have been developed to quantify overlap in flowering time at the level of population or individual plants within a population (e.g., Refs. Primack, 1980; Augspurger, 1983; Marquis, 1988; Mahoro, 2002; Freitas and Bolmgren, 2008). On the contrary, the measurement of FS at the community level has received less attention. Dante et al. (2013) suggested the use of a niche overlap index based on the number of flowering plants per species (Schoener,

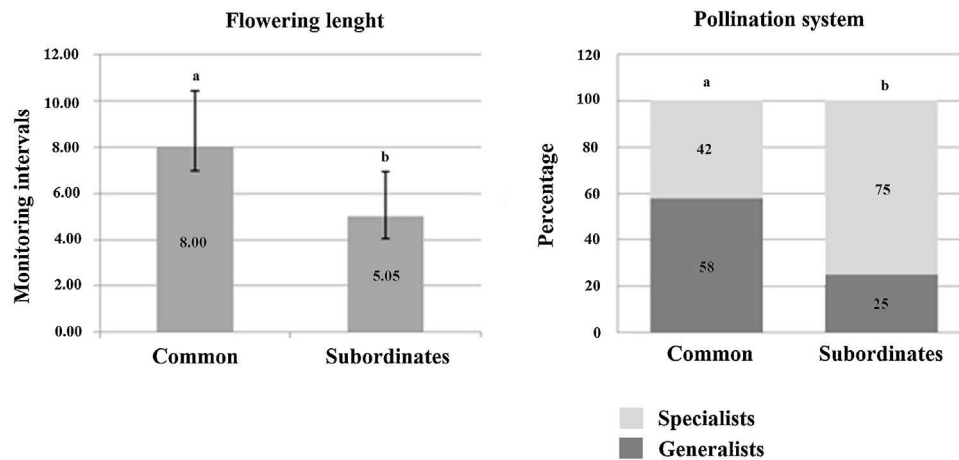


Fig. 2. Flowering length and pollination system of common and subordinate species. Values of the flowering length written on bars are the means of the monitoring intervals \pm standard deviation. Values of the pollination system are the ratio “generalists: specialists” within common and subordinate species. Different letters indicate significant differences according to post-hoc Tukey HSD test.

1970; Pleasants, 1980). As an alternative, we propose to quantify interspecific FS by using a co-occurrence index. Co-occurrence indexes, such as V-score, are commonly used to evaluate the degree to which species co-occur spatially (Lepš and Šmilauer, 2003). We transposed this concept to flowering phenology to assess the temporal co-occurrence of flowering in the studied species. We called this co-occurrence flowering index “CF-index”.

Similarly to V-score, to calculate CF-index we created a presence-absence matrix (flowering matrix), where rows were species (101 entomophilous species) and columns were the monitoring intervals (18 monitoring intervals). Entries represented the presence (1) or the absence (0) of the flowering event. We then calculated the CF-index by correlating the strings of each sampled species to each other through Pearson’s correlation (Lepš and Šmilauer, 2003). As a result, species that tend to flower in the same time interval (i.e. in a completely synchronic pattern) have a positive CF-index ($CF \sim 1$) while species that tend to flower in a diachronic pattern have a negative CF-index ($CF \sim -1$). Finally, no correlation in the flowering span ($CF \sim 0$) arises between those species which show an incomplete FS or no synchrony at all. We then calculate the mean of the CF-indexes for all the species pairs in the matrix in order to assess the mean flowering pattern in our data.

To test for the significance of the observed mean CF-index, we performed a null model test. We built a null model which, in each randomization of the matrix, held constant (constrained) the sum of entries across each rows (“Frequency” null model, Gotelli and Graves, 1996; “picante” R package). In this way, by constraining the sum of entries, the flowering span of each species stayed constant in each randomization. Furthermore, to distinguish early flowering from late flowering species, permutations between columns were allowed only within groups belonging to similar flowering periods. Therefore we constrained the randomization only inside two sub-matrices (from April to July and from July to September). The randomization of each sub-matrix was replicated 999 times, after each time the CF-index and its mean were calculated. We then compared the observed mean CF-index to the distribution of randomized mean CF-indexes in order to assess the statistical significance (p -value) of the mean co-flowering pattern observed.

To reveal fine scale variations in the flowering pattern, we also generated a flowering matrix for each plot. Since no differences in flowering time were observed among individuals of the same

species occurring in different plots, these matrices can be considered as sub-matrices of the general flowering matrix. For each plot flowering matrix, as for the total matrix, the CF-index was calculated between each species pair and averaged over the plot. In this way, we obtained 45 mean CF-indexes, one for each plot, which reflect the degree of FS among species co-occurring in the same plot.

CF-index calculation and null model tests were performed on R statistical environment (R Development Core Team, 2008).

2.3.2. Plot level relationships between FS and total species richness and subordinate and common insect-pollinated species richness

Although extensively used in ecology and conservation biology, a sound definition of the rarity concept is still lacking (Landi and Chiarucci, 2014), since it involves both issues related to threatened or biogeographically rare species and simple abundance/frequency measures. We proceeded dividing species into common and subordinate according to their occurrence in the plots. The threshold level was set at 50% and corresponded to a natural break in species frequencies, in fact few species (12 species) were widespread and present in more than 23 plots (51% of the plots), while most species (89 species) occurred in less than 16 plots (36% of the plots). Accordingly, species with a frequency equal to or below the threshold level (but present in more than one plot) were classified as subordinate, while those which frequency was above the threshold were classified as common.

Spearman’s Rank correlation was used to analyze the influence of the mean CF-index on the total insect-pollinated species richness, and the richness of subordinate and common species.

2.3.3. Flowering pattern and pollination system in subordinate and common species

To test for significant differences in the flowering time length and in the pollination system (generalized vs. specialized) between subordinate and common species, we performed a PERMANOVA test with 9999 randomization (Anderson and Ter Braak, 2003). We used species frequencies (subordinate vs. common) as a grouping variable and the length of flowering span (number of monitoring intervals) and the pollination system as dependent variables. Post hoc Tukey test was performed by STATISTICA software (version 8, 2008) to identify significant differences.

Table 1

Plot level (n = 45) relationships between the CF-index and the total species richness, and the subordinate and common insect-pollinated species richness (Spearman's rank correlation coefficients (Rs) and P-values). Significant results are in bold.

	Rs	P
Total species richness	0.215	>0.05
Subordinate species richness	0.314	<0.05
Common species richness	0.036	>0.05

Table 2

Differences in the flowering length and in the pollinators range, between subordinate and common species, evaluated by PERMANOVA. In bold the significance.

PERMANOVA	
Permutation N:	9999
Total sum of squares:	428.3
Within-group sum of squares:	337.7
F:	30.69
P (same):	<0.001

3. Results

3.1. Flowering synchrony

When all entomophilous species were considered, their mean overlap in flowering span (CF-index) was significantly higher than expected by the null model ($P\text{-value}_{CFobs} > CF_{exp} < 0.001$), showing that entomophilous species tend to synchronize their flowering.

Many species pairs showed almost a complete FS (470 species pairs; $CF > 0.75$), many others shared at least part of their flowering time (1201 species pairs; $CF > 0.50$) whereas, only 10 species pairs showed a complete separate flowering period ($CF < -0.75$). Most common combinations of co-flowering species ($r > 0.75$) were between *Lamiaceae* and *Fabaceae*, *Asteraceae* and *Lamiaceae*, *Geranaceae* and either *Lamiaceae* or *Fabaceae*, *Orchidaceae* and either *Lamiaceae* or *Fabaceae*.

3.2. Plot level relationships between FS and total species richness and subordinate and common insect-pollinated species richness

No significant associations were found between CF-index and the total number of insect-pollinated species per plot (Table 1), demonstrating that richness in entomophilous species and the degree of flowering synchrony are totally independent.

A positive correlation was found between the number of subordinate species and the mean CF-index per plot (Table 1), while no significant correlation was observed for common species (Table 1).

3.3. Flowering pattern and pollination system in subordinate and common species

Significant differences in the flowering length and in the pollination system (generalized vs. specialized species) between subordinate and common species were revealed by PERMANOVA (Table 2). Post hoc Tukey test indicated that subordinate species are characterized by shorter flowering length than the common ones (Fig. 2). Moreover the pollination system was not equally distributed between subordinate and common species. In particular, most subordinate species showed a specialized pollination system (Fig. 2). Conversely common species did not evidence a comparable clear dominance.

4. Discussion

Dry grassland entomophilous species proved to overlap significantly in flowering time, revealing a non-random pattern of flowering, i.e. the degree of convergence in flowering time among species within the community differed from what would be expected if flowering spans were randomly attributed from a uniform distribution.

The observed synchrony may result from a combination of factors. On a broad scale, seasonal timing of flowering has been supposed to possess a phylogenetic component, with repeated patterns in the phenological events across plant lineages (Davies et al., 2013), i.e. the tendency for closely related species to be similar to each other more closely in their flowering phenology than expected by chance. Davies et al. (2013) also described similar phylogenetic patterns in long-term phenological response traits across geographically separated communities. Although phylogenetic studies have shown that especially flowering time is a particularly conserved trait within temperate phylads (Levin, 2006), we may assume that our results are not affected by the phylogenetic effect since the most common combinations of co-flowering species involved different plant families, well spread over published phylogenetic trees of angiosperm (Angiosperm Phylogeny Group, 2009).

A precise phenological timing is certainly linked to climate. Temperate habitats normally show flowering patterns with a strong seasonal bias, which may result from local resource availability peaking within a relatively narrow timeframe (Appanah, 1993; Dominguez and Dirzo, 1995). In particular, most temperate species flower in response to temperature, that defines the start and end of the growing season (Larcher, 2003; Schwartz, 2003; Inouye, 2008). Indeed, flowering pattern at community level broadly resembled the hump-shaped seasonal trend of temperature. Thus, local adaptation to environmental signals might have greater influence on species flowering phenology than taxonomic membership. Furthermore, temperate dry grasslands are mostly semi-natural communities maintained by mowing and grazing which have been proved to regulate species composition and richness (Valkó et al., 2011, 2012). With reference to the flowering time, it could be assumed that the timing of management practices, e.g. the time of hay-making and its regularity over years, could contribute to select species based on their reproductive cycle. However, species categorized upon their frequency evidenced significant differences in the flowering length which was also coupled with a different pollination system, providing further evidence supporting a non-random flowering pattern at community level.

A growing number of studies provided evidence that the flowering phenology is a crucial element of the ecology of plants and an important component of community assembly as it influences not only the relative abundance of species in a given ecosystem, but also their presence or absence (Sargent and Ackerly, 2008; Crimmins et al., 2011). The way in which flowering phenology affects the composition of plant communities is through its effect on species interactions, rising the potential for strong competition, but also facilitation, for pollination resources (Rathcke and Lacey, 1985; Callaway, 1995).

Indeed, several studies have investigated FS among plant species, although findings vary from study to study. Some research described reduced FS in response to competition for pollinators (e.g. Refs. Mosquin, 1971; Pleasants, 1980), to avoid heterospecific pollen transfer (Waser and Fugate, 1986; McLernon et al., 1996; Brown and Mitchell, 2001; Morales and Traveset, 2008) or the spread of pollinator transmitted diseases (Elzinga et al., 2006). Other studies have evidenced convergence in flowering time to ensure pollinator visits (Moeller, 2004; Ghazoul, 2006; Dante et al., 2013). The sharing of the same temporal niche (synchronous

flowering periods) by different plant species may increase the abundance of floral resources. This in turn could enhance the possibility of pollinator sharing to occur by increasing the visitation rate, leading to an amelioration of plant species reproductive success and, eventually, to the establishment of co-flowering species in the community (Waser and Real, 1979; Thompson, 1988; Moeller, 2004). Mutualistic interactions, such as pollination and seed dispersal, are good examples of the complex web of interactions that play a pivotal role in population establishment, reproduction and migration, and community development (Forup and Memmott, 2005). Flowering phenology can thus become a key characteristic in plant reproductive biology by favoring mutualistic interactions, thereby increasing the success of plant reproduction and their long lasting permanence in a site.

We found that the FS does not influence the total species richness, but when considering the richness of species with different frequency, FS turned out to positively influence the richness of subordinate species. Subordinate species are usually under stronger selective pressure to secure pollination than common plants (Gumbert et al., 1999; Dante et al., 2013). Population density is one of the most important spatial components influencing pollen flow and seed set (Morris, 1993; Richards et al., 1999; Brys and Jacquemyn, 2010). Plant density has been proved to influence the visitation frequency of pollinators per plant and the seed production, both of which tend to be lower in lower-density populations (Silander, 1978; Allison, 1990; Ohashi and Yahara, 1998). Moreover, our results revealed that subordinate species are mainly characterized by a specialized pollination system, and that they flower for particularly short time spans. On the other hand, common species showed both generalized and specialized pollination system, and flower for a longer periods than subordinate species. Thus, subordinate species may take advantage of flowering in the same periods of common species, which being visited by a consistent number of functional groups of flower visitors, could increase the attractiveness and the probability of pollination events to occur (McEwen and Vamosi, 2010). Thus, plant-plant relationships for pollinators may contribute to shape dry grassland communities composition, by maintaining subordinate insect-pollinated species which contribute for the most part to the overall diversity in species-rich grassland communities (Wellstein et al., 2014).

5. Conclusions and further perspectives

Species rich ecosystems have long fascinated ecologists. Our findings suggest to regard FS as an important process in influencing the species composition and richness of the community in temperate dry grasslands. Arguably, as plant-pollinator interactions play a central role in the reproduction of the plants and the life histories of the insects they likely have a marked influence in ecological community dynamics and diversity. Thus, the comprehension of the functional relationships between species of different trophic levels, which reciprocally influence each other, is certainly of great importance for the conservation of their populations and the maintenance of the ecosystem services that pollination provides.

Plant-pollinators relationships have been traditionally studied focusing on highly specific interactions between one or few plant species and constrained groups of pollinators. Little information is available at the community level. However, a community wide approach, i.e. in which patterns of interaction among all the plants and pollinators in a given area are studied simultaneously, is essential to account for community level processes (e.g. potential competitive or facilitative effects among species that belong to the plant-pollinator system, or patterns of communities assembly), which are often linked to species diversity. For example, our study suggests that the possibility for a subordinate specialist species to

persist within a diversified community might depend on the sharing of pollination services from generalists.

Moreover, since pollinators establish populations only once their habitat requirements (i.e. food resources, nesting sites and nesting materials) have been met, a community-wide approach can represent a useful tool to define the “conservation status” of a given habitat, a concept that is central to the EC Habitats Directive (EC 92/43). The same approach might also be used for a better understanding of the actual functioning of restored communities and how these compare to a “reference state”. Thus, the inclusion of biotic pollination-related interactions as an element in management or restoration plans could promote the conservation of the whole community, and guide monitoring programs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.flora.2016.04.003>.


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New insights into plants co-existence in species-rich communities: The pollination interaction perspective

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Abstract

Questions: In animal-mediated pollination, pollinators can be regarded as a limiting resource for which entomophilous plant species might interact to assure pollination, an event pivotal for their reproduction and population maintenance. At community level, spatially aggregated co-flowering species can thus be expected to exhibit suitable suites of traits to avoid competition and ensure pollination. We explored the problem by answering the following questions: (1) are co-flowering species specialized on different guilds of pollinators; (2) do co-flowering pollinator-sharing species segregate spatially; and (3) do co-flowering pollinator-sharing species that diverge in anther position spatially aggregate more than those that converge in anther position?

Study Site: Euganean Hills, NE Italy.

Methods: Plant composition, flowering phenology and interactions between each entomophilous plant species and pollinating insects were monitored every 15 days in 40 permanent plots placed in an area of 16 ha. We quantified the degree of flowering synchrony, pollinator-sharing and spatial aggregation between each pair of entomophilous species. We then tested the relationship between the degree of co-flowering, pollinator-sharing and spatial aggregation, and between spatial aggregation and anther position.

Results: Entomophilous species converged, at least partially in flowering time, and the phenological synchronization of flowering was significantly associated with the sharing of pollinator guilds. Co-flowering pollinator-sharing species segregated spatially. Furthermore, co-flowering pollinator-sharing species that diverged in anther position aggregated more than those that converged in anther position.

Conclusions: Reproductive traits that facilitate the co-existence of co-flowering species include specialization on different pollinator guilds and a phenological displacement of the flowering time. Furthermore, in circumstances of increased competition due to phenological synchronization, pollinator-sharing and spatial aggregation, the chance of effective pollination might depend on differences in anther position, resulting in a divergent pollen placement on pollinator bodies. One of the most interesting results we obtained is that the presence of one mechanism does not preclude the operation of others, and each plant species can simultaneously exhibit different strategies. Although more studies are needed, our results can provide additional information

about plant–plant interactions and provide new insights into mechanisms allowing the co-existence of a high number of plant species in local communities.

KEYWORDS

anther position, assembly rules, dry grasslands, flowering synchrony, pollinator-sharing, spatial aggregation/segregation

1 | INTRODUCTION

Temperate semi-natural dry grasslands are known for the high biodiversity they host. At small spatial scale, they stand out for the outstanding diversity of vascular plants (Purschke, Sykes, Reitalu, Poschlod, & Prentice, 2012; Wellstein et al., 2014; Wilson, Peet, Dengler, & Pärtel, 2012). Beside plants, they provide habitat for rare species from different taxonomic groups, including butterflies and other invertebrates (Bobbink & Willems, 1988; Fantinato, Del Vecchio, Baltieri, Fabris, & Buffa, 2017; Ssymank, Hauke, Rückriem, & Schröder, 1998; Van Helsdingen, Willemse, & Speight, 1996; Van Swaay, 2002).

Several studies have attempted to pinpoint principles to explain the assembly of local communities and disentangle the complex and puzzling co-existence mechanisms that ensure the persistence of a high species richness. The essential question is how ecologically similar species can co-exist at small spatial scales. In classical community theory, plant community organization is typically assumed to be non-random (Gotelli & Graves, 1996). According to the assembly rules hypothesis (Götzenberger et al., 2012; Wilson, 1999; Wilson & Gitay, 1995), patterns of species co-occurrence are driven by two main processes: (a) habitat filtering, whereby species are selected according to their adaptation to environmental factors (Batalha, Pipenbaher, Bakan, Kaligarič, & Škornik, 2015; Buffa & Villani, 2012; Del Vecchio, Pizzo, & Buffa, 2015; Del Vecchio, Slaviero, Fantinato, & Buffa, 2016; Pierce et al., 2017), and (b) species interaction, with competition considered as a central factor in community assembly (Kraft & Ackerly, 2014; Myers & Harms, 2009), leading to non-random co-occurrence patterns through niche differentiation or specialization (e.g. Carboni et al., 2014; Kelemen et al., 2015; Pierce, Luzzaro, Caccianiga, Ceriani, & Cerabolini, 2007; Tilman, 1988).

Most studies of plant community assembly have focused on direct competitive interactions for space or nutrients (de Jager, Dreyer, & Ellis, 2011; Sargent & Ackerly, 2008; Slaviero, Del Vecchio, Pierce, Fantinato, & Buffa, 2016). However, interactions among plant species may arise during different stages in their life cycle, comprising both the vegetative growth phase and reproduction, including pollination events and dispersal of seeds (Armbruster, 1995; Hegland, Grytnes, & Totland, 2009). Pollination is the first step of sexual reproduction, determining offspring (seeds) production and directly influencing the persistence of plant species in local communities (Callaway, 2007; Dante, Schamp, & Aarssen, 2013; Ghazoul, 2006).

In the case of animal-mediated pollination, we can assume pollinators are a limiting resource for which entomophilous plant species might interact with each other to assure pollination (Leonard,

Dornhaus, & Papaj, 2012). The most common pollination interactions that can occur are for pollinator attraction and heterospecific pollen transfer (e.g. Campbell & Motten, 1985; Feinsinger, 1987; Hegland & Totland, 2005; Waser & Fugate, 1986). Although case studies have revealed a range of possible outcomes (see Morales & Traveset, 2008 for a review), from no detectable effect to strong fitness reduction, heterospecific pollen transfer can be regarded as a form of competition (Ashman & Arceo-Gómez, 2013; McLernon, Murphy, & Aarssen, 1996; Muchhala, Brown, Armbruster, & Potts, 2010) as it may change the amount and quality of the pollen dispersed between individuals of a given species (Mitchell, Flanagan, Brown, Waser, & Karron, 2009), interfere with newly arriving legitimate pollen on the stigma and determine the loss of ovule receptivity to conspecific pollen (Brown & Mitchell, 2001; De Jong, 2012), resulting in pollen loss and negative effects on interacting species. In particular, in plant communities heterospecific pollen transfer is extremely common (Fang & Huang, 2013) and its fitness costs are assumed to guide the expression of morphological, phenological and physiological traits aimed at reducing them (Ashman & Arceo-Gómez, 2013; Lázaro, Lundgren, & Totland, 2015; Muchhala et al., 2010). Interactions for pollinator attraction, which influence the number of flower visits a plant receives (Mitchell et al., 2009), can span from competition to facilitation, as extremes of a continuum (Lázaro, Lundgren, & Totland, 2009, 2014). Plants may compete for pollinators if a plant species draws away effective visitors from another species, thus limiting its reproductive success (van der Kooij, Pen, Staal, Stavenga, & Elzenga, 2016; Mitchell et al., 2009; Pleasants, 1980). However, also a mutually beneficial pollinator attraction strategy may occur, based on increased floral visitation due to larger floral displays, resource availability and complementation (Ghazoul, 2006; Jakobsson & Ågren, 2014; Podolsky, 1992).

