

## Extensive contemporary pollen-mediated gene flow in two herb species, *Ranunculus bulbosus* and *Trifolium montanum*, along an altitudinal gradient in a meadow landscape

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- **Background and Aims** Genetic connectivity between plant populations allows for exchange and dispersal of adaptive genes, which can facilitate plant population persistence particularly in rapidly changing environments.
- **Methods** Patterns of historic gene flow, flowering phenology and contemporary pollen flow were investigated in two common herbs, *Ranunculus bulbosus* and *Trifolium montanum*, along an altitudinal gradient of 1200–1800 m a.s.l. over a distance of 1 km among five alpine meadows in Switzerland.
- **Key Results** Historic gene flow was extensive, as revealed by  $F_{st}$  values of 0.01 and 0.007 in *R. bulbosus* and *T. montanum*, respectively, by similar levels of allelic richness among meadows and by the grouping of all individuals into one genetic cluster. Our data suggest contemporary pollen flow is not limited across altitudes in either species but is more pronounced in *T. montanum*, as indicated by the differential decay of among-sibships correlated paternity with increasing spatial distance. Flowering phenology among meadows was not a barrier to pollen flow in *T. montanum*, as the large overlap between meadow pairs was consistent with the extensive pollen flow. The smaller flowering overlap among *R. bulbosus* meadows might explain the slightly more limited pollen flow detected.
- **Conclusions** High levels of pollen flow among altitudes in both *R. bulbosus* and *T. montanum* should facilitate exchange of genes which may enhance adaptive responses to rapid climate change.

**Key words:** Elevation, flowering phenology, gene flow, herbs, managed meadows, microsatellites, pollen flow, pollen-pool analysis, *Ranunculus bulbosus*, *Trifolium montanum*.

### INTRODUCTION

Gene flow connects populations, maintains genetic diversity, prevents inbreeding and facilitates the spread of adaptive genes across a species range (Slatkin, 1985, 1987; Ellstrand and Elam, 1993). In plants, the extent and magnitude of gene flow is determined by a variety of pollen and seed dispersal mechanisms. The demography of the species, the mating system, spatial distances and the presence of physical barriers may greatly influence patterns of gene flow (Manel *et al.*, 2003). The genetic composition of populations is influenced by colonization, bottlenecks and gene flow, whose effects can accumulate over multiple generations as well as through selection processes.

Plant species in mountain habitats are thought to be especially sensitive to rapid climatic changes, and upward shifts of plant species distributions are already well documented (Grabherr *et al.*, 1994; Walther *et al.*, 2002; Frei *et al.*, 2010). Climate change might also modify the underlying drivers of gene flow by acting on flowering time and pollinator abundance and behaviour. At the population scale, adaptation to the direct and indirect effects of climate change is likely to be facilitated by population genetic diversity. Thus, understanding how plant populations are connected by gene flow, particularly across altitudinal gradients in mountain regions,

will improve our understanding of how they will respond to future environmental change.

Mountain environments are characterized by strong gradients of temperature, exposition, UV radiation and duration of snow cover. These have a major influence on plant metabolism and physiology and, by extension, on their ability to grow and reproduce (Körner, 2003). Such ecologically relevant gradients are important in shaping patterns of local adaptation in plant species (Byars *et al.*, 2007; Gonzalo-Turpin and Hazard, 2009), and, for example, thermal gradients across altitudes [the adiabatic lapse rate of approx. 0.5 °C per 100 m elevation (Körner, 2003)] can cause differential flowering of populations at different altitudes (Dittmar and Elling, 2006; Larcher, 2006; Ziello *et al.*, 2009). Empirical studies show that differences in flowering periods can influence patterns of genetic structure by constraining pollen dispersal among populations (Stanton and Galen, 1997; Gerber *et al.*, 2004; Kitamoto *et al.*, 2006; Hirao and Kudo, 2008). However, many of these studies focus on species whose flowering time is greatly influenced by the time of snow melt, which does not directly relate to altitudinal difference but rather to differences in micro-topography and exposure (Scherrer and Körner, 2011). It thus remains unclear how phenological differences driven by altitude alone act as a barrier to gene flow within plant species.

In the European Alps, temperatures are expected to increase by about 3 K over the next 100 years (IPCC, 2007). The impacts of these changes for onset of first flowering have been well documented (Walther *et al.*, 2002); however, the implications for flowering overlap among populations at different altitudes remain unexplored. Because pollen-mediated gene flow is often more efficient than seed-mediated gene flow in terms of dispersal distance (Ennos, 1994), it is particularly important to study patterns of contemporary pollen flow in plants occupying altitudinal gradients, especially in the context of land use change.

Studying patterns of contemporary pollen dispersal in species at the landscape scale can be challenging due to the high abundance of conspecifics. Many herbaceous species are aggregated in large populations and, consequently, most empirical studies of contemporary pollen dispersal have focused on relatively small spatial scales within populations (Miyazaki and Isagi, 2000; Hardy *et al.*, 2004b; Scheepens *et al.*, 2012), while landscape-scale studies focused on species with local low-abundance of conspecifics (Buehler *et al.*, 2012). This greatly limits our understanding of patterns of gene flow along altitudinal gradients and over larger scales. For a long time it has been thought that pollen-induced contemporary gene flow only occurs over small distances in herbaceous plants (Hardy *et al.*, 2004b; Pluess and Stocklin, 2004; Gonzales *et al.*, 2006; Ishihama *et al.*, 2006; Kitamoto *et al.*, 2006). More recently, advances in molecular techniques (paternity and pollen-pool analyses instead of pollen analogues and direct observation of pollinators) have enabled us to study these processes at larger scales. For example, a recent study on *Arabis alpina* using exhaustive sampling at the landscape scale (up to 2 km distance and about 350 m elevation difference) by Buehler *et al.* (2012) found that the vast majority of pollen dispersal events were over short distances, but dispersal events can occur beyond 1 km.

In this paper, we investigate patterns of contemporary gene flow by pollen-dispersal and historic gene flow in the two common semi-dry grassland perennial plant species *Ranunculus bulbosus* and *Trifolium montanum*, along an altitudinal gradient located in Grindelwald, Switzerland. These species have similar distributional ranges encompassing colline and sub-alpine belts but have different pollination syndromes and thus they provide a useful study system for exploring contemporary gene flow across altitude; an issue of relevance with respect to likely plant population responses to climate change and consequent elevation shifts. In a recent study of historic gene flow of both these species in the Swiss mountains, Hahn *et al.* (2012) showed that altitude appeared not to influence genetic diversity, and showed little genetic differentiation at the regional scale. However, the patterns of contemporary gene flow in these species across altitude at landscape scales remain unresolved.

