ORIGINAL ARTICLE
Reduced fine-scale spatial genetic structure in grazed populations of *Dianthus carthusianorum*

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Strong spatial genetic structure in plant populations can increase homozygosity, reducing genetic diversity and adaptive potential. The strength of spatial genetic structure largely depends on rates of seed dispersal and pollen flow. Seeds without dispersal adaptations are likely to be dispersed over short distances within the vicinity of the mother plant, resulting in spatial clustering of related genotypes (fine-scale spatial genetic structure, hereafter spatial genetic structure (SGS)). However, primary seed dispersal by zoochory can promote effective dispersal, increasing the mixing of seeds and influencing SGS within plant populations. In this study, we investigated the effects of seed dispersal by rotational sheep grazing on the strength of SGS and genetic diversity using 11 nuclear microsatellites for 49 populations of the calcareous grassland forb *Dianthus carthusianorum*. Populations connected by rotational sheep grazing showed significantly weaker SGS and higher genetic diversity than populations in ungrazed grasslands. Independent of grazing treatment, small populations showed significantly stronger SGS and lower genetic diversity than larger populations, likely due to genetic drift. A lack of significant differences in the strength of SGS and genetic diversity between populations that were recently colonized and pre-existing populations suggested that populations colonized after the reintroduction of rotational sheep grazing were likely founded by colonists from diverse source populations. We conclude that dispersal by rotational sheep grazing has the potential to considerably reduce SGS within *D. carthusianorum* populations. Our study highlights the effectiveness of landscape management by rotational sheep grazing to importantly reduce genetic structure at local scales within restored plant populations.
Heredity (2016) **117**, 367–374; doi:10.1038/hdy.2016.45; published online 6 July 2016

INTRODUCTION
Plants due to their sessile life form are highly sensitive to ongoing trends of habitat loss and fragmentation, likely experiencing low rates of immigration of seed and pollen flow within populations from outside sources (Aguilar *et al.*, 2006; Vranckx *et al.*, 2012). As populations become more isolated, spatially restricted pollen flow and seed dispersal within patches lead to an increase of relatedness among neighboring individuals, considerably increasing fine-scale spatial genetic structure (SGS) (Heywood, 1991; Wells and Young, 2002). SGS can increase homozygosity (Ritland, 2002), which reduces the effective size (N_e) of populations, potentially undermining their adaptive genetic potential. Erosion of genetic diversity can affect plant fitness and population viability, increasing the risk of extirpation (Keller and Waller, 2002; Leimu *et al.*, 2006). Evaluating the circumstances that determine the formation of SGS should be an important target in conservation planning of fragmented plant populations. Moreover, spatial patterns of genetic structure are key for understanding ecological and evolutionary dynamics such as changes in reproductive strategies, progeny fitness and local adaptation (Loveless and Hamrick, 1984; Kalisz *et al.*, 2001; Dick *et al.*, 2008; Zhao *et al.*, 2009).

Empirical studies suggest that the strength of SGS is influenced by seed dispersal mode and mating system. Comparisons across species found that species with seed dispersal by gravity show higher SGS than species whose seeds are dispersed by wind or animal vectors (Hardy *et al.*, 2006; Hamrick and Trapnell, 2011; Chung and Chung, 2013), while SGS is usually stronger in selfing relative to outcrossing species (Ennos, 2001; Vekemans and Hardy, 2004). Insights from computer simulations suggest that seed dispersal can have a stronger influence on SGS relative to the dispersal of pollen, but the interaction between selfing rates, pollen and seed dispersal distances, and plant density are critical for the development of SGS (Epperson, 2007). Other factors such as colonization history (Chung *et al.*, 2011; Helsen *et al.*, 2015), demography (Travis *et al.*, 2004; Jacquemyn *et al.*, 2006), or environmental conditions such as habitat fragmentation (Yamagishi *et al.*, 2007; Yue *et al.*, 2012), habitat quality (Bizoux and Mahy, 2007), and land use history (Smith *et al.*, 2009) can also influence the formation of SGS.