Since pollinators tend to visit nearby plant species, spatially aggregated co-flowering species are likely to experience stronger competition for pollinators and potentially suffer higher pollen loss and fitness decrease due to heterospecific pollen transfer (Fang & Huang, 2013; van der Kooij et al., 2016). Adaptations to minimize competition and pollen loss may include all those changes that reduce the sharing of pollinators, such as specialization on different pollinators (Kipling & Warren, 2014; Muchhala et al., 2010; Ruchisansakun, Tangtorwongsakul, Cozien, Smets, & van der Niet, 2016), shifts in flowering time (Devaux & Lande, 2009; Gleeson, 1981), a segregated spatial distribution (Kipling & Warren, 2014; Mosquin, 1971; Pleasants, 1980); or divergence in floral traits such as the anther position, which allows pollinator-sharing species to avoid pollen loss by placing pollen on different parts of pollinators' bodies (Ruchisansakun et al., 2016).



In a previous study, Fantinato, Del Vecchio, et al. (2016) proved that in temperate dry grasslands, as in all the biomes, animal-pollinated plant species overlap in flowering time mostly due to climatic constraints. Co-flowering species have been widely recognized to share pollinators, suggesting that competition for pollination might be a widespread phenomenon in many terrestrial ecosystems (Cozzolino et al., 2005; Moeller, 2004; Waser, Chittka, Price, & Williams, 1996). Given the high degree of co-flowering and thus generally expected pollinator-sharing in dry grasslands, we can expect that spatially aggregated co-flowering species exhibit suitable suites of reproductive traits that may concur to minimize competition for pollination and pollen loss due to heterospecific pollen transfer, thereby assuring steady co-existence of a high number of animal-pollinated species. In light of these considerations we addressed the problem through the following questions: (1) are co-flowering species specialized for different guilds of pollinators; (2) do co-flowering species sharing pollinators segregate spatially; and (3) do co-flowering pollinator-sharing species that diverge in anther position spatially aggregate more than those that converge in anther position?

2 | METHODS

2.1 | Study area

Field sampling was carried out on semi-natural, oligo- to mesotrophic *Bromus erectus*-dominated dry grasslands in the Euganean Hills (NE Italy). The investigated dry grasslands can be included in the class *Festuco-Brometea* Br.-Bl. & Tx. ex Soó 1947 and the SE European-Illlyrian order *Scorzoneretalia villosae* Kovačević 1959 (= *Scorzonero-Chrysopogonetalia* Horvatić & Horvat In Horvatić 1963; Fantinato, Giovanetti, Del Vecchio, & Buffa, 2016; Mucina et al., 2016). In the study area, dry grasslands cover a total surface of about 16 ha and establish on middle-altitude limestone slopes (average altitude 100 m a.s.l.) on sites characterized by poorly developed, shallow and skeletal calcareous soils, with very low water availability (AWC) and average pH of 7.5 (Bini, 2001; Fantinato, Giovanetti, et al., 2016). Once managed by low-intensive mowing practices, since the 1980s dry grasslands have experienced increasing abandonment. From 2003 onwards they have been mowed every 3 years.

2.2 | Data collection

We randomly placed 40 permanent plots of 2 m × 2 m. To reduce spatial autocorrelation, the minimum distance between plots was set at 25 m (Vaz, Macedo, Alves, Honrado, & Lomba, 2015). In each plot, vascular species composition was recorded and flowering phenology of all entomophilous species (35 species; Appendix S1) was monitored every 15 days (1 April to 12 September 2016) for a total of 11 surveys. Flowering was considered to have begun when the first flower was observed to be open on an individual plant (Pleasants, 1980) and ended when individual plants no longer possessed any flower with anthers (Dante et al., 2013).

Further, during each survey, in 20 out of the 40 permanent plots we also recorded visiting insects, under warm and sunny weather conditions. The visitation frequency was monitored through counting the number of visits to each plant species over 15 min. The observation period was split in 5-min sets distributed over three daily intervals (from 10:00 hr to 12:00 hr; from 12:00 hr to 14:00 hr; from 14:00 hr to 16:00 hr). We considered and counted as pollinators only those insects landing on the flower, visiting it for more than 1 s, and being in direct contact with the floral reproductive organs (Hegland & Totland, 2005).

Furthermore, for the 35 entomophilous plant species we recorded anther position relative to the corolla. Accordingly, plant species were grouped into three categories: plant species with anthers exerting from the bottom of the corolla (e.g. *Geranium sanguineum*, *Scabiosa triandra* and *Ononis natix*); plant species with anthers exerting from the top of the corolla (e.g. *Melampyrum barbatum*, *Stachys recta* and *Thymus pulegioides*); and plant species with anthers inserted near the opening of the corolla tube (e.g. *Campanula rapunculus*, *Muscari comosum* and *Orchis simia*). Anther position can be interpreted as a subtle mechanism to reduce pollen loss due to heterospecific pollen transfer as it leads to differences in pollen placement on a pollinator's body (Ruchisansakun et al., 2016). Hence, in the first group, plant species place pollen mostly on the legs and on the ventral side of the insect body, in the second group mostly on the dorsal side, while in the third group close to the head (e.g. Schiestl & Schlüter, 2009; Westerkamp & Claßen-Bockhoff, 2007).

Pollinators were identified to species or genus (morphospecies) and then grouped into 11 guilds, which allows analysis of pollination interactions from the perspective of function rather than of species identity (e.g. Fang & Huang, 2013; Fontaine, Dajoz, Meriguet, & Loreau, 2006), thus revealing patterns in the functionality in pollination interactions: small solitary bees, large solitary bees, medium social bees, large social bees, beetles, syrphid flies, other flies, butterflies, wasps, ants and bush-crickets. In accordance with previous studies (Arceo-Gomez et al., 2015; Fenster, Armbruster, Wilson, Dudash, & Thomson, 2004; Koski et al., 2015; Moretti, De Bello, Roberts, & Potts, 2009), guilds are based on the visitor's morphology (e.g. body size), energy requirements, flight ability and foraging/feeding behaviour, which can determine the range of flowers they can visit and thus the type of selection they generate. Members of a given guild are thus more similar to each other than to members of other groups (Geslin, Gauzens, Thébault, & Dajoz, 2013; Koski et al., 2015; Rosas-Guerrero et al., 2014). Bush-crickets have been included as a pollinator guild because juveniles were observed to land on flowers and to carry pollen accidentally on their legs and make contact with the floral reproductive organs.

2.3 | Data analysis

2.3.1 | Plant flowering synchronization and spatial assemblage

To quantify the overlap in the flowering time between each pair of plant species we used the co-flowering index (CF index; Fantinato, Del

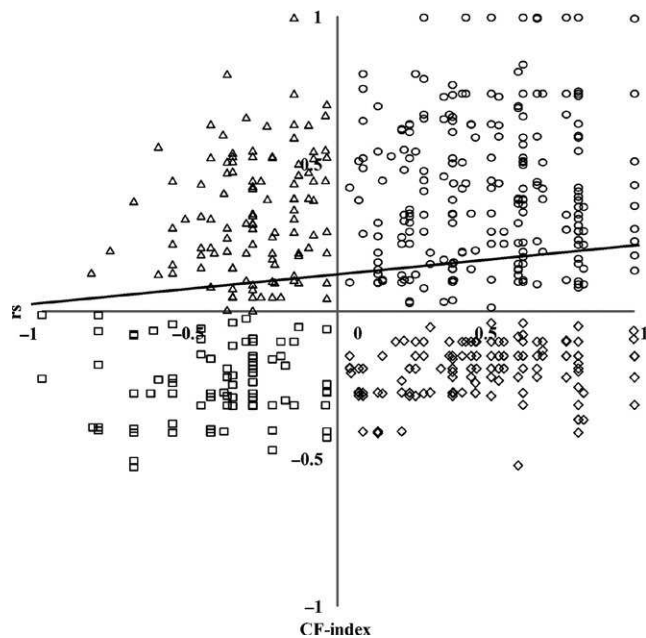


FIGURE 1 Scatterplot of co-flowering values (CF index values) vs. pollinator-sharing values (Spearman's coefficients) calculated through Mantel tests ($p = .001$, $r = .13$). Empty dots represent pairs of co-flowering species that share pollinator guilds; empty rhombus – pairs of species with disjointed flowering periods but sharing pollinator guilds; empty squares – pairs of species with disjointed flowering periods visited by different guilds of pollinator; empty triangles – pairs of co-flowering species visited by different guilds of pollinator

Vecchio, et al., 2016). We created a presence–absence matrix (flowering matrix), where rows were species (35 entomophilous species) and columns were the 11 surveys. Entries represented the presence (1) or the absence (0) of the flowering event. We then calculated the CF index through Pearson's correlation. The value of the CF index is equivalent to the value of the Pearson's correlation coefficient between the presence/absence vectors of two species and can range from -1 (i.e. complete flowering asynchrony) to $+1$ (i.e. complete flowering synchrony; Fantinato, Del Vecchio, et al., 2016). Results were organized in a symmetric matrix (species \times species; hereafter CF matrix).

We performed the same procedure to quantify the spatial association of each pair of species. In this case, we used a species \times plot matrix (35 entomophilous species \times 40 plots), where entries represented the presence (1) or the absence (0) of a species in a plot. For each pair of species we calculated the V score (Lepš & Šmilauer, 2003) through Pearson's correlation. The values range from $+1$ (complete spatial association) to -1 (complete spatial segregation). We obtained a symmetric matrix (species \times species; hereafter V matrix).

2.3.2 | Pollinator-sharing

To quantify the degree to which pairs of plant species share pollinator guilds, we created an abundance matrix where rows were plant species (35 plant species), and columns were insect guilds (11 guilds). In this case, entries represented the number of contacts observed

between plants and insects belonging to a given pollinator guild, calculated as the sum of all the contacts recorded in each monitored plot throughout the entire flowering season. We chose to use abundance data instead of presence–absence data, because abundances allow us to distinguish frequent visits from occasional contacts. For each pair of plant species we compared the abundance of pollinator guilds via Spearman's rank correlation. Spearman's rank correlation was chosen because it provides a reliable comparison of plant species pollinators irrespective of any form of standardization (e.g. for the total number of flowers per species or for the total number of visits).

The values of r_s indicate the trend of a pair of species to share pollinators. A positive correlation coefficient ($r_s \sim 1$) indicates that two plant species share the same guilds of pollinators, while a negative correlation coefficient ($r_s \sim -1$) represents the case in which plant species do not share pollinator guilds. The Spearman's values were organized in a symmetric matrix (PS matrix; namely Pollinator-sharing matrix) of plant species.

2.3.3 | Co-flowering species, pollinator-sharing and spatial assemblage

To assess whether co-flowering species are or are not sharing pollinators, and whether plant species that co-flower (CF index > 0) and share pollinator guilds ($r_s > 0$) are spatially aggregated or segregated, we applied a series of Mantel tests (function “mantel” in the R-based package “Vegan”; R Foundation for Statistical Computing, Vienna, Austria). Specifically, we correlated the CF matrix with the PS matrix; then we correlated the CF matrix with the V matrix by selecting only pairs of species that co-flowered and shared pollinator guilds. The significance of the correlation matrix was assessed by comparing observed values of the Mantel statistic to a random distribution generated through 999 permutations of the rows and columns (Legendre & Legendre, 2012). A positive and significant correlation between the CF matrix and the PS matrix would indicate that co-flowering plant species share pollinator guilds, while a negative value of the Mantel's r would indicate that co-flowering plant species are visited by different guilds of pollinators. A positive and significant correlation between the CF matrix and the V matrix would indicate that co-flowering species are spatially aggregated, while a negative and significant correlation would indicate spatial segregation.

2.3.4 | Anther position

For each pair of co-flowering pollinator-sharing species (i.e. CF index and $r_s > 0$) we tested if anther position differed more between spatially aggregated (V score > 0) plant species than between spatially segregated (V score < 0) plant species. In particular, we assigned 1 to all pairs of plant species in which anther position coincided, and 0 to those in which anther position differed. Significant differences were detected by performing a non-parametric Mann–Whitney U -test. We used spatially aggregated (V score > 0) vs. spatially segregated (V score < 0) plant species as grouping variable, and the coincidence (1) or the difference (0) in anther position as dependent variable. All

calculations performed in the present study were done within the R statistical framework.

3 | RESULTS

Animal-pollinated species converged in flowering time, with 66.2% of species pairs overlapping, at least partially, in their flowering time (394 species pairs; CF index > 0). Overall, 91 species pairs (15.3%) strongly overlapped in flowering time (CF > 0.75), while 115 species pairs (19.3%) showed a moderate overlap ($0.75 > \text{CF} > 0.50$). A total of 201 species pairs (33.8%) showed temporal separation (CF index < 0), with ten pairs of species (1.7%) with a strong phenological displacement of flowering (CF index < -0.75).

The phenological synchronization of flowering was significantly associated with the sharing of pollinator guilds (Mantel test: CF matrix vs. PS matrix; $p = .001$, $r = .13$; Figure 1). Overall, 38.5% of species pairs (229 pairs) showed synchronous flowering periods coupled with pollinator-sharing (CF index > 0 and $r_s > 0$), while 27.7% of species pairs (165 pairs), although flowering synchronously (CF index > 0), relied on pollination from different guilds of pollinators ($r_s < 0$). About 19.5% of species pairs (116 pairs) segregated temporally (CF index < 0) but relied upon the same guilds of pollinators ($r_s > 0$).

Pairs of co-flowering pollinator-sharing species (i.e. CF index > 0 and $r_s > 0$) disclosed a significant negative correlation between the degree of co-flowering and the spatial aggregation (Mantel test: CF matrix vs. V matrix; $p = .036$, $r = -.14$; Figure 2), namely, the higher the flowering synchrony, the lower the spatial aggregation. In particular, 104 species pairs (17.5%) spatially aggregated (V score > 0), while 125 species pairs (21.0%) segregated (V score < 0). All pollinator-sharing plant species that showed a complete flowering synchrony (CF index = 1; 11 species pairs; i.e. 1.8%), were spatially segregated (i.e. $r_s > 0$, CF = 1, V score < 0).

Anther position of co-flowering pollinator-sharing species (i.e. CF index and $r_s > 0$) significantly differed between spatially aggregated (V score > 0) and segregated (V score < 0) species, with spatially aggregated species differing in anther position more frequently than spatially segregated species (Mann-Whitney $Z = -3.18$, $p < .01$).

4 | DISCUSSION

Our results demonstrated that in dry grasslands, entomophilous plant species exhibit different strategies to reduce competition and pollen loss, thereby assuring pollination, a pivotal event for reproduction, offspring production and population maintenance into local communities. Temperate dry grassland entomophilous species revealed a non-random pattern of flowering, with a high degree of convergence in the timing of flowering. In seasonal climates, as in temperate and mediterranean climates, insect-pollinated plants are forced to flower during periods when climatic conditions are most suitable for reproduction (e.g. time available for flowering and seed maturation; Elzinga et al., 2007) and generally match the most favourable season for pollinator

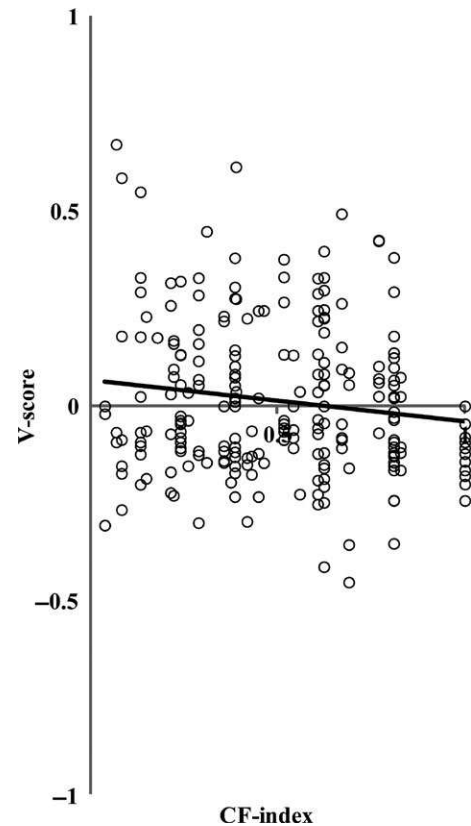


FIGURE 2 Scatterplot of co-flowering values (CF index values) vs. co-occurrence values (V score values) of pairs of species proven to flower synchronously (CF index > 0) and to share pollinator guilds ($r_s > 0$) calculated through Mantel tests ($p = .036$, $r = -.14$)

activity. The flowering synchrony was positively correlated to the sharing of pollinator guilds, a result in line with those of other studies (e.g. Cozzolino et al., 2005; Motten, 1986) stating that pollinator sharing is a widespread phenomenon.

It has been argued that the convergence of flowering phenology among plant species in a community may function as pollinator-mediated facilitation through the improvement of pollinator attraction due to mass flowering (Aizen & Vázquez, 2006; Sargent & Ackerly, 2008) or increased diversity (Ghazoul, 2006). The convergence of flowering periods over the spring months enables the plant community to support a wide range of visitors (Potts, Vulliamy, Dafni, Ne, & Willmer, 2003), by increasing floral resource availability and heterogeneity (e.g. nectar and pollen), which assure the maintenance of pollinators seeking single or multiple resources (Ebeling, Klein, Schumacher, Weisser, & Tschardtke, 2008). However, our results indicated that a notable amount of co-flowering species pairs (27.7%) were specialized to attract different pollinator guilds. Community context and strong interactions with competitors have already been recognized as forces promoting specialization on different pollinators (Jakobsson, Lázaro, & Totland, 2009; Muchhala et al., 2010). Arguably, despite the positive effects that it can have on pollinator attraction, pollinator-sharing by co-flowering species may increase competition for pollinators as well as heterospecific pollen transfer (Carvalho et al., 2014; Potts et al., 2003; Price, 1984), which may result in negative effects on plant

species reproduction (Morales & Traveset, 2008). Co-flowering plant species may compete with each other for pollinator services, thereby affecting each others pollinator visits (Lázaro et al., 2009), and many studies on co-flowering pollinator-sharing species demonstrated increased competition and reduced pollination rate during periods of flowering overlap (van der Kooi et al., 2016; Morales & Traveset, 2008). The positive outcome of specialization becomes clear when considering that plants produce a finite quantity of pollen and selection will favour maximizing the number of grains that reach conspecific stigmas (Harder & Routley, 2006).

Besides specialization on different pollinators, dry grassland entomophilous species also showed patterns of temporal segregation, namely a shift in the flowering period. It has already been suggested (Aizen & Vázquez, 2006) that competition for pollination may cause evolutionary displacement or ecological sorting of flowering phenologies. Being normally at low numbers, early or late flowering species are expected to have fewer competitors in their neighbourhood than are plants flowering at or near the peak of the community (Elzinga et al., 2007). In our case, despite the overriding influence of climate, a non-negligible amount of species pairs (33.8%) showed modulation of flowering time, thus reducing competition by blooming at different times. This result becomes even more interesting when considering that 19.5% of species pairs that segregate temporally shared pollinators. Thus, the phenological displacement of flowering allows species to rely on the same resource for pollination without incurring strong competition, and to maintain the community of pollinators for a longer period of time (Willmer, 2011).

However, Vamosi et al. (2006) suggested that in competitive environments specialization on different pollinators or the displacement of the flowering period may be insufficient to reduce competition. Despite a relative low Mantel correlation coefficient, we found a significant negative correlation between the degree of co-flowering and the spatial aggregation. Especially in dry grassland communities, all those species that evidenced complete flowering synchrony (CF index = 1) and shared pollinator guilds ($r_s > 0$), displayed a striking pattern that involved spatial segregation (V score < 0). Thus, although plant species substantially overlap in flowering time and rely on the same pollinator guild, they are separated in space. Spatial proximity has been shown to possibly affect the intensity of pollination experienced by a given plant (Hegland & Totland, 2005; Hersch & Roy, 2007; Thomson, 1978; Törang, Ehrlén, & Agren, 2006). Lázaro et al. (2009) demonstrated that both the diversity and the composition of pollinators of particular plant species are affected not only by the characteristics of the individual plant species itself and its abundance but also by the identity, diversity and density of the co-flowering neighbour plants. Therefore, we can hypothesize that at small scales the pollination success of plant species, and consequently their reproductive fitness, might increase when individuals are segregated from competitors (Jakobsson et al., 2009; Lázaro et al., 2014). A spatial patchiness of co-flowering pollinator-sharing species may therefore increase the proportion of intraspecific visits made by pollinators, ultimately reducing the chance that where co-flowering pollinator-sharing species co-occur at small spatial scales, either of them would be excluded from the community.

However, further studies on plant fitness would be needed to test our hypothesis and prove that spatially segregated plant species increase their reproductive success.