We sampled individuals and whole seed-families of *R. bulbosus* and *T. montanum* at five meadows along a 1200–1800 m a.s.l. altitudinal gradient stretching over approx. 1 km distance, and genotyped them at seven and 11 neutral nuclear microsatellite markers, respectively. This enabled us to explore patterns of historic gene flow and contemporary pollen dispersal over a landscape scale relevant to predicted climate change. A 600-m elevation range was

chosen because it corresponds to the expected 3 K in temperature increase in the next 100 years (IPCC, 2007). Using population genetics, pollen-pool analysis and ecological surveys of the flowering phenology within this study system we address the following three questions: (1) Do patterns of genetic differentiation and genetic diversity differ across altitude? (2) Does contemporary pollen dispersal link meadows along an altitudinal gradient in *R. bulbosus* and *T. montanum*? (3) Do flowering phenology, demography and mating system influence pollen flow across altitude?

## MATERIALS AND METHODS

### *Plant material*

*Study species.* *Ranunculus bulbosus* L. (Ranunculaceae) and *Trifolium montanum* L. (Leguminosae) are both diploid, perennial herbs that occur mainly on dry calcareous grasslands. They are self-compatible species, yet selfing in *T. montanum* was observed less frequently than in *R. bulbosus* (Schleuning *et al.*, 2009; Matter *et al.*, 2013). The two species differ in their flower morphology and pollination syndromes. *Ranunculus bulbosus* flower heads are made of a single flower with a rotate corolla containing on average 40 carpels. *Trifolium montanum* inflorescences consist of up to 150 zygomorphic flowers each containing one ovule. *Ranunculus bulbosus* is visited by a wide array of generalist pollinators including Diptera (Syrphidae, Muscidae, Anthomyiidae), Coleoptera, small bees (Halictidae), honey-bees (*Apis mellifera*; Harper, 1957) and rarely bumble-bees (Steinbach and Gottsberger, 1994). *Trifolium montanum* is primarily visited by Apidae species such as the honey-bee and bumble-bees (Pettersson and Sjödin, 2000; Schleuning *et al.*, 2009).

*Study site and sampling.* In summer 2009, five meadows located along a single mountain slope at around 1200, 1350, 1500, 1650 and 1800 m a.s.l. in Grindelwald (Switzerland) were sampled for whole seed families (SF; leaf material and seeds from the same individual) of *R. bulbosus* and *T. montanum* (see Fig. 1). The sampling area within each meadow was based upon the abundance of the plants within the focal altitudinal range ( $\pm 40$  m elevation difference). At the lowest elevation the area was clearly delineated by forest boundaries; at the other elevations a shrub layer formed some boundaries, but where meadows were more continuous, our sampling areas were defined by our chosen altitudinal range. Directly following collection, seeds of SF were sown in a 1:1 potting soil:sand mix and germinated in the greenhouse. Fresh *in-situ* sampled maternal leaves as well as fresh seedling leaves were dried in silica-gel for subsequent DNA extraction. Each SF was mapped with a triangular mapping technique including reference points. Measurements were converted into spatial co-ordinates with the Excel-Program Aequometer ([www.aequometer.de](http://www.aequometer.de)).

In *R. bulbosus*, 75 SF (15 per meadow) and in *T. montanum* 85 SF (17 per meadow) with ten offspring each were genotyped. For the assessment of overall genetic variation at the parental level, a further 170 *R. bulbosus* and 149 *T. montanum* individuals, equally distributed among meadows, were sampled, mapped and genotyped (hereafter called ‘additional adults’).

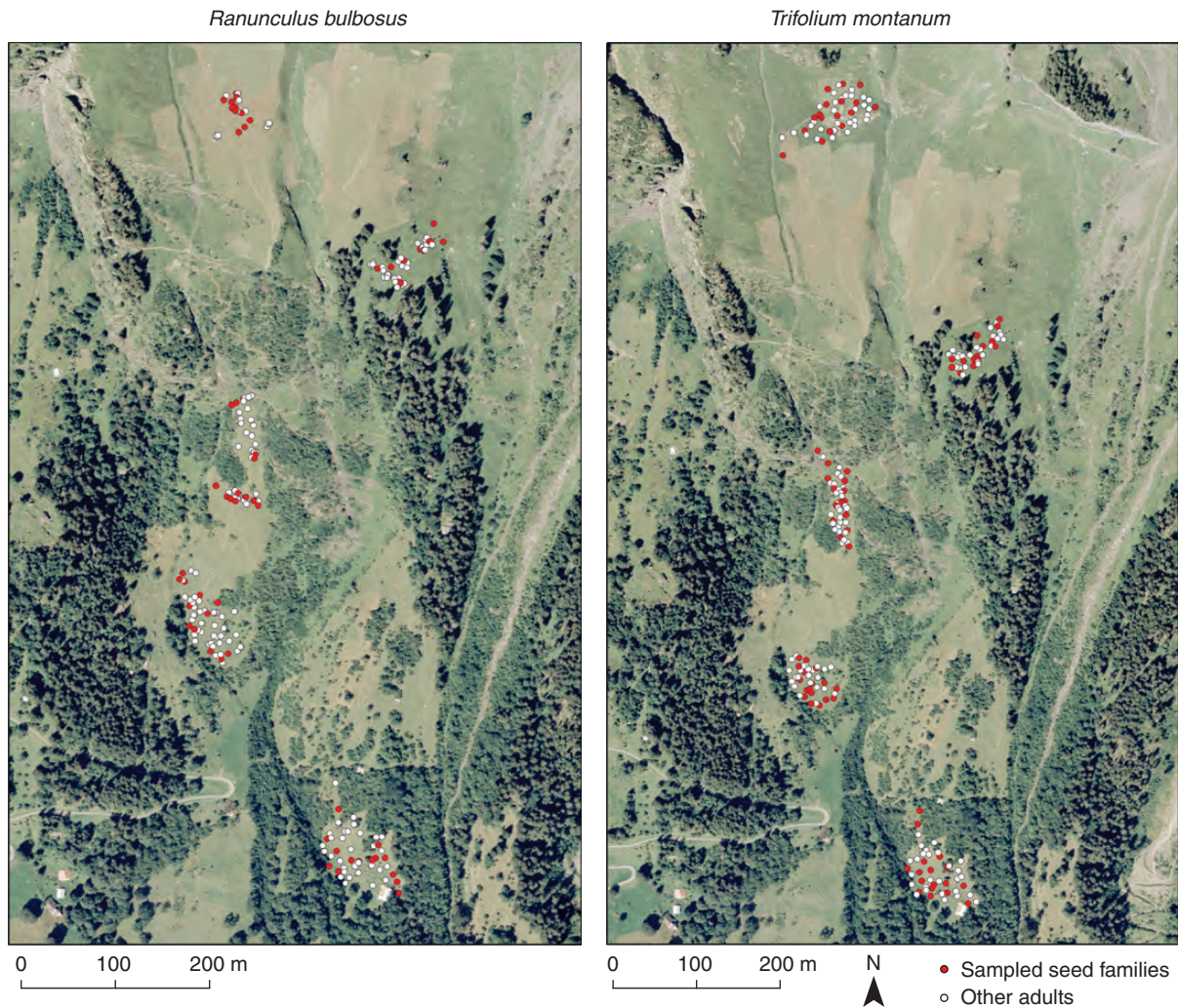


FIG. 1. Location of the sampled seed families and additional adults (as indicated in the key). The meadows at the bottom of the picture lie at approx. 1200 m a.s.l., and the ones at the top lie at approx. 1800 m a.s.l.