In Central Europe, ongoing land use changes had led to a dramatic decline of semi-natural habitats of calcareous grasslands since the 20th century (Poschlod and WallisDeVries, 2002). In particular, the progressive abandonment of rotational grazing by shepherding resulted in numerous extinctions of habitat specialist plants in remnant grasslands due to shrub encroachment and natural reforestation (Butaye *et al.*, 2005). Calcareous grasslands are of high conservation value because they are one of the most species-rich habitats in Central Europe (WallisDeVries *et al.*, 2002). To counteract declines of habitat specialist plants in fragmented grasslands, landscape

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management practices have been implemented to restore their biodiversity (Aviron *et al.*, 2009). One of such practice relies on the implementation of landscape ecological networks where propagule dispersal can be facilitated through linking grassland patches with rotational grazing by shepherding (Mouissie *et al.*, 2005; Wessels *et al.*, 2008; Wagner *et al.*, 2013). Seed dispersal by domestic ungulates, such as sheep, goats or cattle, has been found to facilitate long-distance dispersal for a range of plant species as herds move along the grazing routes connecting spatially fragmented grasslands (Fischer *et al.*, 1996; Manzano and Malo, 2010; Albert *et al.*, 2015). Because rotational sheep grazing can provide effective seed dispersal and gene flow at the landscape-scale (Rico *et al.*, 2012; Rico *et al.*, 2014b), sheep grazing might also influence the development of fine-scale spatial genetic structure within grazed patches. Experimental studies that have quantified the effects of grazing on the strength of SGS suggest that intensive grazing can negatively impact levels of genetic diversity within populations (Ma *et al.*, 2014; Peng *et al.*, 2015). The effect of grazing on SGS in empirical systems has rarely been studied, and results from available studies are mixed. For instance, grazing has been found to promote aggregation of closely related individuals thus increasing fine-scale genetic structure (Kleijn and Steinger, 2002), while the opposite effect has also been reported (Kloss *et al.*, 2011). Discrepancies between studies are likely related to differences in plant species traits, history of population colonization and land use history.

Landscape management by rotational grazing, which involved the shepherding of large flocks of sheep, has been found to increase plant species colonization rates after local extinctions in previously abandoned grassland patches, irrespective of morphological adaptations to zoochory (Rico *et al.*, 2012; Rico *et al.*, 2014a). Genetic theory predicts that if populations are founded by numerous and unrelated colonists from varied sources, the probability of developing SGS would be low compared with the situation where only a few seed sources provided the initial colonists (Slatkin, 1987; Whitlock and McCauley, 1990). Colonization sets up the primary template of SGS on which seed dispersal and pollen flow will further act (Chung *et al.*, 2003; Trapnell *et al.*, 2013). Moreover, colonization success of restored habitats for establishing long-term persistent plant populations might largely depend on their initial genetic make up such as degree of genetic diversity and SGS (Whitlock and McCauley, 1990; Mijangos *et al.*, 2015).

In this study, we investigated the effect of rotational sheep grazing on the strength of SGS within plant populations of recently restored calcareous grasslands in southern Germany. We studied the perennial herb and specialist of calcareous grasslands *Dianthus carthusianorum* (Oberdorfer, 1978), which lacks seed-dispersal traits related to zoochory or anemochory, and does not reproduce vegetatively (Klotz *et al.*, 2002). This landscape-scale network of calcareous grassland patches is an ideal empirical system because it allows us to investigate the intensity of SGS across multiple plant populations within the same landscape and with known management records for all analyzed patches, which previously has not been done. Evaluating if landscape management by rotational sheep grazing can modify the development of SGS in previously fragmented plant populations is relevant to better inform conservation planning. Previous work in the study area for *D. carthusianorum* found that gene flow at the landscape scale and genetic diversity within populations were associated with connectivity facilitated by rotational sheep grazing (Rico *et al.*, 2014a,b). Here, we therefore ask the following: (i) does rotational sheep grazing have a significant effect on patterns of SGS at the local scale, within grassland patches? (ii) Does this effect depend on population size? For grazed grasslands, we further compare SGS and genetic diversity between recently colonized patches (since 1989) and populations

existing before the implementation of the ecological network in our study area. Specifically, we ask (iii) do colonized populations have lower genetic diversity than pre-existing ones, indicating a recent founder event? (iv) Do recently colonized populations exhibit stronger patterns of SGS relative to pre-existing populations?