Some species pairs (17.5%) co-flowered, shared pollinator guilds and were spatially aggregated. This particular situation is expected to increase competition for pollinator attraction as well as pollen loss due to heterospecific pollen transfer (Fang & Huang, 2013; Lázaro et al., 2009). In this regard, it is particularly interesting to observe that in dry grasslands, spatially aggregated plant species diverged in anther position more frequently than spatially segregated species, so that a shared pollinator would obtain pollen on different parts of its body. Flowers with a similar sexual architecture are expected to be particularly likely to exchange pollen due to the increasing overlap in pollinator species and the load of pollen on the same parts of pollinator's body (Montgomery & Rathcke, 2012). Since pollen deposition is related to the placement of pollen on the body of flower visitors, a different anther position mechanically allows species to benefit from living in close proximity and sharing pollinators, while, at the same time, minimizing reproductive interference and pollen loss due to heterospecific pollen transfer (Caruso, 1999; Morales & Traveset, 2008). Additionally, floral similarity may also decrease pollinator constancy (Waser 1986). Thus, differences in anther position among spatially aggregated co-flowering pollinator-sharing species can be regarded as a way to avoid reproductive interference, particularly in circumstances where climatic constraints allow little opportunity for displacement in floral phenology. Several examples of differential pollen placement for congener plant species have been provided (Muchhala & Potts, 2007; Smith & Rausher, 2008; Sprague, 1962). Further evidence was also found between taxonomically distinct flowers (Goldblatt, Manning, & Bernhardt, 1995), yet patterns of pollen placement diversification at community level have so far received less attention (but see Armbruster, Edwards, & Debevec, 1994).

5 | CONCLUSIONS

Plant-pollinator interactions are essential for outcrossing in most flowering plant species, thereby influencing offspring production and in turn the long-term permanence of plant species in the community. Although some correlations we found were weak and need to be supported by further research, our study confirms that pollination interactions contribute to shape patterns of species co-existence by selecting those species that exhibit suitable suites of reproductive traits that are considered to reduce competition. When establishing in a species-rich plant community, the pollination success of a plant species depends on differences between its own phenological and morphological features and those of the co-occurring species. Reproductive traits that facilitate the co-existence of co-flowering species include specialization on different pollinator guilds and phenological displacement of the flowering time. Furthermore, in circumstances of increased competition due to phenological synchronization, pollinator-sharing and spatial aggregation, the chance of effective pollination might depend on



differences in anther position, resulting in divergent pollen placement on a pollinator's body, a method to utilize the same insects as pollinators without incurring significant loss of pollen.

The low Mantel coefficients we obtained might just be due to the fact that there is no dominant mechanism; rather each plant species can simultaneously exhibit different strategies. One of the most interesting results we obtained is that the presence of one mechanism does not preclude the operation of others. Although more studies are needed, our results provide additional information about plant–plant interactions and add new insights into mechanisms allowing the co-existence of a high number of plant species in local communities.

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SUPPORTING INFORMATION

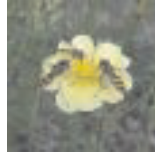
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APPENDIX S1 List of entomophilous species recorded in the studied dry grasslands

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Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only.
It will not be published as part of main article.



Reproductive traits facilitating the coexistence of co-flowering species in species-rich communities include specialization on different pollinators and phenological displacement of flowering. In circumstances of increased competition due to phenological synchronization and pollinator sharing species show spatial segregation. When spatial aggregation occurs, an effective pollination might depend on differences in anther position, resulting in a divergent pollen placement on pollinators' body.

The resilience of pollination interactions: importance of temporal phases

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ABSTRACT

Aims

The loss of species that engage in close ecological interactions, such as pollination, has been shown to lead to secondary extinctions, ultimately threatening the overall ecosystem stability and functioning. Pollination studies are currently flourishing at all possible levels of interaction organization (i.e., species, guild/group and network), and different methodological protocols aimed to define the resilience of pollination interactions have been proposed. However, the temporal dimension of the resilience of pollination interactions has been often overlooked. In the light of these considerations we addressed the following questions: can the temporal approach help to reveal critical moments during the flowering season, when the pollination network may be less resilient to perturbations? Can pollination interactions evaluated at species, guild and network level show different patterns when assessed through time?

Methods

We monitored contacts between plant and pollinator species in dry grassland communities every 15 days during the overall community flowering season (12 surveys). For each survey we built a quantitative plant-pollinator interaction matrix and we calculated two sets of metrics characterizing respectively the “diversity” and the “distribution” of interactions across hierarchical levels. To describe the “diversity” of interactions we calculated partner diversity at the species level, vulnerability at the guild level, and interaction diversity and evenness at the network level. The “distribution” of interactions was characterized by calculating selectiveness at the species and the network level, and modularity at the group level. We assessed the temporal variation of partner diversity, vulnerability at the level of plants and pollinators, and species selectiveness by means of LMMs. To investigate the temporal variation of indexes calculated at group and network level we applied simple linear and quadratic regressions after checking for temporal autocorrelation in residuals by performing a Durbin – Watson test.

Important findings

When taking into account the temporal dimension of interactions, the diversity of interactions showed different patterns at different levels of organization. If, at the species level, no relationship was disclosed between partner diversity and time, when assessing the temporal trend of partner diversity separately for the guild of plants and pollinators we could observe an asymmetric structure of interactions. Pollination interactions showed to be asymmetric throughout the flowering season, however evenness of interactions and network selectiveness showed a significant positive relationship with time, revealing a poorer network of interactions at the end of the flowering season. The temporal analysis of pollination interactions revealed a stronger risk of secondary extinctions during the end of the flowering season, due to a lower degree of redundancy and thus of resilience of the overall network.

KEYWORDS

Asymmetry; Complementarity; Ecosystems’ resilience; Redundancy; Species-rich ecosystems

INTRODUCTION

The interactions between plant species and wild animals for pollination are currently an issue of increasing interest worldwide. This essential process is considered one of the key ecosystem functions, necessary to preserve biodiversity and the functioning of both natural terrestrial ecosystems and crops (Fontaine *et al.* 2006; Ollerton *et al.* 2011). In addition, together with other biotic and abiotic factors, pollination interactions can contribute to define plant communities structure and composition (Dante *et al.* 2013; Fantinato *et al.* 2016a; Fantinato *et al.* 2018), thus influencing ecosystem properties.

Mounting evidence exists that pollinators are declining as a consequence of global environmental changes and degradation (Biesmeijer *et al.* 2006; Winfree *et al.* 2009; Potts *et al.* 2010). Anthropogenic changes in land use (e.g. agriculture, industry and urbanization) are ranked among the most important pressures, typically resulting in the loss of native vegetation and changes to its spatial distribution, deterioration of vegetation structure, reduction of species richness and abundance, and changes in species composition (Fahrig 2003; Fischer and Lindenmeyer 2007). As for pollinators, the destruction and fragmentation of natural or semi-natural habitats is expected to cause the loss of forage and breeding habitats, or at least less floral forage over shorter periods of time (Hall *et al.* 2016), population subdivision and the consequent demographic and genetic stochasticity (Fischer and Lindenmayer 2007).

Under human-induced land use changes, several pollination interaction attributes have been recognized to deeply influence the resilience of pollination networks to perturbations (Vázquez and Aizen 2004; Thébault *et al.* 2010; Blüthgen and Klein 2011; Kaiser-Bunbury and Blüthgen 2015). Pollination studies are currently flourishing at all possible levels of organization (i.e., species, guild/group and network), and different methodological protocols aimed to define the resilience of pollination interactions have been proposed (e.g., Tylianakis *et al.* 2010; Kaiser-Bunbury *et al.* 2010; Thébault and Fontaine 2010; Biella *et al.* 2017). Especially, Kaiser-Bunbury and Blüthgen (2015) introduced a set of metrics describing pollination interactions at different hierarchical levels (i.e., species, guild/group and network), highlighting their effectiveness in defining the resilience of pollination interactions because of the ecological characteristics of the indicators, sound empirical and theoretical support, and conceptual similarities to well-established diversity indicators. However, the temporal dimension of the resilience of pollination interactions has been often overlooked (Willmer 2011). Nonetheless, all studies that have explored the temporal dynamics of pollination interactions (e.g., Medan *et al.* 2002; 2006; Lundgren and Olesen 2005; Basilio *et al.* 2006; Olesen *et al.* 2008) evidenced that pollination interactions can strongly change through time. Arguably, pollination interactions can greatly vary during the community flowering season due to changes in both environmental, ecological, biotic and anthropogenic features (e.g., temperature, resources availability, community composition, disturbance etc.), possibly leading to temporal fluctuations of the resilience of mutualistic communities (Vázquez and Aizen 2004; Thébault *et al.* 2010).

In the light of these considerations we addressed the following questions: does the temporal approach help to reveal critical moments during the flowering season, when the pollination network may be less resilient to perturbations? Do pollination interactions evaluated at species, guild and network level show different patterns when assessed through time?

We addressed the problem by assessing temporal dynamic of pollination interactions in dry grassland communities. Dry grasslands are one of the most important semi-natural habitat in Europe (Dengler *et al.* 2014; Habel *et al.* 2013). They have great conservation value (Wellstein *et al.* 2014) for their high biological diversity which includes a variety of rare species from different taxonomic groups, including plants, butterflies and other invertebrates (Bobbink and Willems 1998; Ssymank *et al.* 1998; Van Swaay 2002; Wellstein *et al.* 2014; Fantinato *et al.* 2016 b). Dry grasslands are severely endangered by threats that apply to many other plant communities: changes in land use, through intensification of agriculture as well as abandonment followed by bush encroachment, fragmentation, decreased habitat quality and the consequent decline of the biodiversity they host (Luoto *et al.* 2003; Török *et al.* 2011; Dengler *et al.* 2014). Furthermore,

being characteristic of pastoral and low-intensity managed landscapes (Wellestein *et al.* 2014), dry grasslands can crucially contribute to biodiversity and pollination service retention in agricultural landscapes (Fantinato *et al.* 2018). They can provide a stable habitat available for foraging and life cycle completion of pollinators thus acting as an insurance against fluctuations in wild pollinator abundance. Arguably, fluxes of individuals between natural habitats and agricultural lands can be supposed to have valuable consequences for the fitness of individuals, population dynamics, species interactions and ecosystem services in both natural and agricultural habitats. Therefore, the comprehension of temporal variation of pollination interactions may have important implications for the understanding of dry grassland resilience to perturbations, thus of their long term conservation.

MATERIALS AND METHODS

Site selection and data collection

Field sampling was carried out on semi-natural, oligo- to mesotrophic, *Bromus erectus*-dominated dry grasslands in the Euganean Hills (NE Italy; Fantinato *et al.* 2016b). They establish on middle-altitude limestone slopes, on sites characterized by poorly developed, shallow and skeletal calcareous soils, with an average pH of 7.5 (Bini 2001; Slaviero *et al.* 2016). They are scattered and fragmented in a patchy landscape of arable fields, vineyards and olive groves, intermingled with low-intensive mowed mesophilous grasslands and forests. Local climate data reveal an average annual rainfall of 720 mm (Kaltenrieder *et al.* 2010), with two maximum peaks in April and September and two minimum peaks in July and December. Annual mean temperature is 13.0°C, with a peak mean high temperature in July and a low in January.

Within an area of 16 ha we randomly placed 26 permanent plots of 2 m x 2 m. In each plot, plant species composition was recorded and flowering phenology of all entomophilous species (34) was monitored every fifteen days, from 1st April to 30th September in 2016, for a total of twelve surveys. The twelve surveys were carried out in warm and sunny days (e.g., Goverde *et al.* 2002; Ghazoul *et al.* 2006; Rodriguez-Perez and Traveset 2016). Flowering monitoring started at the opening of the first flower (Pleasants 1980) and ended when individual plants no longer possessed any flower with anthers (Dante *et al.* 2013). Plant species nomenclature follows Conti *et al.* (2005).

Furthermore, during each survey, we recorded visiting insects. The visitation frequency was monitored by counting the number of visits to each plant species during 14 minutes. The observation period was split in 7-min sets distributed during two daily intervals (from 10 a.m. to 1 p.m.; from 1 p.m. to 4 p.m.) to ensure the observation of pollinators showing different daily periods of activity. We considered and counted as pollinators only those insects landing on the flower, visiting it for more than 1 s, and getting in direct contact with the floral reproductive organs (Hegland and Totland 2005). Floral visitors were identified at the level of species or morphospecies (Memmot *et al.* 1999). Tettigoniidae were included as pollinators because juveniles were observed to land on flowers and to carry pollen accidentally on their legs and make contact with the floral reproductive organs.

Data analysis

We built a quantitative plant-pollinator interaction matrix for each survey (12). As interaction weight we used the visitation frequency, expressed as the number of contacts between each plant and pollinator species during a census. To define the resilience of dry grassland pollination interactions we followed Kaiser-Bunbury and Blüthgen (2015). We used the *bipartite* package (Dormann *et al.* 2009) run in R to obtain all the metrics calculated in this study. The pollination interactions attributes were described through two sets of metrics characterizing respectively the diversity and the distribution of interactions across hierarchical levels (i.e., species, guild/group and network level; Bunbury and Blüthgen 2015). To describe the diversity of

interactions we calculated partner diversity (PD) at the species level, vulnerability (V; i.e., mean partner diversity across (i) plant and (ii) pollinator species) at the guild level, and interaction diversity (ID) and evenness (IE) at the network level. The distribution of interactions was characterized by calculating selectiveness at the species and the network level (d' and H'_2 , respectively; Blüthgen *et al.* 2006), and modularity at the group level (Q; Dormann and Strauss 2014). In order to carry out robust comparisons among networks not affected by neither sampling effort nor network size (Lázaro *et al.* 2016; Traveset *et al.* 2017), observed modularity (Q) was corrected (Ballantyne *et al.* 2017) using the mean values resulting from 1000 randomly generated matrices of the same size as the focal one, using a null model that fixes total number of interactions (function `nullmodel`; method; `r2d`; R-based package *bipartite*). Standardized z-scores were calculated for each group and network level metric ($z = [\text{observed} - \text{null mean}] / \text{null } \sigma$) to test for significant difference from the null model distribution.

We assessed the temporal variation of partner diversity (PD), vulnerability (V) at the levels of plants and pollinators, and species selectiveness (d') by means of LMMs (R-based package *nlme*). We regressed each metric with respect to a quadratic trend and a random factor represented by the identity of species. Possible temporal dependences among the different surveys were taken into account using a first order autoregressive model for the random errors.

To investigate the temporal variation of indexes calculated at group and network level we applied simple linear and quadratic regressions. Especially, we applied simple linear regressions to assess the temporal variation of interaction evenness (IE), corrected modularity (Q) and network selectiveness (H'_2); while a simple quadratic regression was calculated to assess the temporal variation of interaction diversity (ID). Simple linear regressions and the quadratic model were firstly checked for temporal autocorrelation in residuals by performing a Durbin – Watson test (function `durbinWatsonTest`; library-based package *car*).

RESULTS

Diversity of interactions

Overall, we identified 34 plant and 77 pollinator species. At the species level, partner diversity did not show a significant relationship with time (PD; $t = -0.321$; $P = 0.748$; Fig. 1A). At the guild level, vulnerability of plants showed a significant U shaped relationship with time (V; $t = 2.056$; $P = 0.046$; Fig. 1C); while, vulnerability of pollinators showed a significant hump-shaped trend (V; $t = -2.439$; $P = 0.016$; Fig. 1D). Values of interaction diversity (ID; Table 1) and interaction evenness (IE; Table 1) did not show temporal autocorrelation (ID; $P = 0.068$; IE; $P = 0.756$). Interaction diversity (ID) showed a significant hump-shaped relationship with time ($R^2 = 0.537$; $P = 0.031$), while interaction evenness (IE) increased significantly through time ($R^2 = 0.356$; $P = 0.040$).

Distribution of interactions

The degree of species selectiveness (d') did not show a significant relationship with time ($t = -1.774$; $P = 0.078$; Fig. 1B). Values of corrected modularity (Q; Table 1) and network selectiveness (H'_2 ; Table 1) did not show temporal autocorrelation (Q; $P = 0.98$; H'_2 ; $P = 0.089$). No significant relationship could be detected between corrected modularity and time ($R^2 = 0.034$; $P = 0.56$). Contrarily, network selectiveness (H'_2) followed a significant positive trend ($R^2 = 0.846$; $P = 0.001$).

Table 1. Group and network level metrics. Significant differences of observed values from null model predictions were indicated in bold ($P < 0.001$).

Survey	Group level metrics		Network level metrics		
	observed Q	corrected Q	ID	IE	H ₂
1	0.416	0.371	1.798	0.417	0.394
2	0.574	0.334	2.968	0.638	0.226
3	0.562	0.402	3.464	0.585	0.392
4	0.746	0.594	3.345	0.610	0.467
5	0.548	0.343	3.364	0.620	0.217
6	0.587	0.382	2.967	0.583	0.384
7	0.646	0.410	2.128	0.547	0.604
8	0.518	0.153	2.677	0.619	0.393
9	0.442	0.172	2.112	0.800	0.543
10	0.506	0.065	2.303	0.677	0.451
11	1.000	0.799	2.043	0.590	0.812
12	1.000	0.748	1.778	0.716	0.747

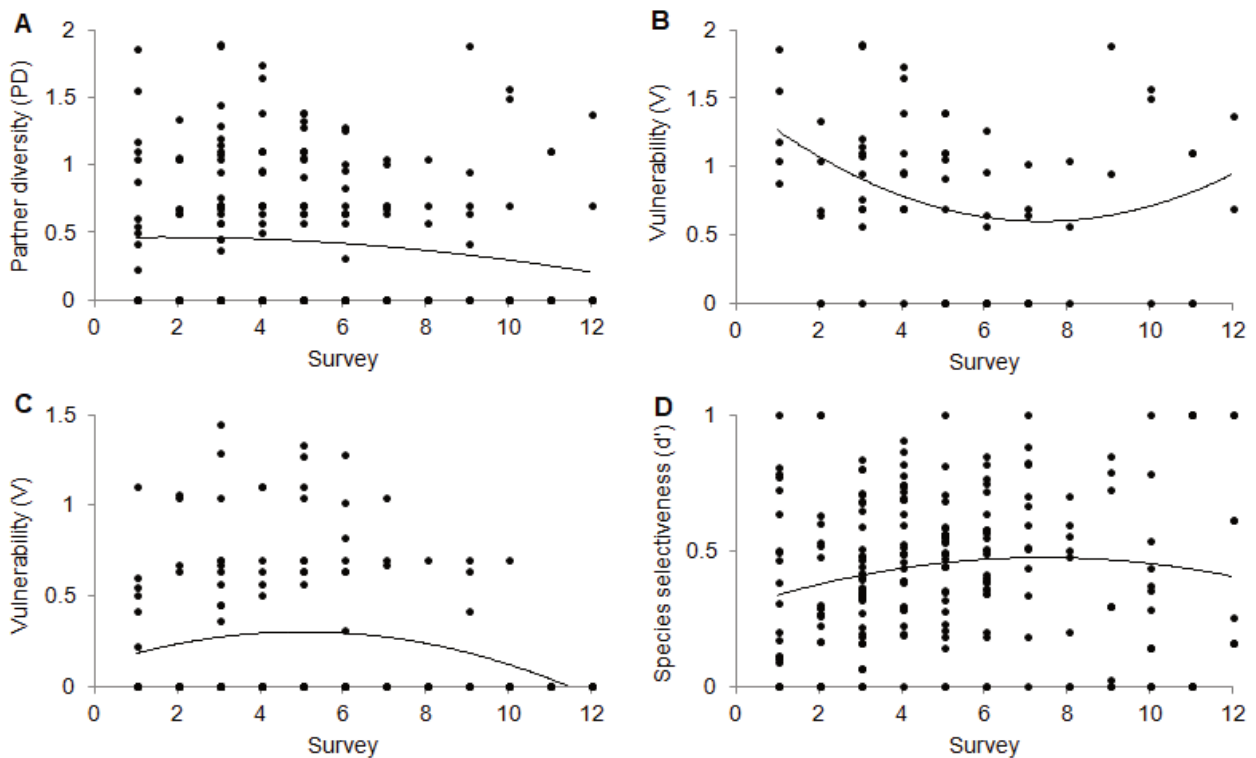


Figure 1. Relationships between time and (A) species partner diversity (PD), (B) plant species vulnerability (V), (C) pollinator species vulnerability (V), and (D) species selectiveness (d'). Lines represent the estimates of the models corrected from temporal dependences among the different surveys.

DISCUSSION

Pollination interactions in dry grassland communities showed to have a strong temporal dynamic. The application of Kaiser-Bunbury and Blüthgen (2015) methodology on a time series of pollination interactions allowed us to highlight critical moments, in which pollination interactions result more vulnerable, and so less resilient to perturbations.