*Census density and overall numbers of individuals.* Since counting open flower heads in the field is far more time efficient than counting individuals, we estimated the density of individuals (i.e. individuals  $\text{m}^{-2}$ ) based on (a) the maximum number of open flower heads (OFH) yielded by their exhaustive count in each meadow at 13 time points in summer 2010 (see preceding paragraph for how the meadow area was delineated and the next section for the OFH count), (b) the mean number of OFH per individual (estimated based on flower counts in 2–190 and 72–141 individuals per meadow in *R. bulbosus* and *T. montanum*, respectively), and (c) the area of the sampled meadows, which was subsequently measured from aerial pictures of SwissTopo<sup>®</sup> using ImageJ (<http://rsbweb.nih.gov/ij/index.html>) and corrected for the slope (Table S1). The resulting estimations of densities at the time point with the maximal numbers of OFH were then used to extrapolate the ‘population’ sizes of *R. bulbosus* and *T. montanum* along the whole altitudinal transect to highlight the abundance of the focal species in the study area and variation among meadows. Thereby, all areas up to approx. 3 km distance of the

sampled meadows and covered by calcareous grasslands containing the species of interests, as indicated in the database of dry grassland sites of national importance in Switzerland (Eggenberg *et al.*, 2001), were considered.

*Quantifying flowering overlap along the altitudinal gradient.* We quantified the intensity of flowering overlap along the altitudinal gradient among meadows to investigate its relationship with pollen flow. We counted open flower heads (OFH) weekly from 28 May 2010 to 20 August 2010 (13 time points) in each meadow. Per census, OFH was measured by walking parallel transects spaced by 8–10 m each and counting OFH present within 4–5 m right and left of the transect. The degree of flowering overlap was thus quantified by estimating the number of simultaneously opened flower-heads (NSO-FH) between meadows, which has the advantage of taking into account potential differences in the species abundance. Previous studies have focused solely on overlapping flowering period which ignores this important demographic parameter (Robledo-Arnuncio *et al.*, 2006). We determined

curves of number of OFH over time and calculated the NSO-FH of each pair of meadows defined by the area of overlapping curves divided by the number of days during which both meadows were simultaneously flowering (see Supplementary Data Fig. S1). As a result, NSO-FH represents the average number of flower heads in each meadow, which were open during the whole flowering period overlap.

#### Laboratory methods and genetic dataset

DNA was extracted from approx. 10 mg dried leaf tissue with DNeasy 96 Plant Kits (Qiagen, Hombrechtikon Switzerland). Samples were genotyped using seven (*R. bulbosus*: Rb204, Rb206, Rb302, Rb306, B127, B129 and B145) or 11 (*T. montanum*: ats002, ats006, ats029, ats032, Tm10, Tm12, Tm13, Tm16, Tm17, Tm21 and Tm24) nuclear microsatellite markers as described in the two Primer Notes by Matter et al. (2012a, b).

In *R. bulbosus*, repeatability of the genotyping was assessed based on 52 (markers Rb204, Rb206, Rb302 and Rb306), 77 (B129 and B145) and 139 individuals (B127), and proved to range between 94.2% and 100% (mean = 97.4%). Individuals with at least six (out of seven) loci typed were included in the analysis, resulting in a final dataset of 924 samples [72 SF with 6–10 (median = 10) individuals each,  $N = 686$ ; 72 maternal plants and 166 additional adults]. Missing data across the dataset were 3.7%.

In *T. montanum*, repeatability was assessed on 18–41 individuals per marker. The overall repeatability rate was 99.7% (97.6% for Tm17 and 100% for all other markers). Individuals with at least nine (out of 11) loci typed were included in the analyses resulting in a final dataset of 1031 samples [82 SF with 8–10 (median = 10) individuals each,  $N = 808$ ; 82 maternal plants and 141 additional adults]. Missing data across the dataset were 1.5%.

#### Statistical analyses

**Patterns of genetic differentiation among meadows.** Since the presence of null alleles in some of the markers has previously been shown (Matter et al., 2012a, b), the degree of inbreeding was estimated with INEst which simultaneously estimates the null allele frequencies and the inbreeding coefficient (Chybicki and Burczyk, 2009), using the Population Inbreeding Model; standard errors were obtained with the jack-knifing procedure accounting for inbreeding. The genetic diversity and the genetic differentiation were estimated with FSTAT (Goudet, 2001). A spatial clustering analysis was done with BAPS 5.3 (Corander et al., 2007) using 25 runs allowing for a varying number of clusters  $K$  (max.  $K = 20$ ) and two spatial co-ordinates. Spatial genetic structure (SGS) was investigated with SPAGEDiv1.3 (Hardy and Vekemans, 2002), yielding correlograms of Loiselle's kinship coefficients (Loiselle et al., 1995) against spatial distance between individuals, calculated based on three spatial co-ordinates. For correlograms across all five meadows, 20 distance classes were set containing about the same number of pairs each; for correlograms on each meadow separately, ten such distances classes were set. Confidence intervals of the estimated kinship coefficients were calculated with jack-knifing over loci; and

confidence intervals of the expectation of no spatial genetic pattern were based on 999 permutations of individual locations among all individuals.

**Indirect estimates of contemporary pollen flow.** Pollen dispersal parameters were estimated using the program KINDIST (Robledo-Arnuncio et al., 2006; Robledo-Arnuncio et al., 2007), which implements the pollen-pool approach. The among-sibships correlated paternities estimated after Loiselle et al. (1995) were used to fit an exponential power function (Austerlitz et al., 2004; Robledo-Arnuncio and Gil, 2005; Klein et al., 2008; Oddou-Muratorio et al., 2010; Field et al., 2011) with a non-linear least squares model. A key assumption made by the program is that among-sibship correlated paternities decay with increasing distance. We tested this with a Mantel test in the R-package 'Vegan' (Oksanen et al., 2012) using Spearman's rank correlation coefficient  $\rho$  and 9999 permutations on the kinship matrix. A further assumption of the program to calibrate kinship coefficients is that, beyond a given threshold distance, pollen pools are unrelated, indicated by a stabilization of the among-sibships correlated paternity estimates (plotted against distance) at a slightly negative value. We tested several threshold distances (ranging between 1 m and the maximum distance between two maternal plants) to find the best-fitting model, indicated by the smallest least-squares residuals (LSR; as in Field et al., 2011). Based on the best fitting kernel, the average pollen dispersal distance ( $\delta$ ) was estimated. To test the robustness of the kernel fitting we repeated one to two analyses per dataset or sub-dataset. Note that KINDIST handles two spatial co-ordinates only and uses the allele frequency of the maternal plants to estimate the correlated paternities.