MATERIALS AND METHODS
Study landscape and species
The study was conducted in the Southern Franconian Alb near Weissenburg, Bavaria, Germany covering an area of $\sim 10 \times 15$ km. Calcareous grasslands of the Gentiano-Koelerietum pyramidatae vegetation association (Oberdorfer, 1978) are mainly located on the steep slopes between the Upper Franconia Jura plateau and the valleys. In our study area, management of calcareous grasslands through rotational sheep grazing was initiated in 1989 to reconnect grassland patches that had been abandoned at least since 1960 with grasslands where grazing had been continued throughout the 20th century (core areas; Wagner *et al.*, 2013). Flocks of 400–800 ewes with their lambs are herded along defined routes in three non-overlapping shepherding systems. Since the start of the project in 1989, out of 62 previously abandoned grassland patches, 26 were subjected to grazing three to five times annually throughout the season, 13 were grazed only toward the end of the season or only during a few years at the beginning of the project, whereas 23 remained ungrazed (Rico *et al.*, 2012). In the study area, sheep are kept in designated paddocks for rumination as prescribed by calcareous grassland conservation management. Consumed seeds are thus unlikely to be deposited in grassland patches, so that dispersal might depend mostly on epizoochory (Rico *et al.*, 2014b).

D. carthusianorum (Caryophyllaceae) is a perennial herb of 30–45 cm in height. It is predominantly outcrossing and does not form a persistent seed bank (Bloch *et al.*, 2005). According to Hensen (1997), *D. carthusianorum* reproduces sexually, and its clonal growth is of the 'phalanx' type that lacks lateral spread. Klotz *et al.* (2002) specify the clonal growth form as 'rhizome-like pleiocorm', where adventitiously rooted shoots may lose their connection with the primary roots. Flowering occurs from June to October and pollination is carried out by specialized Lepidoptera species (Bloch *et al.*, 2005). The seeds lack morphological adaptations to dispersal by wind or animals (Klotz *et al.*, 2002).

Sampling and laboratory procedures
Detailed sampling information and microsatellite genotype data at 11 polymorphic loci are described in Rico *et al.*, 2014a,b (available on DRYAD doi:10.5061/dryad.jn1v7). In brief, we collected leaf material from all adult plants in patches with less than 40 individuals (small populations), whereas 30–40 adult plants were sampled from patches with more than 40 individuals (large populations). The latter implied that sampling was spread out within patches of large populations. During the field sampling collection, we did not observe differences in the density or spatial aggregation of individuals between small and large populations. To maintain comparability, we excluded patches with <10 individuals, one patch with a minimum distance between neighboring individuals >10 m, and four patches that were grazed only during a few years but remained ungrazed for the last 15 years. Geographic coordinates of each sampled individual were recorded using a Trimble GeoXT 2008 (Westminster, CO, USA) GPS receiver with sub-meter resolution based on differential GPS post-processing. The final data set included the genotypes of 1304 adult individuals of 49 patches (referred here as populations; Figure 1).

Quantification of SGS and genetic diversity
To evaluate the patterns of SGS, we calculated a genetic distance matrix using the multilocus measure of the pairwise kinship coefficient F_{ij} of Ritland (1996), which included all individual pairwise comparisons in the whole-data-set without specifying spatial distance classes or groups, as implemented in SPAGEDI (Hardy and Vekemans, 2002). The F_{ij} coefficient measures the probability that two alleles are identical by descent. Negative values of F_{ij} can occur if allele frequencies between two compared individuals differ more than expected at random from the entire data set. To assess the strength of SGS within each patch, we estimated the Sp statistic of Vekemans and Hardy (2004). The Sp statistic has been widely applied in empirical studies of SGS since it fits a linear model that estimates SGS under isolation by distance. The Sp statistic is
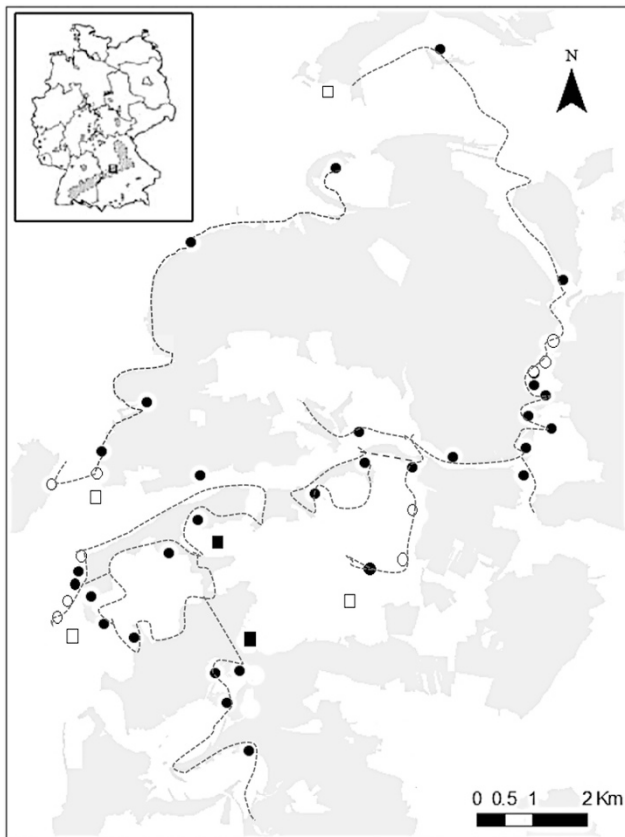
Figure 1 Schematic representation of the spatial configuration of patches analyzed for SGS in *D. carthusianorum*. Black circles denote larger populations and white circles correspond to small populations (<40 individuals). Dashed lines indicate rotational sheep grazing routes connecting calcareous grassland patches in three non-overlapping grazing systems. Ungrazed populations are showed as white (small populations) or black (large populations) squares and without connecting lines. Gray shaded areas in the background show forested areas. The inset map shows the distribution of calcareous grasslands (gray areas) in Germany and the location of the study area (map modified from Beinlich and Plachter, 1995).