When taking into account the temporal dimension of interactions, the diversity of interactions showed different and even contrasting patterns at different levels of organization (species, guild and network). If, at the species level, no relationship was disclosed between partner diversity (PD) and time, when assessing the temporal trend of partner diversity separately for the guild of plants and pollinators (i.e., vulnerability; V) we could observe two opposite temporal trends. Generalist plants (i.e., plant species interacting with many pollinator species) prevailed during the start and the end of the community flowering season and were pollinated by specialist pollinators (i.e., pollinator species interacting with few plant species), while specialist plants (i.e., plant species interacting with few pollinator species) flowered during the peak of the community flowering season, and were pollinated by generalist pollinators (i.e., pollinator species interacting with many plant species), resulting in an asymmetric structure of interactions. An asymmetric structure has been proven to have important implications for mutualistic communities (Vázquez and Aizen 2006; Thébault *et al.* 2010; Abramson *et al.* 2011). The way in which pollination interactions are structured, i.e. the type of interactions developed among species (symmetric or asymmetric), might affect their own stability and function, communities organization as well as the entire system functioning (Bascompte and Jordano 2006; Krause *et al.* 2003; McCann *et al.* 2005). The structure of interactions is also supposed to influence the mutualistic community responses to disruptions, i.e. resilience to perturbations such as the extinction of species if, for example, generalist partners buffer the loss of specialist ones (Memmott *et al.* 2004) or, conversely, fragility, if the most-connected partners disappear. Contrarily to symmetry, asymmetry in species interactions has been proven to confer higher resilience to mutualistic communities, preventing the occurrence of secondary extinctions (Vázquez and Aizen 2004; Thébault *et al.* 2010). Furthermore, Ashworth *et al.* (2004) theorized that asymmetric interactions would mitigate the effects of habitat destruction and fragmentation on the reproductive success of specialist plants, which have been predicted to be more sensitive to the loss of their specific mutualist partners than generalist ones.

Despite pollination interactions in dry grasslands showed to be asymmetric throughout the flowering season, interaction diversity at the network level followed a significant hump-shaped trend. Lower values of interaction diversity (ID) during the start and the end of the community flowering season may imply a lower network resilience (Blüthgen *et al.* 2008; Lázaro *et al.*, 2016; Traveset *et al.*, 2017). However, given the positive trend of interaction evenness (IE) we expect that the resilience of the pollination network might be especially low at the end of the community flowering season. In fact, according to Kaiser-Bunbury and Blüthgen (2015) when values of interaction evenness (IE) are coupled with low values of interaction diversity (ID), they may not represent the spread of more uniform connections among plants and pollinators but rather the development of a poorer network of interactions.

Interactions distribution evaluated at the network level through the analysis of network selectiveness (H'_2), confirmed the pattern of vulnerability arose from the temporal assessment of the network diversity of interactions. A high degree of selectiveness in pollination networks occurs when two or more species improve the overall pollination network by adding further original interactions (Blüthgen *et al.* 2006). High selectiveness requires a certain degree of specialization of each interaction, while high generalization is associated with high pollinator sharing and thus redundancy. Redundancy implies that species are mutually substitutable in terms of a given ecological function. In a mutualistic community it confers a high resilience, acting as a buffer against species loss. Conversely, a high complementarity entails a high dependence of plant diversity to flower visitor diversity (and vice versa), thereby resulting in less resilience to perturbations (Blüthgen and Klein 2011).

Thus, despite a comparable functional composition of generalist plant and specialized pollinator species at the start and at the end of the flowering season, the degree of selectiveness of the pollination network revealed a stronger risk of secondary extinctions during the end of the flowering season, due to a lower degree of redundancy and thus of resilience of the overall network. From a conservation perspective, this implies that the loss of plant species flowering during the end of the community flowering season can put the ecosystem functioning more in danger than the loss of plant species flowering during the start and the peak of the community flowering season. In natural landscapes the loss of one of them may force the related pollinator species to forage elsewhere, damaging the integrity of the pollination network. However, in fragmented habitats surrounded by a hostile matrix, which represents an increasingly common situation for dry grasslands, effects may be even more pronounced. The loss of one of the late flowering plant species would indeed lead to the secondary extinction of specialized pollinators observed to forage exclusively on them, because incapable to find foraging habitats nearby, ultimately compromising the integrity of the mutualistic community also for the upcoming flowering seasons.

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Co-occurring grassland communities: the functional role of exclusive and shared species in the pollination network organization

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ABSTRACT

The importance of semi-natural grasslands for the provision of pollination services in agri-environments has led several studies to investigate which conditions mostly contribute to the maintenance of plant and pollinator species richness. Different grassland communities were found to support different species, however the gamma diversity resulting from the co-occurrence of different grassland communities at the landscape scale has to be clarified. The present study aimed at evaluating the effects of co-occurring dry grassland communities (pioneer vs. late-successional) on the pattern of pollination interactions at the landscape scale. Especially, we assessed the functional role of exclusive and shared plant and pollinator species within pollination networks (keystone vs. peripheral species). Pollination interactions were monitored in grassland communities of the Euganean Hills (NE Italy) from April to July (7 surveys) and the amount of pollen and nectar produced by each animal-pollinated species was quantified. For each survey we calculated quantitative descriptors of pollination networks at both landscape and species level. We used PERMANOVAs and post hoc Tukey tests to compare functional roles (and pollinators feeding preferences) of exclusive and shared (i) plant and (ii) pollinator species. To compare similarities between pioneer and late-successional grasslands in the temporal pattern of floral resources we performed two cross-correlations. Dry grassland communities showed to be highly interconnected, resulting in a highly resilient pollination network at the landscape scale. Functional roles of exclusive and shared plant and pollinator species were asymmetric, with exclusive plants being keystone species and shared plants peripherals, while shared pollinators were keystone species, and exclusives peripherals. Feeding preferences of exclusive and shared pollinator species followed grasslands temporal complementarity in the pollen and nectar production, with shared pollinators being pollen and nectar feeders, while exclusive pollinators were either mostly pollen or nectar feeders. Our results highlighted that the co-occurrence of different grassland communities plays a key role in the maintenance of pollination network stability at the landscape scale and in the sustainment of species requiring their co-occurrence to survive.

KEYWORDS

Agri-environment; Asymmetry; Dry grassland; Landscape; Network; Pollination

INTRODUCTION

Pollinator populations are declining all over the world (Winfree et al., 2009) with unavoidable consequences for the pollination service delivery. Around 60% of both wild and cultivated plant species are expected to potentially suffer from pollination limitation (Aizen et al., 2008), thereby undergoing a decrease of fruit or seed set.

Both domesticated and wild pollinator populations are affected by a range of environmental changes, such as habitat loss and climate change (Traveset et al., 2017). Local and global environmental degradation, and the loss of natural and semi-natural habitats appear to be of greater importance than natural factors (Kosior et al., 2007) in determining the decline of pollinator populations. Furthermore, several studies suggest that the

reduction in wildflowers, which determines a drop of floral resources (i.e., nectar and pollen), plays a major role in the pollinators decline represents (Biesmeijer et al., 2006; Fitzpatrick et al., 2007; Potts et al., 2010). The decline of floral resources can, in fact, trigger a negative feedback on mutualistic communities, leading to local pollinator extinctions, which can in turn lead to the decline in pollination services for wild plants, thereby further reducing floral resources for the pollinators (Potts et al., 2010). If pollinator-dependent plants do not set seeds, the integrity of natural and semi-natural habitats will be destroyed (Lázaro et al., 2016; Traveset et al., 2017).

Given the economic implications of reduced pollination service, the decline of pollinator populations in agri-environment has received substantial attention. Human disturbance, in the form of large-scale farming and urbanization, typically results in the loss of native vegetation, changes in species composition and reduction of floral resources (Fahrig, 2003; Fischer and Lindenmeyer, 2007). However, the long history of low-intensity agricultural land use in Europe has also created unique and species-rich plant assemblages, including semi-natural grassland communities. Semi-natural grasslands shaped by traditional farming practices such as grazing, haymaking and with little impact of artificial fertilizers and ploughing, are among the most species-rich habitats in Europe (Dengler et al., 2014; Török et al., 2016). Beside the high diversity of plant species, sometimes over 50 species per m² (Wilson et al., 2012), they also provide habitat for rare species from different taxonomic groups (Slaviero et al., 2016), including thousands of pollinator species, such as bees, flies, beetles, butterflies (Bastian, 2013; Zulka et al., 2014) and other invertebrates.

Thus, the presence of semi-natural grasslands may represent a key point for the retention of pollinators in agricultural landscapes. In fact, although some pollinator-dependent crops can provide local temporal concentration of floral resources, their relatively short flowering period cannot assure the continuity in forage provision needed for long-lived pollinators such as many social and solitary bees (Corbet, 1991).

Grasslands importance for plants and pollinators maintenance in agri-environments has led several studies to attempt to find which ecological condition and management practices mostly influence the richness of plants and pollinators. Findings proved that different grassland communities support different plant and pollinator species (Dover and Settele, 2009; Öckinger et al., 2012; Valkó et al., 2012), and that only a high spatial heterogeneity of grasslands differing in the disturbance history as well as in water and nutrient supply, can meet the requirements of a high gamma diversity (van Klink et al., 2015; Török et al., 2016). However, these studies ascribed a high gamma diversity to the presence of different grassland communities with different species pools, while they did not always clarify their contribution to the network of pollination interactions.

Arguably, plant-pollinator relationships might be particularly influenced by the co-occurrence of different communities (Moreira et al., 2015); in fact, some species might be exclusive to a particular community, while others might be shared by different communities, thus differently contributing to the structural organization of the overall network of interactions (i.e., showing different ecological functions; Bascompte et al., 2003; Vázquez and Simberloff, 2003; Yoshihara et al., 2008; Saavedra et al., 2011; Vanbergen et al., 2014).

In the light of these considerations, the present study aimed at evaluating the effects of co-occurring dry grassland communities differing in disturbance history as well as in water and nutrient supply (pioneer vs. late-successional grasslands) on the patterns of plant-pollinator interactions. We addressed the problem by answering the following questions: (1) Do species exclusive to one grassland community show different ecological functions compared to those in common between the two grassland communities? (2) Do differences in the temporal pattern of floral resources (i.e., nectar and pollen) between pioneer and late-successional grasslands contribute to maintain pollinator species richness at the landscape scale?

MATERIALS AND METHODS

Study area

The study took place in the Euganean Hills (NE Italy), a group of 81 volcanic hills ranging from 27 m a.s.l. (Mt. Monticelli) to 601 m a.s.l. (Mt. Venda) (Fantinato et al., 2016 a). Average annual rainfall is of 720 mm (Kaltenrieder et al., 2010), distributed according to an equinoctial pattern; two peaks, in correspondence to the months of April and September, are intermingled with two minimum, in the months of December and July. Annual mean temperature is 13.0°C; the highest average temperature values can be found in July, while the lowest in December. Bioclimatic classification (Rivas-Martínez, 2008) shows a Temperate-Oceanic type, with the exception of the southern part of the study area where a Temperate-Continental bioclimate can be observed.

The history of human influence on the land use of the Euganean Hills dates back to the Neolithic; from 4300 - 4400 cal. BP sedimentary pollen analysis proved pasture and grassland species, together with *Castanea*, *Juglans* and *Olea*, to constantly occur in the study area (Kaltenrieder et al., 2010). The presence of humans since ancient times resulted in a fine-scale landscape structure with arable fields, orchards, groves, and semi-natural grasslands intermingled with natural habitats, such as forests and rocky outcrops in a complex agricultural landscape.

Data collection

The study was conducted on semi-natural, oligo- to mesotrophic, dry grasslands. Altogether 26 plots of 2 m × 2 m were selected using a stratified random sampling design on two co-occurring dry grassland communities. 13 plots were placed on open pioneer dry grasslands, while 13 plots on dense late-successional dry grasslands. Open pioneer dry grasslands are scantily covering and dominated by low-growing dwarf shrubs (chamaephytes) (e.g., *Artemisia alba*). They establish on poorly developed calcareous soils characterized by low water retention capacity and high leaching rates (Bini, 2001; Fantinato et al., 2016 b). Dense late-successional dry grasslands are dominated by perennial herbaceous plants (hemicryptophytes) (e.g., *Bromus erectus*), and establish on more developed calcareous soils, with higher water retention capacity (Bini, 2001). Historically, the two grassland communities were maintained through different management practices. The low productivity of open pioneer dry grasslands made them suited for grazing, while late-successional dry grasslands are mowed for hay making.

For each plot we recorded the number of floral displays per entomophilous species every 15 days during the peak of the community flowering season from the 15th of April to the 14th of July. Depending on the plant species, a floral display consisted of a single flower, a flowering head (e.g., *Hieracium pilosella* inflorescence) or of a group of flowers occurring together in a recognizable visual unit (e.g., *Onobrychis arenaria*; Hegland and Totland, 2005; Fantinato et al., 2016b). Furthermore, during each survey we censused and identified pollinators under warm and sunny weather conditions. The observation period was split in 7-min sets distributed during two daily intervals (from 10 a.m. to 1 p.m. and from 1 p.m. to 4 p.m.) for a total of 14 minutes. We considered and counted as pollinators only those insects landing on the flower, visiting it for more than 1 s, and getting in direct contact with the floral reproductive organs (Hegland and Totland, 2005). Floral visitors were identified at the level of species or morphospecies and subsequently grouped on the basis of their foraging preferences according to the literature (i.e., pollen-feeding, nectar-feeding and both pollen and nectar-feeding; Willmer et al., 2011). Plant species nomenclature follows Conti et al. (2005).

Species ecological function

To account for phenological forbidden links (Olesen et al. 2010), we created 7 quantitative pollination interaction matrices (one for survey), in which rows represented pollinator species, columns plant species and entries the number of contacts between each pair of plant and pollinator species.

For each pollination interaction matrix we calculated two quantitative descriptors of pollination networks as a necessary step in the analysis of species ecological function (in the R-based package “Bipartite”; Dormann et al. 2008). To describe network properties we used the following indices (Table 1): weighted nestedness (WNDOF) and quantitative modularity (Q). The significance of weighted nestedness and quantitative modularity were assessed against a null model obtained by constructing 1000 randomized networks with identical margin totals as the empirical network, and comparing the indices value between observed and random networks using the null model ‘r2d’ (Guimerà and Amaral, 2005).

To determine the ecological function of each plant and pollinator species, we calculated the following metrics (Table 1): species selectiveness (d'), strength (S), weighted closeness centrality (wCC), contribution to nestedness (n_i), standardized connection (c) and participation values (z). Species showing a high z and a low c value can be regarded as module hubs of the pollination networks; species with low z but high c value as connectors; species with high values of both c and z as network hubs; while all the other species (with low c and z values) as peripherals (Guimerà and Amaral, 2005; Olesen et al., 2007).

Table 1. Description of metrics used to determine network properties and species ecological function.

Community-level network properties			
Metric	Description	Range	References
WNDOF	Measures the degree of nestedness (i.e., how much specialists interact with generalists, while generalists interact with both specialists and generalists), weighted by sample size.	From 0 to 100. A low value indicates chaos while a high value perfect nestedness.	Galeano et al., 2009
Q	Measures the degree to which a network is structured in subgroups of highly connected nodes (modules).	From 0 to 1. A low value indicates that the community has no more links within modules than expected by chance (low modularity), while a high value that the network is divided into modules.	Dormann and Strauss, 2013
Species-level network properties			
Metric	Description	Range	References
d'	Measures the exclusiveness of a species partner spectrum compared with other species in the network.	From 0 to 1. A low value indicates complete partners sharing, while a high value no sharing of partners with other species of the same trophic level.	Blütgen et al., 2006
S	Measures how important a species is in the network (i.e., how much the other species in the network depend on the selected species).	From 0 to positive infinity. A low value indicates that the other species only partially rely on the selected species, while high values indicate a high level of dependence.	Bascompte et al., 2006
wCC	Measures of the centrality of individual species within the topography of the network.	From 0 to 1. Low values are typical of marginal species, while high values of generalised keystone species with more linkages to other centrally-located species.	Ballantyne et al., 2017
n_i	Determines how individual species' interactions change the community nestedness compared to a random null model.	From -1 to 1. Negative values identify species negatively contributing to nestedness, while positive values indicate species positively contributing.	Saavedra et al., 2011
c	Defines the degree of connection of a species with species of other modules.	From 0 to 1. Low values are typical of species connected only with species of their own module, while high values of species with links equally distributed among modules.	Olesen et al., 2007
z	Defines the position of a species within its own module.	From negative infinity to positive infinity. Low values are representative of scarcely linked species, while high values of species highly connected.	Olesen et al., 2007

Floral resources

For each entomophilous plant species ($n = 28$) we quantified the amount of floral resources, nectar and pollen, on ten to twenty floral displays of different individuals. Nectar and pollen were collected as standing crop from freshly opened flowers from 8 a.m. to 10 a.m.. We measured nectar volume (μl) by using microcapillary tubes (Drummond Scientific Company, Broomal, PA, USA). Nectar was drawn into the microcapillary tubes by means of capillary action (Kearns and Inouye, 1993). The volume of withdrawn nectar was then determined by measuring the column of nectar within the tube and calculating the proportion of the entire column ($2 \mu\text{l}$) filled. Where no nectar was detected with the microcapillary tubes, flowers were examined with a magnifying glass to check for small amounts of nectar (Carvalho et al., 2014). The number of pollen grains was quantified by visually counting them under the microscope (Kearns and Inouye, 1993). Dehisced pollen was suspended in 70% ethanol, and the number of pollen grains in a known volume aliquot was counted on a haemocytometer slide. Total pollen grain number was calculated through a proportion. Food- and sexual-deceptive orchid species were excluded from the resource analysis because orchid pollinia bear coherent masses of pollen grains that pollinators are unable to actively eat or collect (Johnson and Edwards, 2000; Fantinato et al., 2017).

For each survey, we quantified the mean number of pollen grains and the mean nectar volume produced by entomophilous plant species harbored in pioneer and in late-successional grasslands. In particular, for each plot in each survey we multiplied the mean number of pollen grains and the mean nectar volume for the number of visual displays per species and we averaged results among plot belonging to the same grassland community, thereby obtaining two time series for each grassland community (pioneer vs. late-successional) for the number of pollen grains and the nectar volume, respectively.

Statistical analysis

To identify differences between pioneer and late-successional grasslands in the composition of plant and pollinator species we reported all temporal observations into two presence/absence matrices: a plant species \times plot matrix and a pollinator species \times plot matrix. Differences in the composition of plants and pollinators between pioneer and late successional grasslands were tested by conducting two one-way PERMANOVAs (one for plants and one for pollinators; Anderson and ter Braak, 2003) with 9999 permutations using the R-based package *vegan* (version 2.4; Oksanen et al., 2016). Plant and pollinator species were used as dependent variables, sampling plots as replicates and the grassland communities as fixed factor.

In order to detect differences in the ecological function among plant species exclusive to either pioneer or late-successional grasslands, and among those in common between the two grassland communities we performed a one-way PERMANOVA with 9999 permutations, followed by post hoc Tukey test. We used metrics describing species ecological function as dependent variables, plant species as replicates and species exclusiveness as fixed factor. If a plant species was recorded in more than one survey we took the mean value of each metrics. We followed the same procedure to compare ecological function metrics (i.e., S , d' , wCC , n_i , c and z) and feeding preferences (for nectar, pollen or both) between exclusive and shared pollinators.

To compare similarities between pioneer and late-successional grasslands in terms of temporal patterns of floral resources, we performed two cross-correlations (i.e. one for pollen and one for nectar; Venables and Ripley, 2002). The significance of cross-correlations was set at the 5%. Floral resources time series were previously tested for stationary by using the autocorrelation function and visually interpreting autocorrelation correlograms. In stationary process autocorrelation r_k decays quickly to zero (Venables and Ripley, 2002). All calculations were done within the R statistical framework (R-Development-Core-Team, 2012).

RESULTS

Overall, we recorded 411 contacts between 28 plant and 66 pollinator species. Pollination networks were significantly nested and modular throughout the flowering season (WNDOF and Q; $P < 0.001$; Table 2).

Table 2. Network parameters for each survey. H'_2 : complementary specialization; WNDOF: weighted nestedness; Q: quantitative modularity. (***) $P < 0.001$.

Survey	1st	2nd	3rd	4th	5th	6th	7th
WNDOF	17.342 ^{***}	10.655 ^{***}	2.391 ^{***}	5.857 ^{***}	7.842 ^{***}	5.952 ^{***}	7.692 ^{***}
Q	0.421 ^{***}	0.534 ^{***}	0.705 ^{***}	0.562 ^{***}	0.585 ^{***}	0.667 ^{***}	0.494 ^{***}

Species ecological function

Composition of both plant and pollinator species widely differed between pioneer and late-successional grassland communities (Table 3) showing only narrow groups of shared species. Especially, we found 9 plant species exclusive to pioneer grasslands, 12 exclusive to late-successional grasslands and 7 shared by both grassland communities. Among plant species families (13 families), Fabaceae, Lamiaceae, Rubiaceae and Scrophulariaceae were shared by both grassland communities. Cistaceae, Convolvulaceae, Orchidaceae and Plantaginaceae were exclusive to pioneer grasslands, while Dipsacaceae, Gentianaceae, Geraniaceae, Liliaceae and Linaceae to late-successional grasslands. As for pollinator species, 27 were exclusive to pioneer grasslands, 29 to late-successional grasslands, while only 8 species were present in common. Even though orders of pollinators did not show a clear association with one grassland community, shared pollinator species were for 75% Hymenoptera.

Table 3. Differences in the composition of plants and pollinators between pioneer and late-successional grasslands, evaluated by PERMANOVAs.

PERMANOVA		
	Plant species composition	Pollinator species composition
Permutation N:	9999	9999
F:	4.147	2.754
P (same):	< 0.001	< 0.001

Significant differences in the ecological function metrics were revealed by PERMANOVA ($F = 3.636$; $P < 0.01$) among plant species exclusive to pioneer and late-successional grasslands, and those shared by the two grassland communities. Especially, post hoc Tukey test indicated that exclusive plant species exhibited a significantly higher strength (S) ($P < 0.05$) than shared plant species (Fig. 1). Furthermore, both standardized connection (c) and participation values (z) were significantly higher in exclusive plant species (c and z ; $P < 0.05$) than in shared species (Fig. 1).