Pollen pool differentiation (global  $\varphi_{ft}$ ) and, subsequently, number of effective pollen donors ( $N_{ep}$ ) were calculated with TWOGENER (Austerlitz and Smouse, 2001, 2002). The global  $\varphi_{ft}$  is a measure of correlated paternity among all sibships within a given dataset and is calculated via an AMOVA based on allele frequencies of the maternal plants and additional adults, as suggested by Robledo-Arnuncio et al. (2007). To estimate  $N_{ep}$  from the global  $\varphi_{ft}$ , the latter has to be corrected for parental inbreeding ( $F_P$ ) and selfing ( $s$ ) (Austerlitz and Smouse, 2001; Burczyk and Koralewski, 2005). Given  $F_P$ ,  $\varphi_{ft}$  transforms to  $\varphi'_{ft} = \varphi_{ft}/(1 + F_P)$ ; and given  $s$  as  $(1 - t_m)$  with  $t_m$  being the multilocus outcrossing rate,  $\varphi'_{ft}$  transforms to  $\varphi''_{ft} = (2\varphi'_{ft} - s^2)/[2(1 - s)^2]$ .  $N_{ep}$  was calculated as  $1/(2\varphi''_{ft})$ .  $F_P$  and  $t_m$  were estimated with MLTR (Ritland, 2002) using the expectation-maximization method, pollen gene frequencies inferred from each seed-family and inference of parentage with parents chosen at random.

## RESULTS

#### Census density and overall population size

Census density differed among meadows and species (Fig. 2 and Supplementary Data Table S1): the density of *R. bulbosus* individuals tended to decrease with altitude (Pearson's correlation coefficient of  $-0.843$ ;  $P = 0.0729$ ), while there was no correlation in *T. montanum* ( $-0.286$ ,

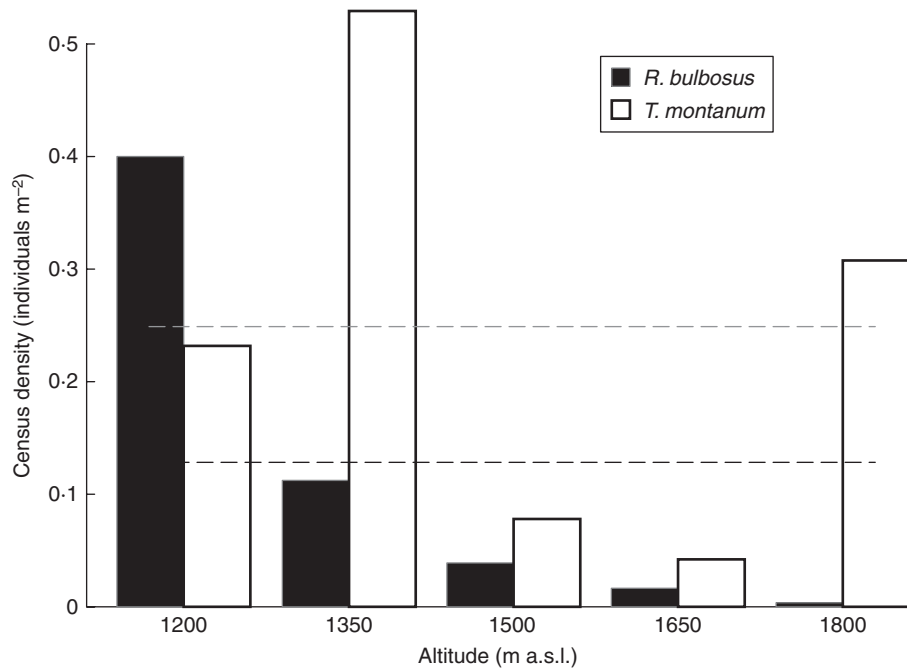


FIG. 2. Census plant density of *Ranunculus bulbosus* and *Trifolium montanum* in function of altitude. The dashed lines indicate the average census density.

$P = 0.640$ ). Given the census density, the average numbers of open flower heads per individual and the area containing the focal species along the altitudinal transect under study (respectively, 26 and 49 ha for *R. bulbosus* and *T. montanum*, according to Eggenberg *et al.*, 2001; see Materials and Methods), the overall numbers of individuals ('population' size) was about 29 000 individuals in *R. bulbosus* and 81 000 individuals in *T. montanum*.

#### Inbreeding, genetic diversity and genetic differentiation

Both *R. bulbosus* and *T. montanum* showed no significant inbreeding coefficients, even when computed separately for each meadow. Allelic richness in each meadow (corrected for sample size) ranged between 4.30 and 4.55 in *R. bulbosus* (minimum sample size = 27) and 4.25 and 4.39 in *T. montanum* (minimum sample size = 37) and did not significantly differ among meadows (1000 permutations, two-sided test;  $P = 0.555$  in *R. bulbosus* and  $P = 0.746$  in *T. montanum*). Differentiation among the five meadows was very low in both species:  $F_{st} = 0.0099$  [95 %CI = (0.006; 0.016)] in *R. bulbosus* and 0.0068 [95 %CI = (0.003; 0.011)] in *T. montanum*.

There was no evidence for spatial clustering: all individuals of the five meadows got assigned to a single genetic cluster in both species. Moreover, SGS across all five meadows was very weak: only the two first distance classes (i.e. up to 38 m) showed a kinship coefficient significantly different from zero in both species (Supplementary Data Fig. S2a). Due to these indications of SGS at small distances, i.e. within meadows, we repeated the analyses at the meadow level. There was a lack of SGS in all but the upper-most *R. bulbosus* meadow ('Ran1800'), where individuals in the shortest distance class were significantly related (Supplementary Data Fig. S2b).