very robust to sampling scheme and definitions of distance classes, which allows comparisons among plots and among species (Vekemans and Hardy, 2004). If F_{ij} decrease linearly with the logarithm of distance, the strength of SGS can be quantified by the Sp statistic as $b_F/(1-F_1)$, where b_F represents the regression slope of the pairwise F_{ij} relatedness coefficients and F_1 is the mean F_{ij} between pairs of individuals at the shortest distance class (Vekemans and Hardy, 2004). Since we have differences in the sampling of individuals between small and large populations, distance classes were not defined per patch, instead F_1 was defined as the regression intercept, that is, the value where the logarithm of distance is 0, which corresponds to the value at a distance of 1 m. Here, F_1 represents the expected degree of the relatedness coefficient between individuals 1 m distance apart. Higher values of the Sp statistic indicate a high rate of distance-decay of kinship. Significance of the regression slope in each population was tested by permuting the pairwise F_{ij} coefficients within populations 1000 times. Analyses were implemented using R (R CoreTeam, 2014). Genetic diversity indices including allelic richness (A_r), observed (H_o) and unbiased expected heterozygosity (H_e) were estimated for each population using GENALEX (Peakall and Smouse, 2012). We corrected the measures of allelic richness for the effect of sample size using the rarefaction procedure in HP-RARE (Kalinowski, 2005). F_{ST} indices of genetic differentiation among populations within patches of the same type (for example, colonized vs pre-existing) and their s.d. were estimated by jackknifing loci over populations using SPAGEDI (Hardy and Vekemans, 2002).

Statistical analysis

We used a two-way analysis of variance (ANOVA) to test for main effects and interaction of the two factors: grazing (two classes: grazed and ungrazed) and population size (two ordinal classes: <40 individuals vs ⩾40 individuals) on each of five response variables: the three genetic diversity indices (A_r, H_o and H_e) and the F_1 and Sp statistics. To account for an unbalanced design, we used type II sums of squares. To normalize the distribution of residuals, we applied square-root transformation to F_1 and Sp statistic, while genetic diversity indices were not transformed. Negative values of the Sp statistic were set to zero. Model assumptions of normal distribution of residuals and homogeneity of variances were visually checked. In addition, we tested if there were differences in the degree of spatial isolation between ungrazed vs grazed patches using the Welch two-sample *t*-test of a S_i patch connectivity index estimated in Rico *et al.* (2012).

Since population history (that is, presence of *D. carthusianorum* before reintroduction of sheepherding 1989) is not confirmed for all core patches, statistical analysis of population history was only performed for populations with known history: populations present at 1989 survey (pre-existing populations, $n = 7$) and new populations (colonized patches, $n = 9$) which were absent in 1989 but present in the 2009 survey. Owing to reduced sample size, population history was analyzed separately but not included as an additional factor in the two-way ANOVA with the factors grazing and population size. All statistical analyses were performed in R (R CoreTeam, 2014).

RESULTS

Strength of SGS

SGS in *D. carthusianorum* was significant in 26 out of 49 populations (Table 1). The pairwise kinship coefficient F_{ij} showed a distance decay in all treatment groups (Supplementary Figure S1). The strength of SGS as quantified by the Sp statistic was significantly related to both grazing and population size, and without a significant interaction (two-way ANOVA: sheep grazing: $F_{1,45} = 4.74$, $P = 0.035$; population size: $F_{1,45} = 4.28$, $P = 0.044$; and interaction: $F_{1,45} = 0.003$, $P = 0.954$). Grazed populations showed significantly weaker SGS than ungrazed populations (that is, lower Sp values), and independent of grazing, small populations showed significantly higher Sp values than large populations (Figure 2). Moreover, the regression slopes b_F of all ungrazed patches, independent of population size, were significant, while for grazed grasslands, small populations showed a higher number of significant cases of SGS than large populations (small: 6 out of 11, large: 13 out of 32 populations; Table 1).