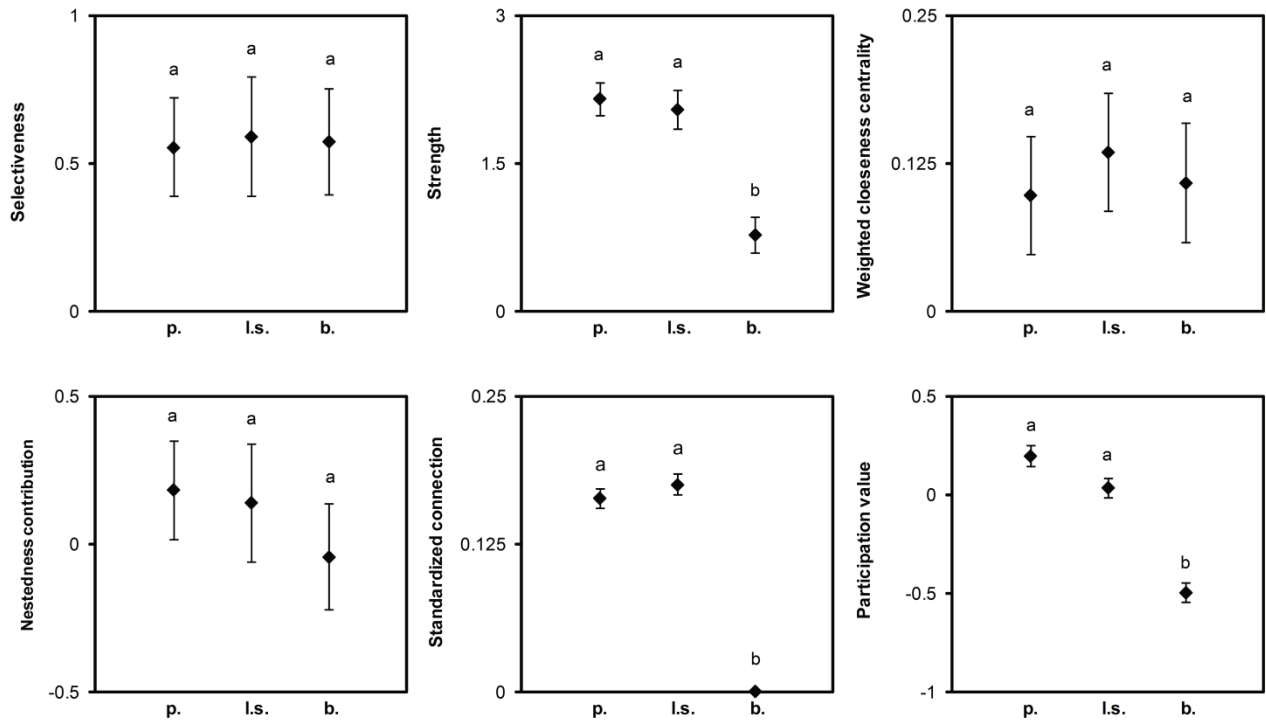


Figure 1. Mean (\pm SD) values of selectiveness (d'), strength (S), weighted closeness centrality (wCC), nestedness contribution (n_i), standardized connection (c) and participation values (z), of plant species exclusive to pioneer and late-successional grasslands, as well as shared by both grassland communities. Different letters indicate significant differences among plant species groups. Abbreviations: p., pioneer grasslands; l.s., late-successional grasslands; b., both grasslands.

Ecological function metrics differed significantly among pollinator species exclusive to pioneer and late-successional grasslands, and those shared by the two grassland communities (PERMANOVA; $F = 4.402$; $P < 0.0001$). Post hoc Tukey test revealed that shared pollinator species had a significantly higher strength (S) ($P < 0.0001$) than exclusive plant species (Fig. 2). Moreover, both standardized connection (c) and participation values (z) were significantly higher for shared than for exclusive pollinator species (c and z ; $P < 0.001$; Fig. 2). Contrarily to plant species, shared pollinator species connected species within and between modules significantly more than the exclusives. Furthermore we observed a significant difference in the feeding preferences between exclusive and shared pollinator species ($P < 0.05$). Especially, the majority of shared pollinators fed of both pollen and nectar while exclusive pollinators were much more specialized to the pollen or the nectar resource (Fig. 2).

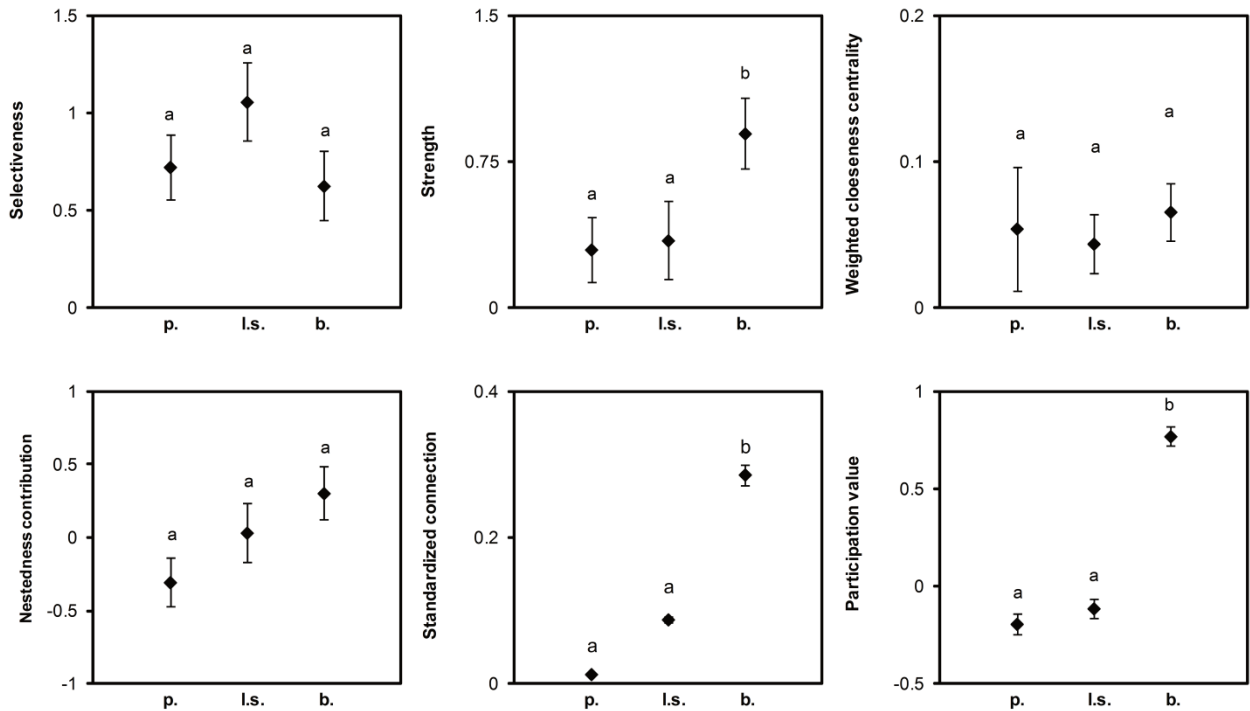


Figure 2. Mean (\pm SD) values of selectiveness (d'), strength (S), weighted closeness centrality (wCC), nestedness contribution (n_i), standardized connection (c) and participation values (z), of pollinator species exclusive to pioneer and late-successional grasslands, as well as shared by both grassland communities. Different letters indicate significant differences among pollinator species groups. Abbreviations: p., pioneer grasslands; l.s., late-successional grasslands; b., both grasslands.

Floral resources

Correlograms suggested that all time series were stationary, with autocorrelation coefficients r_k decaying quickly to zero. Cross-correlations between pioneer and late-successional grasslands disclosed temporal mismatches in the peak of pollen and nectar production. Cross-correlation at the 5% level of significance were found at lag 2 for the pollen grain number and at lag 3 for the nectar volume. In fact, in pioneer grasslands pollen grain number peaked at the 4th survey, while at the 6th survey in late-successional grasslands (Fig. 3 A). On the contrary, nectar volume peaked at the 2nd survey in late-successional grasslands, and at the 5th survey in pioneer grasslands (Fig. 3 B).

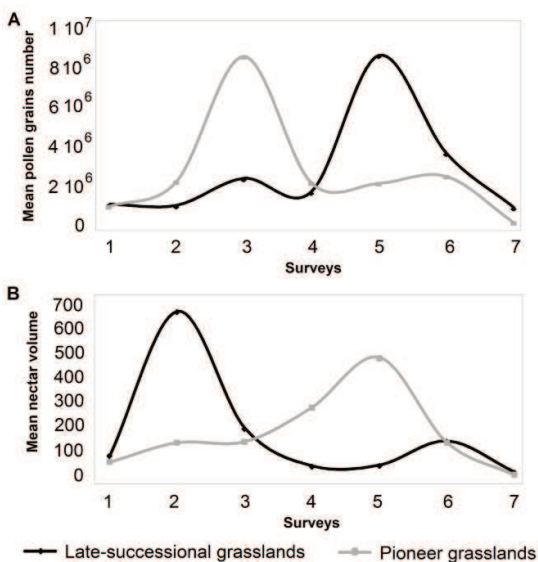


Figure 3. Temporal trend of mean pollen grain number (A) and of mean nectar volume (B) per plot, in late-successional and pioneer grasslands.

DISCUSSION

The two dry grassland communities we examined showed to be highly interconnected. The two communities have in common both plant and pollinator species, resulting in a highly resilient pollination network at the landscape scale. A nested organization of pollination interactions (WNODF; $P < 0.001$) is expected to increase system robustness by decreasing the probability of local extinction of specialists, which are considered the most vulnerable network members (Fortuna and Bascompte 2006; Joppa et al. 2010). Furthermore, a modular network (Q; $P < 0.001$) has been recognized to slow the spread of disturbances further increasing the stability of pollination networks (e.g., Krause et al., 2003). However, we interestingly demonstrated that exclusive plant species, which are responsible for the heterogeneity of dry grassland communities, accounted more than shared species for the stability of the pollination web and for the maintenance of the overall network of interactions at the landscape scale. Exclusive plant species showed a significantly higher strength than shared plant species (S; $P < 0.05$), indicating that pollinator species depended more on plant species growing either in pioneer or in late-successional grasslands than on those growing indistinctly in both grassland communities. Furthermore, shared and exclusive plant species significantly differed in the standardized connection (c) and participation (z) values ($P < 0.05$), with exclusive plant species playing a much important functional role in the pollination web at the landscape scale than shared plant species, because more connected with both plant species belonging to their own module and with those of the other modules.

Shared pollinator species, on the other hand, showed to account more than exclusive pollinator species for the sustainment of dry grassland pollination webs at the landscape scale. In dry grassland communities shared pollinators were almost coincident with the network hub species found by Olesen et al. (2007) (i.e., large social bees like *Apis mellifera* and *Bombus* spp., large solitary bees, such as *Anthidium manicatum* and *Osmia* spp., and the syrphid fly *Episyrphus balteatus*), revealing a repeated pattern of species functional role in different plant communities. Moreover, in heterogeneous landscapes, they might exert a stabilizing effect by widening the range of interactions, and thus, the redundancy of the resulting pollination web (Blütghen et al., 2008; Rooney et al., 2008; Blütghen et al., 2011; Kaiser-Bunbury et al., 2015). In fact, we demonstrated that shared pollinator species showed a significantly higher strength (S; $P < 0.001$) than those exclusive to either grassland community, meaning that animal-pollinated species depended more on shared pollinators than on the exclusives. Furthermore, shared pollinators connected species within and between network modules more than the exclusive ones (z and c values; $P < 0.001$).

In summary, the functional roles of exclusive and shared plant and pollinator species were asymmetric, with exclusive plants being hub species and shared plants peripherals, while shared pollinators were hub species, and exclusive pollinators peripherals. The observed asymmetric pattern might critically contribute to the maintenance of the pollination web stability at the landscape scale, by increasing the degree of redundancy, thus of resilience, of the network of interactions (Ashworth et al., 2004; Kaiser-Bunbury et al., 2010). If, for example, both shared plant and pollinator species were hub species, we might expect a progressive homogenization of grassland communities, with an increased dominance of shared species. On the contrary, if both exclusive plant and pollinator species were hub species, we might expect the maintenance of a strong isolation between communities. In both cases the landscape pollination webs would possibly have shown a high compartmentation (i.e., low redundancy), with one compartment in the first case and two distinct compartments in the second case, resulting in a lower pollination web resilience at the landscape scale.

Furthermore, pioneer and late-successional grasslands showed a high temporal complementarity of floral resources, with the peak of pollen in pioneer grasslands approximately coinciding with the peak of nectar in late-successional grasslands and vice-versa. Since pollinator species usually show periods of activity that overcome the flowering length of each single plant species (Willmer et al., 2011; Senapathi et al., 2015), a sequential patterning of floral resources between co-occurring dry grassland communities might help to maintain the pollinator community at the landscape scale, including insects seeking different or multiple

resources. In fact, beside pollinators feeding exclusively of pollen or nectar, the positive effects exerted by co-occurring grassland communities on landscape richness of pollinator species become even more outstanding as far as pollinator species feeding of both pollen and nectar are concerned. Almost all pollinator species shared by surveyed communities sought both pollen and nectar, suggesting that pollinator species richness at the landscape scale was higher than the sum of species harbored exclusively by each grassland community, because of the biodiversity resulting from their co-occurrence.

The co-occurrence of grassland communities offering complementary floral resources may, in turn, increase pollinator species richness, which would result in a higher chance for entomophilous plant species to be pollinated (Albrecht et al., 2012). In fact, a higher richness of pollinator species has been proven to exert positive effects on plant communities by increasing both fruit and seed set of animal-pollinated plant species (Johnson et al., 2003; Ghazoul et al., 2006).

CONCLUSIONS

Mosaic of different plant communities has far been recognized to crucially contribute to maintain species with different requirements at the landscape scale. By focusing on pollination interactions, we found that the co-occurrence of different dry grassland communities plays a key role not only for the conservation of exclusive species, but also because they simultaneously contribute to the sustainment of the biodiversity resulting from their co-occurrence. Dry grasslands are among the most important semi-natural habitats in Europe. However, they are severely endangered by changes in land use, both through intensification of agriculture as well as abandonment followed by decreased habitat quality and the consequent decline of the biodiversity they host. Being characteristic of agricultural landscapes dry grassland communities can crucially contribute to biodiversity and pollination service retention by providing a stable habitat available for foraging and life cycle completion of pollinators. Enhancing wild pollinator populations through the conservation of different dry grassland communities can thus be especially important at the level of entire agricultural landscape, meeting both biodiversity conservation and agricultural production goals.

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Are food-deceptive orchid species really functionally specialized for pollinators?

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Abstract Food-deceptive orchid species have traditionally been considered pollination specialized to bees or butterflies. However, it is unclear to which concept of specialization this assumption is related; if to that of phenotypic specialization or of functional specialization. The main aim of this work was to verify if pollinators of five widespread food-deceptive orchid species (*Anacamptis morio* (L.) R.M. Bateman, Pridgeon & M.W. Chase, *Anacamptis pyramidalis* (L.) Rich., *Himantoglossum adriaticum* H. Baumann, *Orchis purpurea* Huds. and *Orchis simia* Lam.) predicted from the phenotypic point of view matched with the observed ones. We addressed the question by defining target orchid phenotypic specialization on the basis of their floral traits, and we compared the expected guilds of pollinators with the observed ones. Target orchid pollinators were collected by conducting a meta-analysis of the available literature and adding unpublished field observations, carried out in temperate dry grasslands in NE Italy. Pollinator species were subsequently grouped into guilds and differences in the guild spectra among orchid species grouped according to their phenotype were tested. In contradiction to expectations derived from the phenotypic point of view, food-deceptive orchid species were found to be highly functionally generalized for pollinators, and no differences in the pollinator guild spectra could be revealed among orchid groups. Our results may lead to reconsider food-deceptive orchid pollination ecology by reevaluating the traditional equation orchid-pollination specialization.

Keywords Dry grasslands · Food-deceptive orchids · Functional specialization · Pollination · Specialization trade-offs

Introduction

Specialization in plants may arise as an adaptive response to a particular environmental state defined by a narrow combination of environmental parameters, such as abiotic factors (e.g., soil moisture or climate parameters; Pierce et al. 2017), structural community features (e.g., above-ground cover density and vertical structure), or interspecific interactions (Sburlino et al. 2008; Buffa and Villani 2012; Vamosi et al. 2014). Specialization is considered as the ultimate response of plant species to the environment, with ‘advanced’ plants being more specialized than ‘primitive’ plants, because more effective in the provision of needed resources under different as well as original environmental conditions (Ollerton 1999; Gómez and Zamora 2006). However, at least in pollination interactions, several authors proved that the ultimate response may be also an increased degree of generalization (Armbruster et al. 2000; Tripp and Manson 2008). In fact, being a specialized species often claims cost: specialized species are under greater extinction risk than generalist species, especially when they totally depend on a restricted combination of environmental resources (Gumbert et al. 1999; Dante et al. 2013).

The linkage between specialization and rarity has been proven also for orchids, with almost half of the extinct orchid species being temperate terrestrial perennials (IUCN 1999). In terrestrial orchids, specialization has been mostly found to pertain processes related to seeds germination and biotic pollination (Selosse et al. 2002; Otero et al. 2004; Anderson et al. 2005). Orchid seeds may have very specific ecological requirements to overcome dormancy and initiate the growth of the embryo (Baskin and Baskin 2014). In many terrestrial orchids, seed germination and growth is subordinate to

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mycorrhizal associations (Warcup 1973; Rasmussen 2002), which can extend to adulthood, when mature plants remain colonized by mycorrhizal fungi (Batty et al. 2002; Rasmussen and Whigham 2002). Moreover, the establishment of some terrestrial orchid species is affected by the structure of the surrounding vegetation, which would constrain both seed germination and seedling establishment (Slaviero et al. 2016).

Orchid specialization becomes even more outstanding when examining pollination processes. In fact, orchids are widely believed to have the highest degree of pollinator specialization when compared with other families of flowering plants (Tremblay 1992; Ibisch et al. 1996). In plant-pollinator interactions, specialization of plant species for pollinators can be defined according to three different approaches. First, by counting the number of interacting pollinator species, thus defining specialized plants as those that attract a particular subset of the available animal species. This rigorous partner selection has been defined as ecological specialization, to distinguish it from phenotypic specialization (Ollerton et al. 2007). Phenotypic specialization (second definition) assigns plant species degree of specialization a priori according to a particular set of morphological traits (e.g., length of the corolla tube) by following the pollination syndrome approach (Fægri and van der Pijl 1979). Accordingly, one of the major features of flowering plants is that particular suites of floral characters can be associated with a specific functional group of animal pollinators which, however, may or may not be confirmed by observations (Stebbins 1970; Fægri and van der Pijl 1979; Pellmyr 2002; Fantinato et al. 2016a). Phenotypic specialization is often associated with functional specialization (third definition), that occurs when a plant species is observed to be visited exclusively by species of functionally similar pollinators (namely, pollinator guilds), for example butterflies (Fenster et al. 2004).

The majority of orchid species rely on biotic pollen vectors to reproduce (Nilsson et al. 1992), often by establishing highly ecological specialized interactions. It is supposed that the 60% of orchids are pollinated by only one or few pollinator species (Tremblay 1992). However, much uncertainty remains over, because in many cases the pollinator has never been observed. Orchids pollination is even more controversial because about the 30% (Ackerman 1986) employ among the most complex deception systems known in angiosperms to secure pollination without offering rewards (Tremblay et al. 2005; Jersáková et al. 2006). Visitors are deceived by various means; most commonly the deception is based on sexual advertisements or on a general resemblance to rewarding producing plants (Tremblay et al. 2005). Sexual deception occurs in 18 orchid genera (Jersáková et al. 2006), in which flowers resemble the females of the pollinating species and are pollinated by the males during attempts at mating (pseudocopulation; e.g., Schiestl et al. 2003).

However, in non-rewarding orchids, food-deception is reported as the most common pollination mechanism (reported in 38 genera; Jersáková et al. 2006). Food-deceptive orchids exploit pollinators' innate food-foraging behavior (Nilsson 1980; Dafni 1983) by miming typical rewarding flower traits, such as inflorescence shape, flower colour, nectar guides, spurs and pollen-like papillae (Gumbert and Kunze 2001; Galizia et al. 2005). Originally, Darwin (1885), reinforced by Müller's (1873) and Delpino's (1868–1875) observations, asserted that “bees (...) should persevere in visiting flower after flower of the above named Orchids, (...) in the hope of obtaining nectar which is never present”. Hence, from Darwin's time onwards, food-deceptive orchid species have been generically considered to be visited by a narrow functional group of pollinators (e.g., Inda et al. 2012; Schiestl 2012). Literature data report that, contrarily to sexual-deceptive orchids, food-deceptive orchids are ecologically generalized because they are visited by a relatively diverse group of pollinators (Cozzolino et al. 2005), but that all pollinators belong to the guild of bees (or rarely of butterflies; Cozzolino and Widmer 2005). However, it is not clear to which concept of specialization this assumption is related; if to that of phenotypic specialization or of functional specialization.