#### Indirect estimates of contemporary pollen flow

*Pollen-pool approach and dispersal kernel fitting.* Across all five meadows, the among-sibship correlated paternity estimates showed a weak decay with increasing distance [ $\rho = -0.086$  ( $P = 5.0e-04$ ) in *R. bulbosus* and  $\rho = -0.048$  ( $P = 0.005$ ) in *T. montanum*; Fig. 3]. At the meadow scale, patterns were slightly different. In the upper three *R. bulbosus* meadows 'Ran1500', 'Ran1650' and 'Ran1800' and in the *T. montanum* meadow 'Trif1650', the among-sibships correlated paternity estimates (tended to) decrease stronger with distance than across all meadows:  $\rho = -0.191$  ( $P = 0.033$ ) in 'Ran1500';  $\rho = -0.215$  ( $P = 0.023$ ) in 'Ran1650';  $\rho = -0.18$  ( $P = 0.075$ ) in 'Ran1800'; and  $\rho = -0.139$  ( $P = 0.112$ ) in 'Trif1650' (Fig. 3). In the two lower *R. bulbosus* meadows and in all other *T. montanum*'s meadows there were no negative correlations found.

Given that Robledo-Arnuncio *et al.* (2007) recommends not to run KINDIST when  $\rho > -0.1$  to avoid inflated biases, we estimated the parameters of an Exponential-Power dispersal kernel in the five following datasets only: the single meadows 'Ran1500', 'Ran1650', 'Ran1800' and 'Trif1650' (although the correlation in the two latter meadows was not significant as, for example, in de-Lucas *et al.*, 2008), and also all *R. bulbosus*' meadows pooled (since  $\rho$  was close to  $-0.1$  and highly significant).

The analyses across all meadows as well as within the meadows 'Ran1500', 'Ran1650' and 'Trif1650' reached the best fit (i.e. lowest LSR) when the threshold distance equaled the maximum inter-seed-family distance (Supplementary Data Table S2). The resulting kernels had shape parameters below one, indicating fat tails (Austerlitz *et al.*, 2004) and their average pollen dispersal distances ( $\delta$ ) were 141 m in *R. bulbosus* across all meadows, 297 m within the meadow

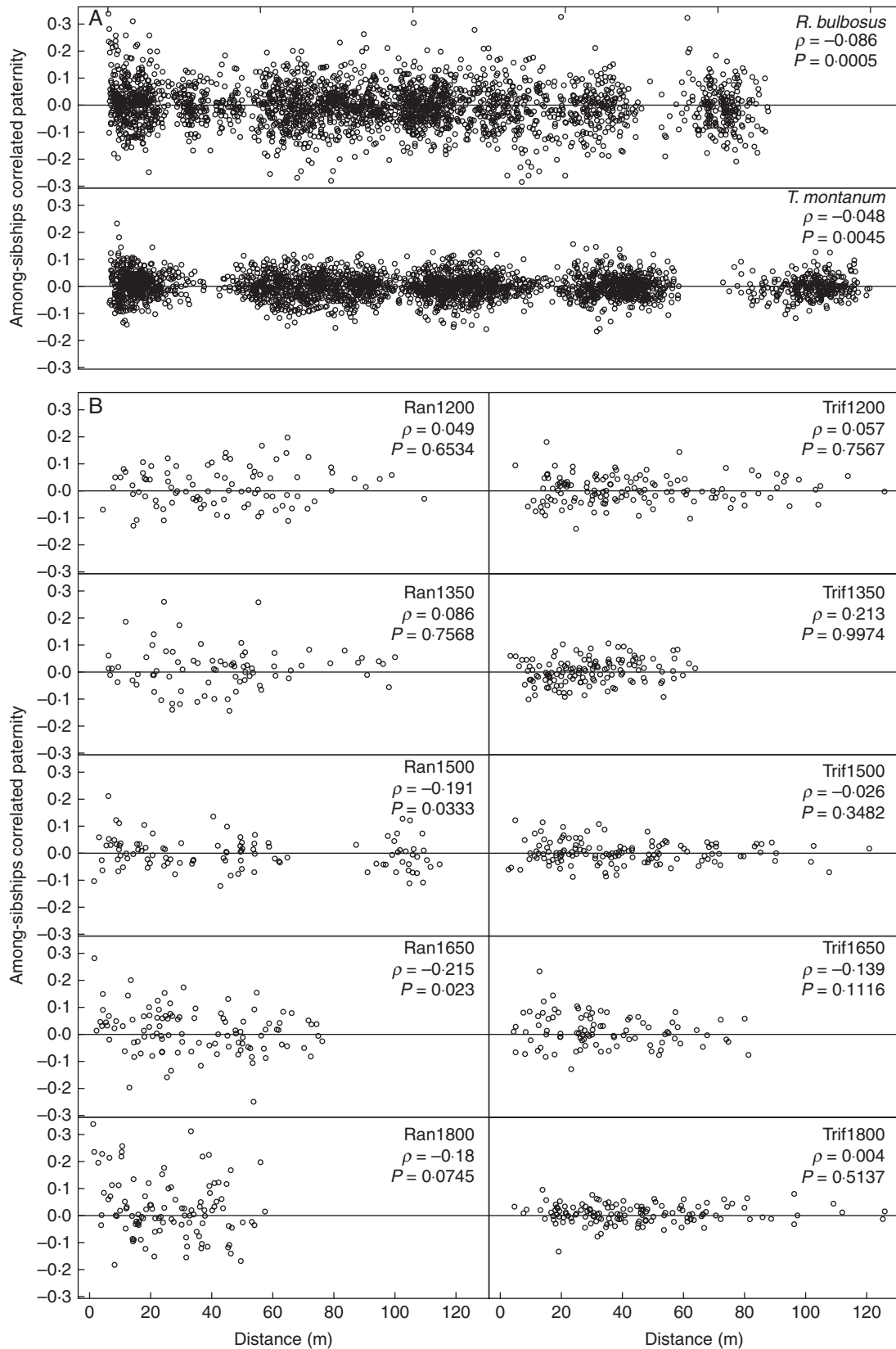


FIG. 3. Among-sibships correlated paternity estimated in *Ranunculus bulbosus* and *Trifolium montanum* against distance: (A) the five meadows pooled; (B) within each meadow separately. The correlations ( $\rho$ ) and associated  $P$ -values are indicated, calculated with a Mantel test using Spearman's rank correlation coefficient and 9999 permutations on the kinship matrix.

TABLE 1. Exponential-power kernel fit on four *Ranunculus bulbosus* samples and one *Trifolium montanum* sample

Dataset	$N_{SF}$	$N_{offs}$	$a$	$b$	$\delta$ (m)
All <i>R. bulbosus</i> meadows	72	686	0.000033	0.1724	140.72
Ran1500	15	141	0.000011	0.1589	296.53
Ran1650	15	143	0.000003	0.1522	225.43
Ran1800	15	143	0.0001	0.1843	109.46
Trif1650	14	136	0.000003	0.1380	3490.57

The kernel was fitted with KINDIST (Robledo-Arnuncio *et al.*, 2007).