In the light of these considerations, our goal was to assess the degree of functional specialization for pollinators of five food-deceptive orchid species native to temperate dry calcareous grasslands (i.e., *Anacamptis morio* (L.) R.M. Bateman, Pridgeon and M.W. Chase, *Anacamptis pyramidalis* (L.) Rich., *Himantoglossum adriaticum* H. Baumann, *Orchis purpurea* Huds. and *Orchis simia* Lam.). Especially, we answered the following questions: (1) does the expected guild of pollinators predict the observed one? (2) are target orchid species functionally specialized for pollinators? We addressed the problem by defining target orchids phenotypic specialization on the basis of their floral traits (sensu Fægri and van der Pijl 1979), and we compared the expected guilds of pollinators with the observed ones.

Materials and methods

Literature search

We carried out an extensive literature search in the ISI Web of Knowledge database using as keyword combinations the scientific name of target orchids followed by ‘pollination’ or ‘pollinators’ in the topic field. We also included insect species reported in Claessens and Kleylen (2011) and not included in the studies found via the ISI Web of Knowledge. We retained only insects identified at species level and referred to as carrying pollinia on their body, and we sorted synonyms.

Site selection and data collection

The field study took place in the Euganean Hills (NE Italy), a group of about 100 individual hills (15,096 ha) isolated in the Po plain, characterized by conic shapes of different elevations (peaks altitude ranges from 13 to 601 m a.s.l.). The hills are of submarine volcanic origin, emerged during two phases occurred between 45 million and 35 million years ago. This led volcanic and calcareous formations to co-exist nearby each other. The site shows a warm-rainy climate (Kaltenrieder et al. 2010). The mean annual temperature is of 13.0 °C with the peak of the mean high temperature in July and the low in January. The average annual rainfall is of 720 mm peaking in April and September, intermingled by two minimum in July and December. Study sites (Table 1) were represented by small- to medium-sized *Bromus erectus*-dominated dry grasslands, extending from 0.27 to 6.95 ha (ranging from 68 to 284 m a.s.l.; Fantinato et al. 2016b). Dry grasslands were chosen because, among natural and semi-natural habitats, they host important orchid populations when low-intensity agricultural regimes are maintained (Pierce and Belotti 2011; Slaviero et al. 2016; Biella et al. 2017). In Europe, dry grassland communities also achieved a legal status as they are listed as endangered habitat in Annex I of the Habitats Directive 92/43 (EEC 1992). Furthermore the habitat is considered a priority type if it is an important orchid site (EEC 2013).

Target orchids are listed in the Annex I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) of Washington (1973; www.cites.org), and in the national and regional red list

Table 1 Localities, coordinates (Decimal degrees) and altitude (m a.s.l.) of the sampled grasslands

Locality	Latitude N	Longitude E	Altitude (m a.s.l.)
Mt Mottolone	45.287431	11.705459	273
Val di Spin	45.280217	11.704192	202
Sassonegro	45.265562	11.699706	104
Mt Cecilia	45.255150	11.699845	112

Table 2 Floral traits collected for the five target orchid species

	<i>A. morio</i>	<i>A. pyramidalis</i>	<i>H. adriaticum</i>	<i>O. purpurea</i>	<i>O. simia</i>
Brightness	Low	High	Low	Low	Low
Colour	Purple	Red	White	Purple	White
Floral symmetry	Bilateral	Bilateral	Bilateral	Bilateral	Bilateral
Flowering length (surveys nr)	2	3	2	2	1
Mean spur length ± SD (mm)	0.80 ± 0.10	1.34 ± 0.11	0.39 ± 0.02	0.61 ± 0.16	0.42 ± 0.07
Mean spur width ± SD (mm)	0.17 ± 0.05	0.10 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
Nectar guides	Present	Absent	Present	Present	Present
Nectar presence	Absent	Absent	Absent	Absent	Absent
Pollen deposition	Head and tongue	Head and tongue	Head and tongue	Head and tongue	Head and tongue

Brightness and colour were derived from the BioFlor database (Klotz et al. 2002); while all the other traits were recorded in the field. *A. morio*, *Anacamptis morio*; *A. pyramidalis*, *Anacamptis pyramidalis*; *H. adriaticum*, *Himantoglossum adriaticum*; *O. purpurea*, *Orchis purpurea*; *O. simia*, *Orchis simia*

of vascular plants (Rossi et al. 2013; Buffa et al. 2016). According to the regional red list of vascular plants (Buffa et al. 2016), *A. morio*, *A. pyramidalis*, *O. purpurea* and *O. simia* were classified as Least Concern (IUCN 2013), while *H. adriaticum* as Near Threatened (IUCN 2013). Even though at regional level, target orchid species were assessed under the threshold of extinction, they are increasingly threatened by the decline of their habitat, which has been listed as Vulnerable in the European Red List of Habitat (Janssen et al. 2016).

Overall, 20 (4 m²) plots were selected through a preferential sampling design (on target orchids populations). *A. morio* occurred in 13 plots with an average number of 33.53 ± 27.00 (mean ± SD) individuals, *A. pyramidalis* in 6 plots with 2.40 ± 1.51 individuals, *H. adriaticum* in 3 plots with 4.33 ± 5.77 individuals, *O. purpurea* in 3 plots with 4.00 ± 0.00 individuals and *O. simia* in 6 plots with 7.83 ± 6.64 individuals. The number of plots was proportional to the target orchids frequency in the study site and in many cases different orchid species co-occurred in the same plot.

For each target orchid species we collected floral traits (Table 2) on five flowers per species. We measured the length and the width of the spur and ascertained the presence of nectar at its bottom by using microcapillary tubes (Kearns and Inouye 1993). Additionally, we recorded floral symmetry and the presence of nectar guides on the labellum in the field, while brightness and colour based on the BioFlor database (Klotz et al. 2002). Floral traits were chosen on the basis of their acknowledged relevance for the identification of pollination syndromes (Fægri and van der Pijl 1979; Willmer 2011).

Target orchids flowering length (Table 2) and pollinators were recorded every 15 days, during the entire flowering season in 2016 (1st April to 15th June), under warm and sunny weather conditions.

Interactions between target orchids and visiting insects were monitored in each plot during 15 min (from 10 a.m. to 1 p.m.) and repeated for 5 surveys to cover the entire flowering season. Orchid visitors were identified to species, and presence and position of pollinia on insects' body after each visit was ascertained (Table 2). Both

pollinators found in literature and those recorded in the field were grouped following the classical pollination syndrome (Fægri and van der Pijl 1979) into five guilds: bees, beetles, butterflies, flies and moths. Furthermore, the pollinator guild of bees, which is traditionally considered as a single guild by the pollination syndrome theory, has been split into three guilds according to bee tongue length, following Fenster et al. (2004) and Scopce et al. (2007). Especially, we identified long tongue bees (>8 mm), medium tongue bees (>6 and <8 mm) and short tongue bees (<6 mm; Goulson et al. 2005; Willmer 2011). The grouping of pollinators into guilds allowed us to highlight interactions from a functional perspective rather than taxonomical (e.g., Fang and Huang 2012; Fontaine et al. 2006).

Data analysis

Overall pattern of floral trait variation and its relation to species' identity were investigated using principal component analysis (PCA; Legendre and Legendre 1998). Since floral symmetry (bilateral), nectar volume (absent) and pollen deposition (head and tongue) were constant for all the target orchid species, we excluded them from

the PCA. For each group of orchid species resulting from the PCA we inferred expected guilds of pollinators according to Willmer (2011).

Furthermore, we tested if observed guilds of pollinators differed significantly among orchid species grouped according to their phenotype by using one-way PERMANOVA with 9999 randomization (Past software; Hammer et al. 2001). Analysis were performed by taking into account pollinators reported in the literature along with insects observed in the field. In both cases, only flower visitors recorded as carrying pollinia on their body were considered. This, according to literature on orchid pollination ecology (e.g., Cozzolino et al. 2005; Jersáková et al. 2016; Braunschmid et al. 2017) allowed us to count them as potential pollinators, and to distinguish them from simple floral visitors ineffective in carrying pollinia.

Results

The ordination of floral traits variation and their relation to target orchid species displayed two clear gradients (Fig. 1; Table 3). The first PCA axis explained the 76.30% of total floral trait variation and discriminated

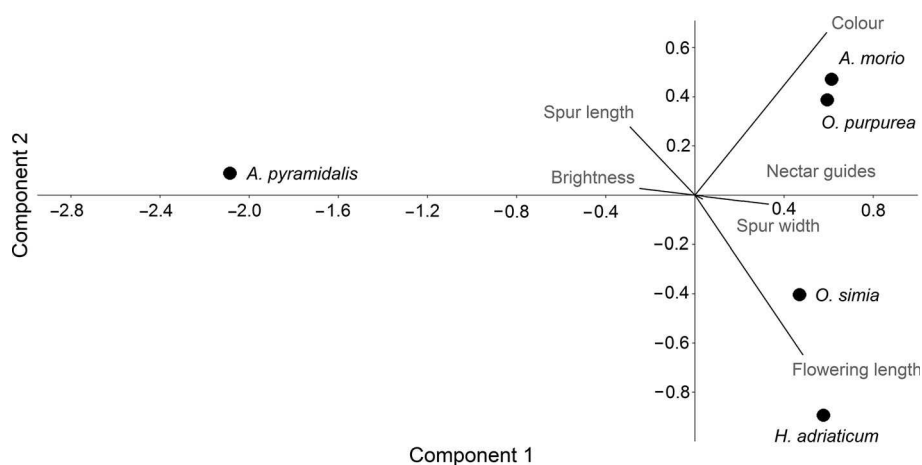


Fig. 1 First two axes of the principal component analysis of target orchid species based on floral traits. For abbreviations of orchid genus name, see Table 2 in the caption

Table 3 Principal component analysis of target orchid species based on floral traits

	Axis 1	Axis 2	Axis 3	Axis 4
Variation explained (%)	76.30	20.88	2.66	1.44
Trait	Loadings			
Brightness	-0.37	0.04	0.37	-0.45
Colour	0.62	0.69	0.27	-0.22
Flowering length	0.49	-0.66	0.56	0.00
Nectar guides	0.37	-0.04	-0.38	0.46
Spur length	-0.28	0.27	0.56	0.71
Spur width	0.03	-0.01	-0.05	-0.15

orchid species according to the flower colour, and the flowering length. The second PCA axis, explaining the 20.88% of trait variation, separated orchid species mainly according to the colour and the spur length. Therefore, according to the phenotypic point of view, target orchid species could be assigned to three distinct groups, which reflected three different pollination syndromes. *H. adriaticum* and *O. simia*, being characterized by a bilateral symmetry, a relative short spur, and light colours coupled with the presence of marked nectar guides were assigned to the syndrome of short tongue bees. Likewise, *A. morio* and *O. purpurea* showed a bilateral symmetry and the presence of nectar guides, however a longer spur made them better described by the syndrome of medium tongue bees. Lastly, *A. pyramidalis* having long-spurred pink flowers resembled the traditional butterfly syndrome.

The meta-analysis allowed us to identify 19 publications from all over Europe (ESM 1). Out of these publications, 6 were about *A. morio*, 11 about *A. pyramidalis*, 3 about *H. adriaticum*, 2 about *O. purpurea* and 3 about *O. simia*. In total, 81 pollinator species were reported in literature: 25 for *A. morio*, 50 for *A. pyramidalis*, 8 for *H. adriaticum*, 2 for *O. purpurea*, and 4 for *O. simia*.

Field survey allowed the observation and identification of 6 pollinia-carrying visitor species for *A. morio* (Table 4), 6 for *A. pyramidalis* (Table 4), 4 for *H. adriaticum* (Table 5), 2 for *O. purpurea* (Table 5) and 2 for *O. simia* (Table 5).

Overall, in contradiction to expectations derived from the phenotypic point of view, we showed that target orchid species interacted with a wide spectrum of guilds of pollinators (Fig. 2). *A. morio* and *A. pyramidalis* were the most generalist, because observed to interact with 6 guilds of pollinators; *H. adriaticum* and *O. simia* with 4 guilds of pollinators; while *O. purpurea* with 3 pollinator guilds. Furthermore, guilds of pollinators were widely shared by orchid species groups; in fact, no significant differences in the proportion of the observed pollinator guilds were revealed by PERMANOVA ($F = 7.99$; $P > 0.05$).

Discussion

We found *A. morio*, *A. pyramidalis*, *H. adriaticum*, *O. purpurea*, and *O. simia* to be more functionally generalized for pollinators than expected from the phenotypic point of view. According to Ollerton et al. (2009), primary pollinators can be predicted by the floral pheno-

Table 4 Identity of pollinators caught on *Anacamptis morio* and *Anacamptis pyramidalis* inflorescences

	<i>Anacamptis morio</i>	<i>Anacamptis pyramidalis</i>
Beetles	* <i>Tropinota hirta</i> (Poda, 1761)	* <i>Tropinota squalida</i> (Scopoli, 1783)
Moths	* <i>Macroglossum stellatarum</i> (L., 1758)	* <i>Zygaena carniolica</i> (Scopoli, 1763) * <i>Zygaena trifolii</i> (Esper, 1783) * <i>Epysirphus balteatus</i> (De Geer, 1776)
Flies		
Short tongue bees	* <i>Dasypoda hirtipes</i> (F., 1793)	
Medium tongue bees	* <i>Bombus pascuorum</i> (Scopoli, 1763)	* <i>Apis mellifera</i> (L., 1758)
Long tongue bees	* <i>Anthophora retusa</i> (L., 1758) * <i>Bombus hortorum</i> (L., 1761)	* <i>Bombus hortorum</i> (L., 1761)
Other visitors	<i>Bombylius major</i> (L., 1758)	<i>Melanargia galathea</i> (L., 1758) <i>Pieris brassicae</i> (L., 1758) <i>Scolia flavifrons</i> (F., 1775) <i>Vanessa cardui</i> (L., 1758)

* Indicate that pollinia were observed on the insect body

Table 5 Identity of pollinators caught on *Himantoglossum adriaticum*, *Orchis purpurea* and *Orchis simia* inflorescences

	<i>Himantoglossum adriaticum</i>	<i>Orchis purpurea</i>	<i>Orchis simia</i>
Beetles	* <i>Tropinota squalida</i> (Scopoli, 1783)	* <i>Tropinota hirta</i> (Poda, 1761)	* <i>Tropinota hirta</i> (Poda, 1761)
Butterflies		* <i>Iphiclides podalirius</i> (L., 1758)	
Short tongue bees	* <i>Anthidium manicatum</i> (L., 1758)		
Medium tongue bees	* <i>Apis mellifera</i> (L., 1758)		* <i>Apis mellifera</i> (L., 1758)
Long tongue bees	* <i>Colletes daviesanus</i> (Smith, 1846)		
Other visitors	<i>Eristalis arbustorum</i> (L., 1758) <i>Scolia hirta</i> (Schrank, 1781)	<i>Bombus terrestris</i> (L., 1758)	<i>Bombylius major</i> (L., 1758) <i>Lasioglossum marginatum</i> (B., 1832) <i>Vanessa cardui</i> (L., 1758)

Asterisks indicate that pollinia were observed on the insect body

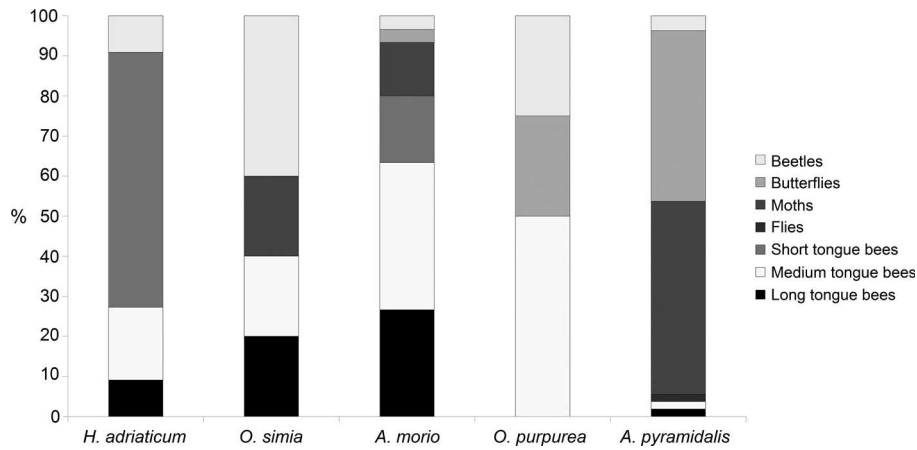


Fig. 2 Relative abundance of pollinator guilds for the five orchid species. For abbreviations of orchid genus name, see Table 2 in the caption

type only for one-third of plant species. It is widely acknowledged that adaptive phenotypic responses of floral traits might arise through selection by the most effective guild of pollinators (Fantinato et al. 2016a; Stebbins 1970), implying that floral characteristics should reflect adaptation to the pollinator that transfers the most pollen (Mayfield 2001; Souza et al. 2017). However, specific floral traits for the most common guild of visitors, or the one most effective in transferring pollen, may not preclude visits by less efficient floral visitors (i.e., secondary pollinators; Stebbins 1970), which in many cases have been proven to contribute to pollination (Rosas-Guerrero et al. 2014). Thus, floral adaptations might be triggered also by secondary pollinators (Aigner 2001, 2006), as well as by antagonistic floral visitors (e.g. Strauss and Armbruster 1997; Strauss and Irwin 2004), or by mixtures of different guilds of pollinators (e.g. Hurlbert et al. 1996; Waser 1998), which can ultimately contribute to generate mismatch between predicted and observed guilds of pollinators. Furthermore, deceptive orchid species often show a remarkable degree of variation in floral traits (e.g., colour; Nilsson 1980; Petterson and Nilsson 1993; Johnson 1994), which might contribute to attract a broader than expected range of pollinator guilds, which often respond to different floral signals. This in turn may ensure pollination events to occur even in the case of fluctuations in pollinator abundance and diversity, thus guaranteeing the reproductive success of at least some individuals within a population.

The effectiveness of a pollinator guild in carrying pollen can greatly influence plant species reproductive success, with most effective pollinators carrying more pollen than less effective ones (Barrios et al. 2016). In most orchid species (including target orchids) pollen is gathered in two compact and coherent masses, the so-called pollinia; thus the effectiveness of an orchid pollinator in carrying pollen depends just on the chance of making contact with them. Pollinia are attached to a sticky plate called ‘viscidium’, which enable them to

adhere to the pollinator body, thus to be entirely removed from the flower. Our results showed insect species belonging to guilds of pollinators unexpected from the phenotypic point of view (e.g., beetles, flies, moths) to actually carry pollinia, thus potentially contributing to target orchid species pollination. In fact, one of the limitations of our study is that our approach allowed us to evaluate only the male fitness component of orchid reproductive success (i.e., pollinia removal), and we cannot conclude that all recorded flower visitors carrying pollinia will proceed to deposit them onto receptive orchid stigmas like effective pollinators. However, previous studies have shown a positive correlation between pollinia removal and the overall effectiveness of pollination (Nilsson et al. 1992, O’Connell and Johnston 1998).

To our knowledge our findings are consistent with those of only one other study (Scopece et al. 2007). In fact, according to the majority of the literature, *A. pyramidalis* should be functionally specialized to butterflies, while *A. morio*, *H. adriaticum*, *O. purpurea*, and *O. simia* to bees (although we should consider at least three different sub-guilds of bees). In the light of our results we hypothesize this assumption to derive mostly from the concept of phenotypic specialization rather than of functional specialization. For example, in the attempt to explore pollen dispersal patterns of *A. pyramidalis*, Lind et al. (2007) focused a priori on butterflies, assuming them as the major guild of pollinators of *A. pyramidalis* on the basis of its floral phenotype. According to the same principle, Cozzolino et al. (2005) chose to investigate the extent to which deceptive orchid species share pollinators by catching just bees and large dipterans. Similarly, other studies (e.g., Johnson et al. 2003; Schiestl 2005; Biró et al. 2015) focused on a single or on narrow groups of pollinators, always composed of medium or long tongue bees. Furthermore, inferences regarding orchid pollinators have been widely advanced because direct observation of orchid pollination events are notoriously difficult to record, especially in orchids

with a deceptive pollination system, since pollination rates are typically low (Neiland and Wilcock 1995).

From an ecological perspective a high degree of generalization for pollinators may enhance the chances of pollination, especially when pollinators are non evenly distributed in space and time. According to Purvis et al. (2000) species that tend to have a high risk of extinction generally show a high dependence on other components of the ecosystem. In the case of interspecific interactions the risk of extinction may be even more pronounced because interactions can involve just two or few more species, thus being highly specialized. Furthermore, the degree of specialization has been highlighted as both cause and consequence of species rarity, acting as a constrain to species frequency and distribution (Dixon 2010; Swarts et al. 2010). As far as pollination is concerned, the presumed high degree of specialization has been often highlighted as cause of orchids rarity (Swarts and Dixon 2009). Food-deceptive orchids have been shown to be highly ecologically specialized (Warcup 1973; Rasmussen 2002; Slaviero et al. 2016). Their ecological specialization pertain to different stages of their life cycle, exposing them to an overall high risk of extinction. A high degree of functional generalization for pollinators may therefore counterbalance their broad ecological specialization, indicating specialization trade-offs during different stages in their life cycle. Costs and benefits of being specialized may lead to modulate the degree of specialization, making specialization advantageous during some stages, while detrimental during others.

Our results may lead to reconsider food-deceptive orchid pollination ecology; the equation orchid-pollination specialization should be reevaluated, and findings may be much more unpredictable than expected.

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Altitudinal patterns of floral morphologies in dry calcareous grasslands

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Abstract

A central goal in vegetation ecology is the identification of processes that influence species assemblage and distribution within a community. Among the wide variety of biotic interactions, plant-pollinator interactions are assumed to have a marked influence on plant communities assemblage and dynamics. The aim of this work was to verify if in dry grasslands there is a non random distribution of different blossom types along an altitudinal gradient, which may exert a selective pressure on both plants and insects, as well as on their mutualistic relationships. We sampled 85 plots in pre-alpine and hilly reliefs of the Veneto Region, finding that different blossom morphologies were patterned along the altitudinal gradient. Wind blossom type was dominant at low altitude while disk shaped flowers prevailed at high altitude. Our study revealed that altitude might affect species assemblage in dry grassland communities not only by selecting plant species according to their tolerance to different environmental conditions, but also according to their floral morphology, evoking the possibility of an indirect pollination filtering to occur.

Key words: blossom types, dry grasslands, habitat filtering, plant species assemblage, pollinators.

Introduction

A central goal in vegetation ecology is the identification of processes that influence species assemblage and distribution within a community. Historically, much consideration has been given to the effects of the environmental conditions in shaping plant communities (Austin, 2007). Indeed, environmental conditions can govern species assemblages by influencing both plants characteristics and adaptations, and the patterns of biotic interactions. Among the wide variety of biotic interactions, authors underlined that pollination is likely to play an important role in structuring natural plant communities (Dante *et al.*, 2013; Fantinato *et al.*, 2016). Most of flowering plants (angiosperms), by some evaluation up to 86% (Hu *et al.*, 2008), show adaptations for pollination by animals, especially insects (39% generalist insects, 27% specialized pollen collecting insects; Hu *et al.*, 2008). Given the high diversity of flowering plants and pollinating insects, pollination by animals can be considered a ubiquitous ecological interaction in virtually all terrestrial ecosystems (Memmot *et al.*, 2004). Although generalist pollination systems are frequent on a global scale, specialization is also common and it has been assumed to influence angiosperm diversification (Johnson & Steiner, 2000). Most angiosperms produce recognizable suites of convergent floral traits and reward patterns that recur in flowers with different evolutionary origin but that share similar pollinators (Rodríguez-Gironés & Santamaría, 2004; Thomson & Wilson, 2008; Wilmer, 2011). According to existing theories (Faegri &

Van der Pijl, 1979), pollinators are assumed to select plant species based on distinctive floral traits or blossom types, giving rise to pollination syndromes, namely different combinations of floral characters (e.g. flower shape, accessibility of floral rewards and flower openness). According to the pollination syndrome theory, combinations of floral traits may be used to infer suites of possible pollinators, thereby determining plant species degree of specialization towards pollinators (Fontaine *et al.*, 2006; Olesen *et al.*, 2007; Westerkamp & Claßen-Bockhoff, 2007). For example, whether blossom types as open disk are accessible to any pollinator, showing a low degree of specialization, bilabiate blossoms are pollinated mainly by bees (order *Hymenoptera*, superfamily *Apoidea*), showing a high degree of specialization (Ramírez, 2003; Westerkamp & Claßen-Bockhoff, 2007). Even if some attention has to be paid when applying these theories to investigate individual plant species reproductive strategies (Giovanetti & Aronne, 2011; Aronne *et al.*, 2012), combination of floral traits may be indicative of prevailing trends.

Generally, pollinators are not spread evenly along environmental gradients (Müller, 1881; Kearns, 1992; Bingham & Orthner, 1998; Devoto *et al.*, 2005). Some insect groups are sensitive to altitudinal changes, which can affect both their activity and reproductive success (Kearns, 1992; Totland, 1992). Most hymenopterans are widespread in warm and dry conditions, which are typical of low altitudes or latitudes (Müller, 1881; Arroyo *et al.*, 1982; Devoto *et al.*, 2005; Lázaro *et al.*, 2008), while dipterans can forage at very high

altitudes or latitudes, where they become predominant pollinators (Faegri & van der Pijl, 1979; Arroyo et al., 1982; Kearns, 1992; Totland, 1993; Elberling & Olesen, 1999). Thus, changes in the proportion of different groups of pollinators might lead to changes in the structure and composition of natural plant communities.

European temperate calcareous dry grasslands provide a particularly suitable example to this specific issue. They occur from the lowland to the mountain level, mostly on calcareous to neutral substrates (Calaciura & Spinelli, 2008). Their biological diversity is high and includes a variety of rare species from different taxonomic groups (Van Helsdingen et al., 1996; Ssymank et al., 1998). Among plant communities, dry grasslands rank as one of the richest in species both at small (<1 m²; Purschke et al., 2012; Wilson et al., 2012; Wellstein et al., 2014) and at large habitat spatial scale. High species richness is also found for butterflies and other invertebrates living in these habitats (Bobbink & Willems, 1998; Van Swaay, 2002). Moreover, in Europe, dry grassland habitats are listed in Annex I of the EU Habitat Directive (CE 43/92) as habitats of European interest.

As plant–pollinator interactions play a central role in plant reproduction, they likely have a marked influence on plant community assemblage and dynamics: for example, the absence of a particular group of pollinators can prevent the establishment of a given plant species in specialized pollination systems (Pellissier et al., 2010). Thus a better knowledge of the patterns of blossom types will add new information that may help preserving the habitat and the ecosystem services that it provides (Bartha et al., 2004; Wellstein et al., 2014). Accordingly, the main aim of this work was to verify if blossom types have a structured pattern in the landscape. Especially, we tested if there is a non random distribution of different blossom types along an altitudinal gradient, which may exert a selective pressure on both plants and insects, as well as on their mutualistic relationships.

Study area

The study was conducted in pre-alpine (Cavallo-Visentin Range, Mt. Grappa and Mts. Lessini) and hilly reliefs (Asolani, Marosticani, Berici and Euganei Hills) of the Veneto Region (NE Italy) (Fig. 1; Table 1).

Venetian Pre-Alps, as well as their foothills (i.e. Asolani and Marosticani Hills), originated from an orogenic thrust front resulting from the collision of the Adriatic and the European plates at the end of the Cretaceous (Cuffaro et al., 2010), which caused the lift of marine sedimentary rocks of calcium carbonate. Berici and Euganei Hills lie further south isolated from the Pre-Alps by the northern fringe of the Po plain. Both

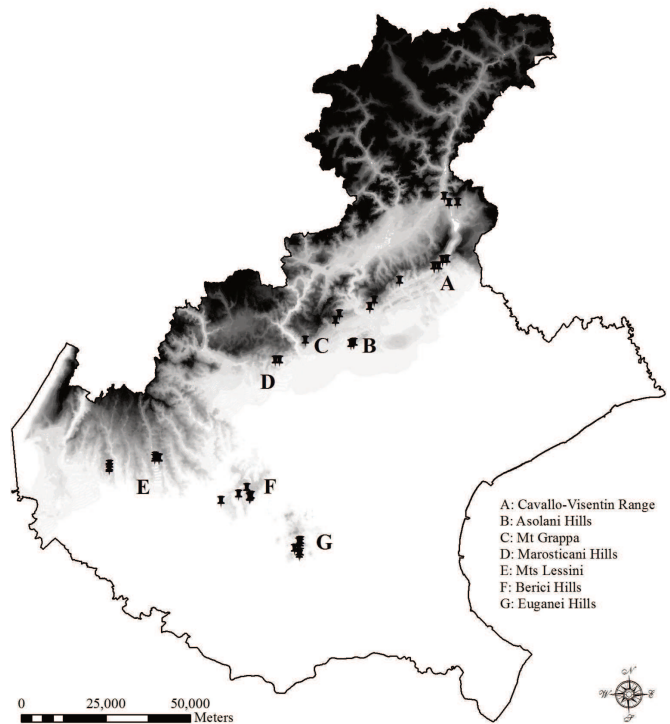


Fig. 1 - Map of the study sites and samples location.

Tab. 1 - Altitude and coordinates (WGS84) of the sampled reliefs.

Relief name	Maximum altitude (m a.s.l.)	Latitude N	Longitude E
Cavallo-Visentin Range	2.472	46.039.224	12.265.774
Mt. Grappa	1.775	45.881.279	11.800.585
Mts. Lessini	1.850	45.546.280	11.044.295
Asolani Hills	496	45.817.382	11.951.327
Marosticani Hills	495	45.772.301	11.653.346
Berici Hills	445	45.443.909	11.516.229
Euganei Hills	601	45.315.955	11.702.056

of them are of volcanic origin (from late Paleocene to late Oligocene; Macera et al., 2003) and are characterized by the co-occurrence of volcanic and calcareous formations. The climate varies from pre-alpine to plain (Barbi, 2012). Cavallo-Visentin range, Mt. Grappa, Mts. Lessini and their foothills (Asolani and Marosticani Hills) show the pre-alpine mesoclimate, with a mean annual temperature of 12°C and mean annual precipitations of 1200 – 1500 mm; while Berici and Euganei Hills show the plain mesoclimate, with a mean annual temperature of 13–14 °C and mean annual precipitations of 800 – 1000 mm (Barbi, 2012).

The study was conducted on semi-natural, oligo-to mesotrophic, *Bromus erectus*-dominated dry grasslands, which establish on poorly developed, shallow and skeletal calcareous soils, characterized by low water retention capacity and high leaching rates (Bini, 2001).

The floristic composition reflects the particular geographic position of the study sites, in a transitional connection area among the Mediterranean, the Alpine and the South-Eastern European phytogeographical regions (Villani *et al.*, 2016). The vegetation is normally dominated by few, highly covering, anemophilous species (e.g. *B. erectus*, *Festuca rupicola*, *Artemisia alba*, *Koeleria pyramidata*) and several, scarcely covering, entomophilous species. The most common insect-pollinated species are *Helianthemum nummularium* ssp. *obscurum*, *Teucrium chamaedrys* and *T. montanum*, *Thymus* sp.pl., *Scabiosa triandra*, *Globularia bisnagarica*, *Stachys recta* ssp. *subcrenata*, *Salvia pratensis* ssp. *bertolonii*, *Fumana procumbens* and *Convolvulus cantabrica*. Based on a recent syntaxonomic revision (Terzi, 2015), the investigated dry grasslands can be included in the class *Festuco-Brometea* Br.-Bl. & Tx. ex Klika & Hadač 1944 and the SE-European-Illyrian order *Scorzoneretalia villosae* Kovačević 1959 (= *Scorzonero-Chrysopogonetalia*), represented by several diagnostic species, with high frequency values (*Chrysopogon gryllus*, *Centaurea jacea* ssp. *gaudini*, *Eryngium amethystinum*, *Plantago holostium*, *S. pratensis* ssp. *bertolonii*, *Sanguisorba minor* ssp. *balearica*, *S. triandra*, etc.). Along the altitudinal range, dry grassland communities can be included in two different alliances. At lower altitude, the community structure is mainly determined by hemicryptophytes and, subordinately, chamaephytes. Given the high percentage of Mediterranean and Illyrian taxa and the phytogeographical and ecological context, the vegetation can be referred to the alliance *Saturejion subspicatae* Tomić-Stanković 1970. At higher altitude, the *Saturejion subspicatae* gives way to the mesoxerophytic alliance *Scorzonerion villosae* Horvatić ex Kovačević 1959. Dry grassland communities of this alliance show a prevalence of hemicryptophytes and of European and Eurasian taxa (Feoli Chiappella & Poldini, 1993; Terzi, 2015) and share many species with the mesophytic meadows of the class *Molinio-Arrhenatheretea* Tüxen 1937 (e.g. *Dactylis glomerata*, *Plantago lanceolata*, *Anthoxanthum odoratum*, *Veronica chamaedrys*).

Materials and Methods

Data collection

Dry calcareous grasslands were sampled in May and June 2014. A total of 85 2x2 m plots (ranging from 82 m a.s.l. to 1453 m a.s.l.) were selected using a stratified random sampling design. During the peak of the community growing season, all vascular plant species were recorded and their percentage cover was visually estimated. Plant nomenclature was standardized following Conti *et al.* (2005; 2007).

For each plot, we recorded altitude and geographi-

cal coordinates (UTM – WGS84) by using a commercial GPS (Garmin GPS62s). All the species recorded were classified on the basis of their floral morphology following Pellissier *et al.* (2010). We used six of the seven blossom categories described by Pellissier *et al.* (2010): a) *wind* (e.g. *B. erectus*, *Carex flacca*), usually green inconspicuous flowers, rewardless and nectarfree. They hang anthers and stigmas outside flowers and produce great amounts of light, smooth pollen grains; b) *disk* (e.g. *F. procumbens*, *Geranium sanguineum*), the simplest and most common flower type among existing insect-pollinated species. Disk blossoms usually have a mass of anthers centrally positioned and exposed nectaries. Thus pollen and nectar are generally easily accessible to visitors; c) *funnel* (e.g. *Campanula rapunculus*, *Colchicum autumnale*), open tubular flowers, with central, strongly united stigma and stamens. Nectaries lie at the base of the corolla tube and visitors selection is expected to apply on the basis of body size; d) *bilabiate* (e.g. *S. pratensis*, *Trifolium scabrum* ssp. *scabrum*), zygomorphic tube- or funnel-shaped flowers, characterized by a small and fixed number of concealed stamens, and by totally hidden nectaries. Hence floral resources (pollen and nectar) are accessible only to a narrow group of visitors, which should have a good perception of the flower shape and adequate handling abilities in order to reach resources (Muller, 1995). Food- and sexually-deceptive orchid species (e.g. *Ophrys sphegodes*, *Orchis morio*) were included within this group; e) *tube* (e.g. *Blackstonia perfoliata*, *Silene vulgaris* ssp. *tenoreana*), long tubular corollas, with a fixed number of stamens and totally concealed nectaries. This design usually favours long tongue pollinators (i.e. butterflies and long-tongue bees); f) *head* (e.g. *Centaurea* sp.pl., *S. triandra*), inflorescences of tiny actinomorphic and/or zygomorphic flowers. These inflorescences usually include two distinctive parts: the outer part, which is made up of ray florets, and the central part, consisting of disk florets. Reproductive organs are generally elongated over the top of corollas, while nectaries are concealed in the bottom. Some head blossoms are constituted by long tubular flowers accessible only to long-tongued insects, whereas others are composed of short flowers that can be easily probed by insects with a short proboscis (i.e. flies and short-tongued bees) (Willmer, 2011).

Data analysis

For each plot we determine the coverage of each blossom type by summing the coverage of plant species belonging to the same blossom category; furthermore, blossom type richness and the evenness (Pielou, 1966) were calculated per plot.

To explore the trend in the distribution of blossom types we performed a Principal Components Analysis

(PCA), based on the relative blossom type coverage matrix.

To highlight possible relationships between blossom types and altitude, Spearman's Rank Correlations were calculated between altitude and the relative coverage of different blossom types, blossom type richness and evenness (Pielou, 1966).

The computations were performed using STATISTICA 8 (StatSoft Inc., 2008; www.statsoft.com).

Results

A total of 254 species of vascular plants were recorded in the 85 sampled plots. The most common species (found in more than half of the plots) were *B. erectus*, *S. minor* ssp. *balearica*, *H. nummularium* ssp. *obscurum*, *T. chamaedrys*, and *Galium verum*. Among the blossom types recorded, disk was prevalent (72 species), followed by bilabiate (70 species), wind (45 species), head (43 species), funnel (15 species) and tube blossoms (9 species). Families were represented by blossom types as follows: 20 families by disk, 11 families by wind, 9 families by bilabiate, 9 families by funnel, 3 families by head, and finally 2 families by tube blossoms. See Table 2 for details about families.

The PCA based on the relative blossom type coverage matrix (Fig. 2) distinguished two groups of plots.

From a floristic point of view, most common species of Group 1 were *B. erectus*, *S. minor* ssp. *balearica*, *T. chamaedrys*, *H. nummularium* ssp. *obscurum*, *S. triandra*, and *G. verum*. The most frequent species present exclusively in Group 1 included *A. alba*, *F. procumbens*, *Dorycnium pentaphyllum* ssp. *herbaceum*, *T. montanum* and *Odontites luteus*. Similarly to Group 1, *B. erectus*, *H. nummularium* ssp. *obscurum*, *G. verum* and *T. chamaedrys* were among the most common species of Group 2. In addition, *Oreoselinum nigrum*, *Stachys officinalis*, *Knautia arvensis*, *F. rupicola*, *P. lanceolata*, *Euphorbia cyparissias*, *Cruciata glabra*, *T. montanum*, *T. pulegioides* and *C. jacea* ssp. *gaudini*, became more common. *K. arvensis*, *Laserpitium latifolium* and *Dianthus monspessulanus*, were the most frequent species present exclusively in Group 2.

The first PCA axis (which explained the 30.67% of the total variance) primarily reflected an altitudinal gradient. In fact, the mean altitude of Group 1 was 332.49 ± 120.1 m a.s.l., while the mean altitude of Group 2 was 997.14 ± 191.59 m a.s.l.. The second axis (17.54% of the total variance) defined a pollination forms gradient, from the prevalence of anemophily at the negative extreme to an increasing presence of entomophily at the positive one. In fact, sampled plots of Group 1 were dominated by highly covering anemophilous species, such as *B. erectus*, *Brachypodium rupestre* and *A. alba*, while sampled plots of Group 2 were more even.

Tab. 2 - Percentage of plant families grouped according to the blossom categories identified in the studied communities.

blossom categories	Family	percentage
Wind	<i>Poaceae</i>	62%
	<i>Cyperaceae</i>	13%
	<i>Plantaginaceae</i>	7%
	Others	18%
Disk	<i>Rosaceae</i>	17%
	<i>Apiaceae</i>	14%
	<i>Brassicaceae</i>	10%
	<i>Euphorbiaceae</i>	9%
	<i>Rubiaceae</i>	9%
	<i>Caryophyllaceae</i>	6%
	<i>Crassulaceae</i>	4%
	<i>Cistaceae</i>	4%
	<i>Geraniaceae</i>	4%
	<i>Ranunculaceae</i>	4%
Others	19%	
Funnel	<i>Campulaceae</i>	20%
	<i>Rubiaceae</i>	20%
	<i>Asparagaceae</i>	13%
	<i>Convolvulaceae</i>	13%
	<i>Amaryllidaceae</i>	7%
	<i>Apiaceae</i>	7%
	<i>Colchicaceae</i>	7%
	<i>Liliaceae</i>	7%
<i>Oleaceae</i>	6%	
Bilabiate	<i>Fabaceae</i>	44%
	<i>Lamiaceae</i>	23%
	<i>Orchidaceae</i>	19%
	<i>Scrophulariaceae</i>	6%
Others	8%	
Tube	<i>Caryophyllaceae</i>	86%
	<i>Primulaceae</i>	14%
Head	<i>Asteraceae</i>	96%
	Others	4%

Spearman's Rank Correlations (Fig. 3) revealed a decreasing dominance of wind blossoms from low to high altitude, coupled with higher values of blossom type evenness.

Among entomophilous blossom types, disk blossoms showed to prevail significantly at high altitude, while no significant correlations were observed for bilabiate, funnel, head and tube blossoms.

The richness of blossom types didn't change significantly with the altitude.

Discussion

Previous studies (Pellissier et al., 2010) already underlined the importance of altitude in defining floral morphologies distribution in western Swiss Alps as a response to climate variations. Indeed, climate heterogeneity has been proven to affect, inter alia, vegetation distribution and composition (Wang et al., 2006; Gould et al., 2006), life-form spectra (Pavón et al., 2000; Klimeš, 2003; Mahdavi, 2012) as well as pollinator distribution (Müller, 1881; Arroyo et al., 1982; Pellissier et al., 2010). Our results at habitat level confirm the observations made by Pellissier et al. (2010).

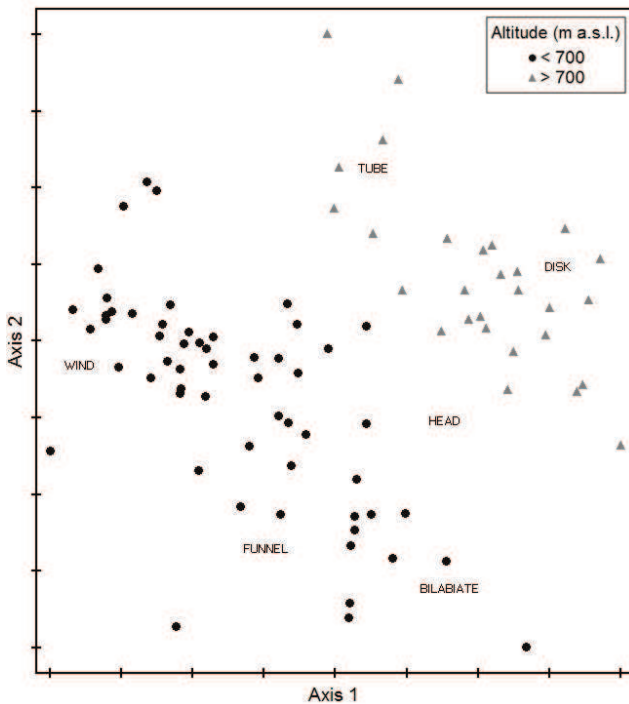


Fig. 2 - PCA ordering of the sampled plots. Sampled plots belonging to different altitudinal groups (threshold level: 700 m a.s.l.) were distinguished with different symbols. Plots belonging to Group 1 (<700 m a.s.l.) were shown as black dots, while plots belonging to Group 2 (>700 m a.s.l.) were shown as grey triangles.