The best-fitting kernel estimates are indicated.

$N_{SF}$ , Number of seed-families;  $N_{offs}$ , total number of offspring;  $a$  and  $b$ , scale and shape parameters of the dispersal kernel (for details, see Austerlitz *et al.*, 2004);  $\delta$ , average pollen dispersal distance.

‘Ran1500’, 225 m within ‘Ran1650’ and 3.5 km within ‘Trif1650’ (Table 1).

Results of ‘Trif1650’ are questionable since the same parameter estimates were obtained regardless of the threshold distance used, excepting the maximum inter-seed-family distance. In the upper meadow ‘Ran1800’, LSR indicated a best fit when the threshold distance was set to 45 m, which is smaller than the maximum inter-seed-family distance of 57 m. However, parameter estimates were similar for six out of ten different threshold distances used in ‘Ran1800’ but changed drastically with threshold distances of 44, 45 and 46 m, resulting in a pollen dispersal distance of 250, 109 and 7.5 m, respectively. As the latter estimate of pollen dispersal would result in a spatial genetic structure, which was absent here, the other two values might be better estimates.

The seven kernel fittings which have been replicated (see Supplementary Data Table S2) yielded exactly the same parameter estimations and LSRs.

In summary, as among-sibship correlated paternity decayed less across all meadows in *T. montanum* compared with *R. bulbosus* and decayed more often with distance in the latter species, our results indicate higher rates of contemporary gene flow in *T. montanum* than *R. bulbosus*.

*Correlated paternity and effective number of pollen donors.* The correlated paternity ( $\varphi'_{ft}$ ) across all meadows was higher in *R. bulbosus* (0.129) than in *T. montanum* (0.042; Table 2). Within individual meadows it was also consistently higher in *R. bulbosus* (range: 0.088–0.155) than in *T. montanum* (range: 0.017–0.064). The effective number of pollen donors  $N_{ep}$  was higher in *T. montanum* than in *R. bulbosus*, both at the whole-sample scale (12.0 vs. 3.9) and at the meadow scale (7.8–29.3 vs. 3.2–5.7).  $\varphi'_{ft}$  and  $N_{ep}$  did not correlate with altitude and neither did  $\varphi'_{ft}$  with plant density (all correlation tests with Spearman’s ranks tests, details not shown).

#### Flowering overlap among altitudes

The flowering peak of two consecutive meadows was delayed by <1 week in *R. bulbosus* and at maximum by 2 weeks in *T. montanum*. The overlapping flowering period between the highest and the lowest meadow, was 3 weeks for *R. bulbosus* and 6 weeks for *T. montanum* (see Fig. 4). The pairwise number of simultaneously opened flower-heads (NSO-FH) between meadow pairs, a potential indicator of

TABLE 2. Correlated paternity, mating system parameters and effective number of pollen donors in five meadows of *Ranunculus bulbosus* and *Trifolium montanum* along an altitudinal gradient

Dataset	Global $\varphi_{ft}$	$F_P$	$\varphi'_{ft}$	$s$	$\varphi''_{ft}$	$N_{ep}$ (individuals)
<i>R. bulbosus</i>						
All meadows pooled	0.128	0.011	0.127	0.010	0.129	3.9
Ran1200	0.097	0.000	0.097	0.007	0.099	5.1
Ran1350	0.124	0.000	0.124	0.000	0.124	4.0
Ran1500	0.086	0.000	0.086	0.011	0.088	5.7
Ran1650	0.141	0.034	0.136	0.013	0.140	3.6
Ran1800	0.157	0.054	0.149	0.020	0.155	3.2
<i>T. montanum</i>						
All meadows pooled	0.043	0.038	0.041	0.002	0.042	12.0
Trif1200	0.066	0.041	0.064	0.000	0.064	7.8
Trif1350	0.045	0.025	0.044	0.000	0.044	11.3
Trif1500	0.029	0.031	0.028	0.005	0.029	17.4
Trif1650	0.042	0.065	0.039	0.000	0.039	12.7
Trif1800	0.017	0.021	0.017	0.006	0.017	29.3

Global  $\varphi_{ft}$ , pollen-pool differentiation parameter (calculated with TwoGener);  $\varphi'_{ft}$  and  $\varphi''_{ft}$ , pollen-pool differentiation parameter corrected for parental inbreeding and selfing (see text);  $F_P$ , inbreeding coefficient of the parental generation;  $s$ , selfing rate;  $N_{ep}$ , number of effective pollen donors.

pollen flow, was higher in all pairs of *T. montanum* meadows (144–2355, median = 372,  $n = 10$  pairs) compared with *R. bulbosus* (17–411, median = 40; see Table 3). This measure of pairwise flowering overlap did not correlate with the pairwise altitudinal difference [Mantel test using Spearman’s  $\rho$  and 9999 permutations:  $\rho = -0.152$  ( $P = 0.335$ ) in *R. bulbosus* and 0.1758 ( $P = 0.764$ ) in *T. montanum*].

## DISCUSSION

This study provides new insights into pollen-mediated contemporary gene flow in abundant mountain herbs across an altitudinal gradient. Based on neutral genetic markers, our evaluation of contemporary pollen dispersal and genetic differentiation among five meadows ( $F_{st} = 0.01$  in *R. bulbosus* and 0.007 in *T. montanum*) indicate extensive contemporary and historic gene flow. Our results suggest that plant density and degree of flowering period overlap among meadows could be important drivers for shaping the patterns of pollen dispersal. Pollen flow appears to be slightly greater in *T. montanum* than *R. bulbosus*. The tail of the pollen dispersal curve extended well beyond the scale of our study area, supporting the idea that contemporary gene flow by pollen is extensive in these species in mountain meadow systems. Below we discuss the processes which may lead to the observed patterns and their implications for maintenance of genetic diversity and adaptive variation in mountain plants facing rapid and unprecedented climate change.