Even though the richness of blossom types remained constant when moving from low to higher altitude, two blossom types (wind and disk) changed significantly their relative coverage within dry grassland communities. We found a clear dominance of wind blossoms at low altitude. This result is consistent with the observations made by Pellissier *et al.* (2010), who have hypothesized that precipitation, which increases with increasing altitude, may negatively influence pollen availability and dispersion. Indeed, precipitation in pre-alpine reliefs of the Veneto Region are abundant, making anemophily not be the best pollination option. Our results also match the observations made by Carli *et al.* (2012), who recorded graminoid species (anemophilous) as more abundant in the lowlands, whereas non-graminoid species (mostly entomophilous) were more common in the highlands of Matese dry grasslands. However, in this regard, it is also worth considering the poor knowledge existing on the exploitation of wind blossoms by insects, and their potential contribution to pollination of so-called wind pollinated species (Giovanetti & Aronne, 2011; Aronne *et al.*, 2012). If this hypothesis will be verified, wind blossoms distribution may not be driven exclusively by precipitations, but also by pollinators availability.

Disk blossoms were found to be prevalent at high al-

titude, where climatic stressful conditions may limit insect activity. In such a situation, a strategic offer of easily accessible pollen and nectar may guarantee pollination by a wider variety of insects, among which short tongue bees and flies (Pellissier *et al.*, 2010). Indeed, despite being considered generally less efficient than hymenopterans, flies are important pollinators of many plants at high altitudes (Arroyo *et al.*, 1982; Walker-Larsen & Haarder, 2001). This pattern was observed also by Poldini (1989), who showed an increasing frequency of fly pollinated species in more mature and mesophilous associations, in our case referable to the alliance *Scorzonerion villosae*.

From a broader point of view, the observed blossom patterns may be the result of a combination of processes. Temperate dry grasslands are mostly semi-natural communities maintained by mowing and grazing, which have been proved to regulate species composition and richness (Valkó *et al.*, 2011; 2012). Much evidence has been found to indicate that dry grasslands abandoning in the early successional stages results in increasing dominance of grasses such as *B. erectus* or *B. rupestre* and, as a consequence, in decreasing species diversity (Bobbik & Willems, 1987; Bonanomi & Allegrezza, 2004; Biondi *et al.*, 2006). Regular mowing in general affects the competitive relationships in grasslands. In particular, restraining dominant graminoids competitors, mowing has been proven to enhance the richness in characteristic grassland forbs. Thus, the abandoning of management practices would lead to an increased dominance of wind blossoms against entomophilous blossom types, which contribute for the most part to the overall diversity in species-rich grassland communities (Wellstein *et al.*, 2014; Fantinato *et al.*, 2016). Indeed, at higher altitude, dry grassland communities showed an increased evenness of blossom types. This coupled with a greater presence of species of the class *Molinio-Arrhenatheretea* suggests that management activities are still implemented. However, only the most generalist blossom type, i.e. disk, evidenced a significant increase in the relative coverage with altitude, providing further evidence supporting the hypothesis that an indirect pollination filtering exerted by altitude may occur.

Our results highlight the importance of considering the functional relationships between species to gain a deeper knowledge about ecosystem structure and processes. The knowledge of the role played by such interactions could improved management practices or restoration plans, leading to the promotion of the functional conservation of the whole ecological community and ensuring its maintenance over time.

The relationships between pollinators and the reproductive success of flowering plants have been mostly documented at the species level, traditionally focusing on highly specific interactions between one or few

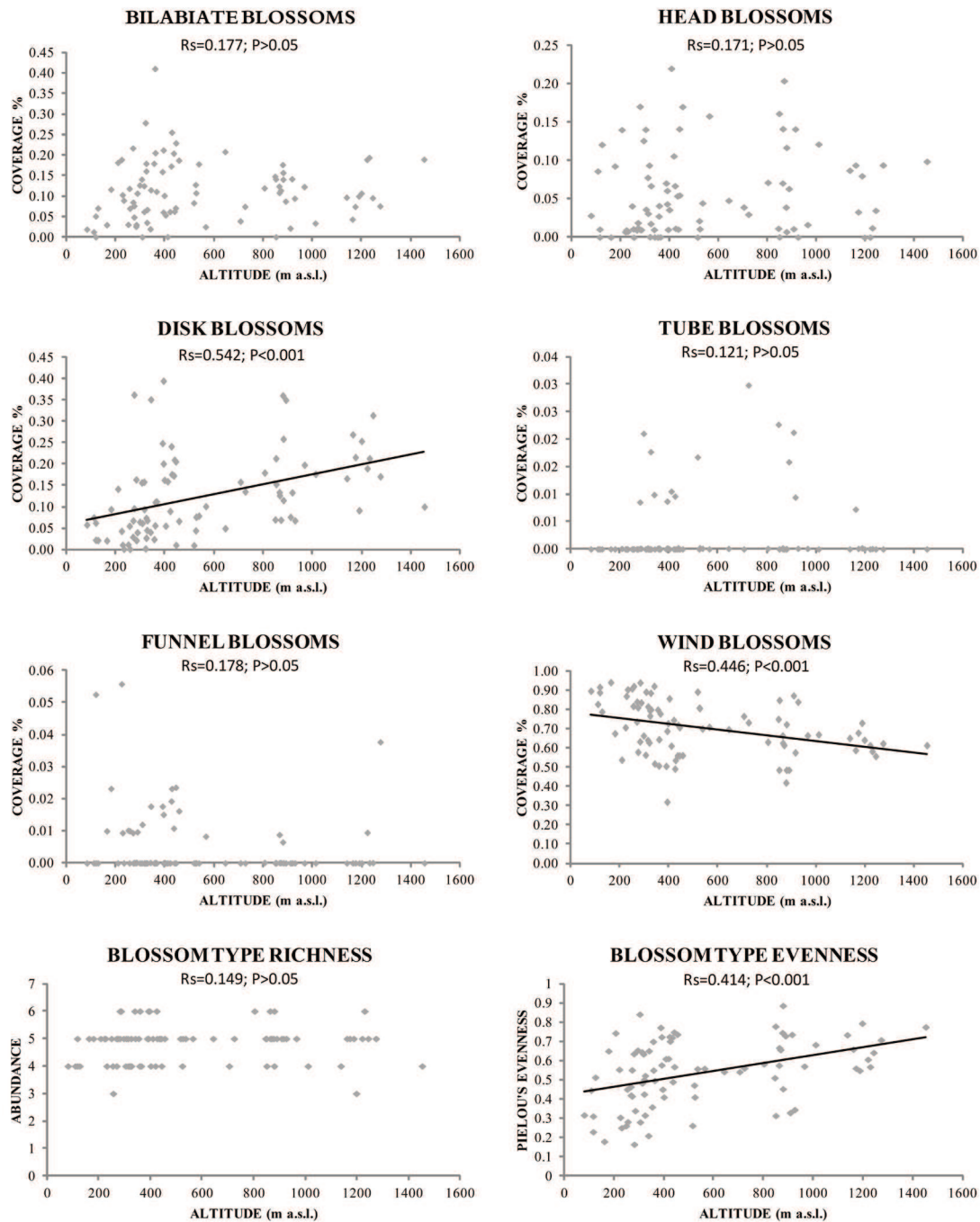


Fig. 3 - Relationships between altitude and blossom type relative coverage, richness and evenness (Spearman's rank correlation coefficients (R_s) and P values). Tendency lines were added to the significant correlations.

plant species and constrained groups of pollinators. Little information is available at the community level, since the study of plant community assembly has rarely considered the role of plant-animal interactions.

However, given the strong dependence of entomophilous species upon pollinators availability for reproduction, we may assume a filter, a selective pressure to the assemblage of plants (Sargent & Ackerly, 2008). Our study suggests that besides the environmental ef-

fect, which selects plant species according to their tolerance to different environmental conditions, altitude might affect the species assemblage in dry grassland communities also through indirect pollination filtering. Studying these interactions may turn out to be essential to disentangle community level processes, e.g. potential competitive or facilitative effects among species, or patterns of community assemblages.

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Conclusions and further research perspectives

The understanding of ecological processes involved in the maintenance of ecosystem integrity over time represents the main purpose of several disciplines dealing with plant ecology and vegetation science. The concept of 'ecosystem integrity' was originally introduced by Leopold (1944) to define the set of requirements needed by biotic communities for the maintenance of their own stability. Subsequent definitions revised this concept on the light of the ecological self-organization theories (e.g. non-equilibrium thermodynamics, network development and succession theory; Jørgensen et al., 2007), recognizing that biotic communities organize from spontaneous processes, or functions (Kandziora et al. 2013), which ensure the long-term stability of ecosystems (Karr, 1981; Woodley et al., 1993; Barkmann et al., 2001). The maintenance of the ecological structure and functions responsible for the ecosystem's self-organizing capacity becomes even more crucial as far as the provision of the ecosystem services is concerned (Haines-Young and Potschin, 2010). In fact, ecosystem integrity has been proven to positively influence the provision of particular ecosystem services (Cardinale et al., 2012), while ecosystem disruption, driven by human induced global changes, has been linked to their loss (Hooper et al., 2012).

The present research project proved that pollination processes are involved in the assemblage of dry grassland communities and in the maintenance of the biodiversity they host. Further studies should be implemented to test hypothesis arose during the development of the present research project and new frontiers should be investigated. Two key aspects need to be explored more in depth; i.e., the spatial and the temporal components of pollination interactions. Especially, the effects of landscape mosaic on dry grasslands pollination networks should be taken into account. Furthermore, methodological issues on the spatial scale and the sampling size might be addressed by constructing webs from data collected at different spatial scales (first issue) and in plots of different size (second issue), thus examining similarities and differences.

Results may help us to better understand the contribution of pollination interactions in plant communities development and organization, ultimately improving our efforts in biodiversity conservation. In fact, pollination interactions have often been overlooked in conservations plans and restoration practices, however more successful results might be gained if pollination interactions will be considered, ultimately encouraging natural regeneration processes involved in the self-organization of plant communities.

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Appendix S1. Supporting Information to the paper Fantinato, E., Del Vecchio, S., Giovanetti, M., Acosta, A.T.R., Buffa, G. Space or time: plant-plant interactions for pollination in species rich plant communities. *Journal of Vegetation Science*. Appendix S1. List of entomophilous species recorded in dry grasslands. For each animal-pollinated plant species we provide flowering time, anther position relative to the corolla and the percentage of each visiting pollinator guild calculated on the number of visits recorded. Abbreviations: LSolB, Large solitary bees; MsocB, Medium social bees; OF, Other flies; Butt, Butterflies; B-C, Bush-cricket; LSyrF, Large syrphid flies; SSolB, Small social bees.

Plant species	Flowering time	Anther position	Pollinator guilds (%)											
			Beetles	LSolB	MSocB	LSocB	OF	Butt	B-C	LSyrF	SSolB	Wasps	Ants	
<i>Blackstonia perfoliata</i>	middle May to middle June	Bottom	50	.	50
<i>Bupleurum baldense</i> ssp. <i>gussonei</i>	middle May to middle July	Bottom	67	33
<i>Campanula rapunculus</i>	middle May to early June	Opening	.	100
<i>Centaurium erythraea</i>	middle May to early June	Bottom	100
<i>Convolvulus cantabrica</i>	early May to middle June	Opening	44	21	9	.	.	18	3	6
<i>Crepis sancta</i>	early April to middle June	Bottom	50	.	25	25	.	.
<i>Crepis vesicaria</i> ssp. <i>taraxacifolia</i>	middle April to middle May	Bottom	74	.	11	16	.	.
<i>Cytisus hirsutus</i>	middle April to early June	Bottom	33	67
<i>Dorycnium pentaphyllum</i> ssp. <i>herbaceum</i>	early June to middle June	Bottom	.	19	13	3	13	52	.
<i>Eryngium amethystinum</i>	early July to middle August	Bottom	29	4	13	.	.	.	4	.	.	.	13	38
<i>Euphorbia cyparissias</i>	early April to middle May	Bottom	39	23	.	2	.	.	26	11
<i>Fumana procumbens</i>	early May to early September	Bottom	.	.	100
<i>Galatella linosyris</i>	middle August to early September	Bottom	13	19	.	25	19	6	.	19
<i>Galium verum</i>	middle May to middle July	Bottom	100
<i>Geranium sanguineum</i>	middle April to early June	Bottom	49	10	13	3	1	.	.	21	.	.	1	.
<i>Globularia bisnagarica</i>	early April to middle May	Bottom	15	5	16	4	2	3	51	3
<i>Helianthemum nummularium</i> ssp. <i>obscurum</i>	middle April to early September	Bottom	77	3	7	7	7
<i>Hieracium pilosella</i>	middle April to middle May	Bottom	100
<i>Hypericum perforatum</i>	middle April to middle May	Bottom	22	11	33	33
<i>Hippocrepis comosa</i>	middle May to early June	Bottom	.	50	10	.	.	.	30	.	.	10	.	.
<i>Linum tenuifolium</i>	middle May to early July	Bottom	.	10	.	3	73	10	.	.	.	3	.	.
<i>Medicago minima</i>	middle April to early July	Bottom	.	100
<i>Melampyrum barbatum</i>	early May to middle June	Top	.	.	.	100
<i>Muscari comosum</i>	early May	Opening	.	100
<i>Ononis natrix</i>	middle May to early July	Bottom	.	.	.	100
<i>Orchis simia</i>	early April to early May	Opening	25	25	50
<i>Peucedanum cervaria</i>	early July to early September	Bottom	3	17	.	.	3	3	.	73
<i>Potentilla hirta</i>	early May to early June	Bottom	100
<i>Potentilla pusilla</i>	middle April to middle May	Bottom	9	11	43	.	11	.	28
<i>Scabiosa triandra</i>	early June to early September	Bottom	6	10	2	8	14	37	.	18	.	.	6	.
<i>Stachys recta</i>	middle May to middle June	Top	.	.	.	100
<i>Teucrium chamaedrys</i>	middle May to middle June	Top	.	.	.	80	.	20
<i>Thymus glabrescens</i> ssp. <i>decipiens</i>	middle April to middle June	Top	.	33	.	17	.	50
<i>Thymus pulegioides</i>	early May to middle June	Top	.	.	18	.	.	82
<i>Viburnum lantana</i>	early April to middle April	Bottom	52	.	2	1	10	.	33	1

Appendix S2. Ecological Research - Title: Are food-deceptive orchid species really functionally specialized for pollinators?

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ESM 1. Lists of insects observed on target orchids inflorescences as reported in the literature.

Butterflies	Moths	Short tongue bees	Medium tongue bees	Longue tong bees	References
<i>Anacamptis morio</i>			<i>Apis mellifera</i> (L., 1758)		
<i>Anthocharis euphenoides</i> (Staudinger, 1869)			<i>Bombus confusus</i> (Schenck, 1859)		
			<i>Bombus humilis</i> (Illiger, 1806)		
	<i>Cucullia santonici</i> (Hübner, 1813)	<i>Andrena albopunctata</i> (Rossi, 1792)	<i>Bombus lapidarius</i> (L., 1758)	<i>Anthophora plumipes</i> (Pallas, 1772)	Barile et al. 2006
	<i>Hada plebeja</i> (L., 1761)	<i>Andrena ovatula</i> (Kirby, 1802)	<i>Bombus lucorum</i> (L., 1761)	<i>Bombus muscorum</i> (L., 1758)	Johnson et al. 2003
	<i>Zygaena lonicerae</i> (Scheven, 1777)	<i>Halictus patellatus</i> (Morawitz, 1874)	<i>Bombus pascorum</i> (Scopoli, 1763)	<i>Bombus ruderatus</i> (F., 1775)	Cozzolino et al. 2005
		<i>Lasioglossum xanthopus</i> (Kirby, 1802)	<i>Bombus pratorum</i> (L., 1761)	<i>Bombus sylvarum</i> (L., 1761)	Darwin 1885
			<i>Bombus rupestris</i> (F., 1793)	<i>Eucera hungarica</i> (Friese, 1896)	Schiestl 2005
			<i>Bombus terrestris</i> (L., 1758)	<i>Eucera longicornis</i> (L., 1758)	Vöth 1987
			<i>Bombus vestalis</i> (Geoffroy in Fourcroy, 1785)		
			<i>Osmia bicornis</i> (L., 1758)		

Beetles	Butterflies	Moths	Medium tongue bees	References
		<i>Adscita statures</i> (L., 1758)		
	<i>Aglais urticae</i> (L., 1758)	<i>Agrotis catalaunica</i> (L., 1758)		
	<i>Aporia crataegi</i> (L., 1758)	<i>Apamea sublustri</i> (Esper, 1788)		
	<i>Arginnis aglaja</i> (L., 1758)	<i>Caradrina alsines</i> (Goeze, 1781)		
	<i>Brenthis ino</i> (Rottemburg, 1775)	<i>Caradrina blanda</i> (Denis & Schiffermüller, 1775)		
	<i>Euphydryas aurinia</i> (Rottemburg, 1775)	<i>Eubolia mensuraria</i> (L., 1758)		
	<i>Fabriciana adippe</i> (Denis & Schiffermüller, 1775)	<i>Euclidia glyphica</i> (L., 1758)		
	<i>Glaucopsyche alexis</i> (Poda, 1761)	<i>Hadena monoglypha</i> (Hufn. 1776)		Barile et al. 2006
	<i>Lycaena phlaeas</i> (L., 1761)	<i>Heliothis marginata</i> (Hufnagel, 1766)		Berger 2006
	<i>Lysandra bellargus</i> (Rottemburg, 1775)	<i>Hemaris fuciformis</i> (L., 1758)		Bournérias et al. 1998
	<i>Maniola jurtina</i> (L., 1758)	<i>Leucania lithargyria</i> (Esper, 1788)		Claessens and Kleinen 2011
	<i>Melanargia galathea</i> (L., 1758)	<i>Lithosia complana</i> (L., 1758)		Darwin 1885
<i>Anacamptis pyramidalis</i>	<i>Trichius fasciatus</i> (L., 1758)	<i>Mythimna sicula</i> (Treitschke, 1835)	<i>Apis mellifera</i> (L., 1758)	Démars 2000
	<i>Melitaea athalia</i> (Rottemburg, 1775)	<i>Shargacucullia caninae</i> (Rambur, 1833)		Grasso 1994
	<i>Melitaea cinxia</i> (L., 1758)	<i>Siona lineata</i> (Scopoli, 1763)		Lind and Lindeborg 1989
	<i>Melitaea didyma</i> (Esper, 1778)	<i>Sitochroa palealis</i> (Denis & Schiffermüller, 1775)		Lind et al. 2007
	<i>Melitaea parthenoides</i> (Keferstein, 1851)	<i>Toxocampa pastinum</i> (Treitschke, 1826)		Reinhard et al. 1991
	<i>Melitaea phoebe</i> (Denis & Schiffermüller, 1775)	<i>Tyta luctuosa</i> (Denis & Schiffermüller, 1775)		Vöth 1999
	<i>Syrichthus alveolus</i> (L., 1758)	<i>Zygaena corsica</i> (Boisduval, 1828)		
	<i>Thymelicus lineolus</i> (Ochsenheimer, 1808)	<i>Zygaena filipendulae</i> (L., 1758)		
	<i>Thymelicus sylvestris</i> (Poda, 1761)	<i>Zygaena loniceriae</i> (Scheven, 1777)		
	<i>Ochlodes venatus</i> (Bremer & Grey, 1853)	<i>Zygaena minos</i> (Denis & Schiffermüller, 1775)		
	<i>Plabejus argus</i> (L., 1758)	<i>Zygaena purpuralis</i> (Brünnich, 1763)		
	<i>Pyrgus carthami</i> (Hübner, 1813)	<i>Zygaena trifolii</i> (Esper, 1783)		
	<i>Pyrgus malvae</i> (L., 1758)	<i>Zygaena viciae</i> (Denis & Schiffermüller, 1775)		

Beetles	Moths	Short tongue bees	Medium tongue bees	Longue tong bees	References
<i>Himantoglossum adriaticum</i>		<i>Andrena carbonaria</i> (L., 1767) <i>Andrena nigroaenea</i> (Kirby, 1802) <i>Andrena haemorrhoa</i> (F., 1781) <i>Andrena potentillae</i> (Panzer, 1809) <i>Colletes similis</i> (Schenck, 1853)	<i>Apis mellifera</i> (L., 1758) <i>Osmia caerulescens</i> (L., 1758)	<i>Megachile melanopyga</i> (Cockerell, 1909)	Biró et al. 2014 Teschner 1980 Vöth 1990
<i>Orchis purpurea</i>			<i>Apis mellifera</i> (L., 1758) <i>Bombus terrestris</i> (L., 1758)		Berger 2006 Vöth 1990
<i>Orchis simia</i>	<i>Cidnopus pilosus</i> (Leske, 1785)	<i>Hemaris fuciformis</i> (L., 1758)	<i>Apis mellifera</i> (L., 1758)	<i>Eucera longicornis</i> (L., 1758)	Démares 2000 Schatz 2006 Vöth 1999

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