*Do patterns of genetic differentiation and genetic diversity differ across altitude?*

Patterns of historic gene flow are important because these may underlie patterns of local adaptation in plant species

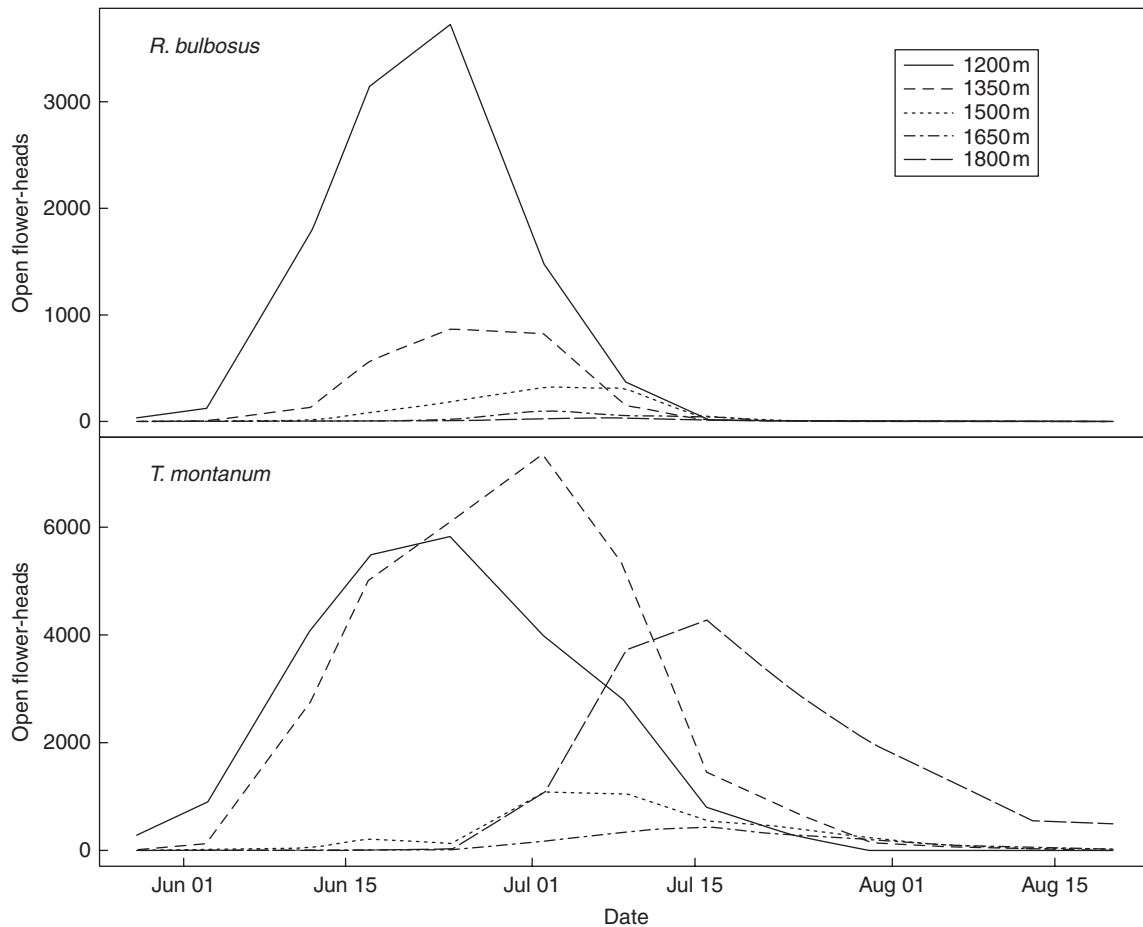


FIG. 4. Numbers of open flower-heads in *Ranunculus bulbosus* and *Trifolium montanum* in five meadows at 1200, 1350, 1500, 1650 and 1800 m a.s.l. at 13 weekly census points between 25 May and 20 August 2010.

TABLE 3. Pairwise numbers of simultaneously opened flower-heads (NSO-FH) of five *Ranunculus bulbosus* (lower diagonal) and five *Trifolium montanum* (upper diagonal) meadows

	M1200	M1350	M1500	M1650	M1800
M1200	–	2345	460	183	841
M1350	411	–	407	172	944
M1500	169	162	–	145	337
M1650	41	42	40	–	144
M1800	20	20	18	17	–

Each meadow is referred to as ‘M’ with its approximate altitude.

(Gonzalo-Turpin and Hazard, 2009). The absence of any genetic clustering, the very low  $F_{st}$  values and similar levels of genetic diversity among meadows reflect weak genetic differentiation at our study site. This is consistent with the findings of Hahn *et al.* (2012) who studied the same two species at 1200 and 1800 m a.s.l., i.e. similar upper and lower altitudes, but over a much larger spatial scale and using different molecular markers. It is interesting to note that, although both studies observed little differentiation, contrary to our study Hahn *et al.*

(2012) observed greater differentiation in *T. montanum* ( $F_{st} = 0.118$ ) than in *R. bulbosus* ( $F_{st} = 0.071$ ). This difference can be explained by several factors, including much greater sampling scale (about 200 km), fewer individuals per site sampled ( $n = 20$ ), and different markers (AFLP vs. microsatellites). In contrast, in *Arabis alpina* sampled over very similar spatial scales to our study, very strong genetic structuring was observed (Buehler *et al.*, 2012). We attribute these differences to the predominantly selfing mating system of *Arabis alpina*.

#### Does contemporary pollen dispersal link meadows along an altitudinal gradient in *R. bulbosus* and *T. montanum*?

Recent advances in population genetic statistics and modeling have greatly enhanced our capacity to indirectly evaluate pollen flow, i.e. without exhaustive sampling of populations (Robledo-Arnuncio *et al.*, 2006). However, such approaches do have limitations. First, they require adequate genetic structuring over the scale of the investigation (Robledo-Arnuncio *et al.*, 2006; Rong *et al.*, 2010). Second, over-interpretation of the shape of the dispersal kernel derived from such data should be avoided (Robledo-Arnuncio *et al.*, 2006). Without baseline studies it remains difficult to determine what the appropriate scale of sampling is. Our study provides a baseline



analysis and demonstrates that the scales of contemporary gene flow are greater than the scale of sampling in *T. montanum* and *R. bulbosus* (1 km horizontal and 600 m of altitudinal difference). This point is particularly evident by the Least-Squares Residuals of the pollen pool analyses, which in most datasets indicate that the best fit of the dispersal kernels is reached at the maximum sample distance. In the seven datasets which were not suited for pollen-pool analysis, and, especially in *T. montanum*, the lack of a prominent structuring in the pollen pool is likely a consequence of extremely low differentiation of the parental generation. Indeed, since individuals are genetically similar over the whole sampling area, the pollen produced by paternal plants will also be similar across potential pollen donors. Yet, combining information on mating system, flower morphology and phenology, we can infer some additional important factors shaping pollen dispersal.

*Do flowering phenology, demography and mating system influence pollen flow across altitude?*

**Flowering phenology.** The overall phenology patterns illustrated in Fig. 4 are consistent with the lack of genetic differences as shown by KINDIST in *T. montanum*. Its greater population size and the greater proportional flowering overlap (6 weeks of simultaneous flowering for the highest and lowest meadow) among meadows appears to contribute to the more extensive pollen flow. In contrast, in *R. bulbosus*, flowering overlap across all meadows is clearly much smaller (maximum 3 weeks of simultaneous flowering between the highest and the lowest meadow). Indeed, average pollen dispersal distance proved to be smaller than 300 m in *R. bulbosus*, which corresponds to a dispersal over three consecutive meadows. Although patterns of genetic differentiation may be influenced by seed dispersal, the tail of the pollen dispersal kernel and average dispersal distances, our results imply that dispersal between the highest and the lowest meadow is more limited, although not excluded, in *R. bulbosus* compared with *T. montanum*. This is further supported by the consistently larger number of simultaneously open flower-heads (NSO-FH) per meadow pair in *T. montanum* compared with *R. bulbosus*, suggesting that the probability of pollen flow in *T. montanum* is greater than in *R. bulbosus*. The fact that NSO-FH does not correlate with pairwise altitudinal difference among meadows, which was unexpected, is attributable to the large differences in number of individuals per meadow. This is evident in *R. bulbosus*, in which the largest meadow contains over 150 times more OFH than the smallest meadow which is the uppermost location under study.

Gerber *et al.* (2004) studied patches of *Ranunculus alpestris* in Switzerland and found that flowering phenology delays induced by time of snow-melt only weakly affected genetic differentiation of sub-populations along the gradient, and that distance was more important for structuring populations. In contrast, Hirao and Kudo (2004) found that genetic distance was correlated with phenological distance in *Veronica stelleri* and *Gentiana nipponica* of northern Japan. In the alpine buttercup *Ranunculus adoneus* in Colorado, USA, Stanton *et al.* (1997) found that populations showed slight but significant genetic differentiation among the different snow-melt zones. As both pollen and seed dispersal are relevant to the observed

patterns of genetic structure, our results suggest that, between altitudinal extremes, seed dispersal may be more important when phenology constrains pollen-mediated gene flow.

**Flower morphology and population demography.** Additionally to phenology, flower morphology and population density also contribute to determining the diversity of the pollen pool sampled by seed families (Hardy *et al.*, 2004a). Global correlated paternity ( $\varphi_{it}$ ) was higher in *R. bulbosus* (0.128) than in *T. montanum* (0.043) and the effective number of pollen donors ( $N_{ep}$ ) was lower in *R. bulbosus* ( $N_{ep} = 3.2-5.7$ ) than in *T. montanum* ( $N_{ep} = 7.2-29.3$ ). These interlinked metrics reflect how these species ‘sample’ the background pollen pool and are influenced by co-dispersal and limited mate availability (Hardy *et al.*, 2004a). Hardy *et al.* (2004a) report values of effective number of pollen donors ranging from  $N_{ep} = 1.2-8.8$  in eight herb species from five different families. The values reported are comparable to those found for *R. bulbosus*, but much smaller than those found for *T. montanum*. One explanation for this difference may be the floral morphology of the species. *Ranunculus bulbosus* has a single corolla circling many stigmas; in contrast the inflorescence of *T. montanum* is composed of 100–150 corollas each containing only one stigma. This is consistent with the eight species mentioned above, where the by-far highest  $N_{ep}$  value is reported for *Centaurea solstitialis*, whose inflorescences are also composed of several long and tubular corollas. The probability that several stigmas receive pollen from the same individual through foraging insects is thus greater in *R. bulbosus*; a pattern already described by Matter *et al.* (2013). Additionally, limited mate availability could result from the lower density of conspecifics with increasing altitude in *R. bulbosus* and lower flowering overlap (see preceding paragraph) between meadows of different altitudes. In contrast larger population densities, greater overlap in flowering and floral morphology facilitated a reduced correlated paternity in *T. montanum*.

**Mating system.** Generally pollen dispersal distance is thought to be negatively correlated with the selfing rate in plants (Ennos, 1994; Chauvet *et al.*, 2004). In *Arabis alpina* 84 % selfing rates were detected using maximum likelihood-based paternity analysis while the direct estimate of pollen dispersal showed only a tiny proportion of mating events occurring over distances >1000 m (Buehler *et al.*, 2012). In our study using the same maximum likelihood approach, we observed 25 % selfing in *R. bulbosus* and 4.3 % in *T. montanum* (results not shown). While we treat these estimates with caution (due to the limited power to assign paternity in field conditions with many un-sampled potential donors), the slightly lower pollen dispersal in *R. bulbosus* compared with *T. montanum* based on this paternity analysis and on the pollen-pool approach is consistent with the idea that selfing may reduce the frequency of long-distance dispersal.

*Implications of pollen dispersal among altitudes for mountain perennial herbs in the face of climate change*

The implications of climate change for species and habitat conservation is the subject of global concern (Theurillat and Guisan, 2001; Thomas *et al.*, 2004; Hannah *et al.*, 2005). The ability of species to survive these changes at their present location will depend on their adaptive potential.

Our findings suggest that populations occurring across an altitudinal gradient have the capacity to exchange genes by pollen dispersal. This ensures population connectivity, large effective population sizes and reduced probability of inbreeding. Extant adaptive variation should thus be maintained and transferable among altitudes in response to climate change.

Limited local adaptation is often attributed to high levels of gene flow and because only very few dispersal events are required to spread advantageous alleles (Morjan and Rieseberg, 2004). However, in perennial herb species patterns of local adaptation appear to be more commonly observed over large scales but seem to be rarer at small spatial scales (Clausen *et al.*, 1940; Snaydon and Davies, 1976; Scheepens *et al.*, 2010). Some studies also show local adaptation at small scales specifically associated with altitude, e.g. in *Poa hiemata* (Byars *et al.*, 2007) and in *Festuca eskia* (Gonzalo-Turpin and Hazard, 2009). In such species extensive gene flow might facilitate the exchange of advantageous genes to higher elevations which may enhance population resilience to climatic change.

Perhaps of more immediate threat to mountain species is rapid land use change. Abandonment of traditional management in dry grassland meadows has been reported over the last 60 years across Switzerland (Stöcklin *et al.*, 2007). Such habitat fragmentation is predicted to reduce genetic connectivity. However, our estimates of contemporary pollen dispersal in *T. montanum* and *R. bulbosus* suggest that recent land use change has little consequence for contemporary gene flow for some herbaceous semi-dry grassland species. In conclusion, these common semi-grassland herb species are likely to be highly resilient to anthropogenic habitat change provided that sufficient meadow habitat is maintained.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: characteristics of plant numbers and densities in *Ranunculus bulbosus* and *Trifolium montanum* in five meadows along an altitudinal transect and estimated at 13 weekly census days over the growing season 2010. Table S2: exponential-power Kernel fit on four *Ranunculus bulbosus* and one *Trifolium montanum* samples with KINDIST. Fig. S1: estimating the number of simultaneously opened flower-heads between two given meadows. Fig. S2: spatial genetic structure in *Ranunculus bulbosus* and *Trifolium montanum*.

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