There were marginally significant effects of grazing ($F_{1,45} = 3.47$, $P = 0.068$) and no significant effects on population size ($F_{1,45} = 1.87$, $P = 0.17$) on F_1, without significant interaction ($F_{1,45} = 0.15$, $P = 0.701$). F_1 values varied across populations (Table 2) and overall, F_1 mean values were small for all treatment groups (large grazed: $F_1 = 0.027$, small grazed: $F_1 = 0.040$, small ungrazed: $F_1 = 0.074$, large ungrazed: $F_1 = 0.048$), which indicated that the nearest neighbors (at 1 m distance) were not highly related. On the other hand, there were no significant differences in the degree of spatial isolation between populations of ungrazed and grazed grassland patches (df = 10.7, $t = 1.61$ and $P = 0.13$). None of the models showed unusual patterns in the residuals distribution or influential outliers.

Within grazed grasslands, the strength of SGS (Sp statistic) was higher on average in pre-existing populations relative to colonized populations (Figure 3), but the difference was not statistically significant (ANOVA, $F_{1,14} = 0.7$, $P = 0.416$). The difference remained non-significant when accounting for population size (two-way ANOVA: population history $F_{1,12} = 4.03$, $P = 0.067$; population size: $F_{1,12} = 2.7$, $P = 0.127$; interaction: $F_{1,12} = 1.26$, $P = 0.282$). Similarly, F_1 values were not statistically significantly different between pre-existing and colonized populations (ANOVA, $F_{1,14} = 3.09$, $P = 0.587$).

Table 1 Estimates of spatial genetic structure SGS across 49 populations of *D. carthusianorum*

Population	Minimum distance	N	Connectivity index S_i	Grazing	Population size	Population history	F_1	Slope b_F	Sp	P-value
A03	0.3	29	0.943	Grazed	Small	Colonized	0.064	−0.0126	0.0135	**0.001**
A05	0.3	26	1.497	Grazed	Small	Colonized	0.054	−0.0127	0.0134	**0.001**
A08	0.5	23	1.599	Grazed	Small	Colonized	0.011	0.0003	−0.0003	0.564
A26	0.3	26	2.346	Grazed	Small	Colonized	0.032	−0.0062	0.0064	**0.026**
A31	0.3	23	2.773	Grazed	Small	Colonized	0.050	−0.0099	0.0104	**0.001**
A33	0.4	35	1.805	Grazed	Small	Colonized	0.097	−0.0180	0.0200	**0.002**
A45	0.7	15	1.262	Grazed	Small	Colonized	0.021	−0.0020	0.0021	0.272
C08	0.3	30	0.819	Grazed	Small	Unknown	0.025	−0.0015	0.0015	0.203
A12	0.3	12	1.325	Grazed	Small	Pre-existing	0.055	−0.0150	0.0159	0.148
A25	0.5	15	1.572	Grazed	Small	Pre-existing	0.103	−0.0444	0.0495	**0.001**
E07	2.2	15	0.092	Grazed	Small	Pre-existing	−0.068	0.0328	−0.0307	0.91
A06	0.5	30	1.673	Grazed	Large	Colonized	0.038	−0.0100	0.0104	**0.017**
A07a	2.0	30	1.207	Grazed	Large	Colonized	0.008	0.0019	−0.0019	0.678
N05	0.5	28	0.119	Grazed	Large	Colonized	0.021	−0.0016	0.0016	0.334
A14	0.4	16	1.714	Grazed	Large	Pre-existing	0.128	−0.0346	0.0397	**0.001**
A18	1.2	29	1.560	Grazed	Large	Pre-existing	0.081	−0.0125	0.0136	**0.005**
A28	0.4	33	2.574	Grazed	Large	Pre-existing	0.014	0.0004	−0.0004	0.558
A25	0.5	15	1.572	Grazed	Small	Pre-existing	0.103	−0.0444	0.0495	**0.001**
A29	0.8	28	2.644	Grazed	Large	Pre-existing	0.012	−0.0034	0.0034	**0.012**
A38	2.4	30	1.041	Grazed	Large	Pre-existing	0.010	−0.0006	0.0006	0.456
E01	0.3	36	0.456	Grazed	Large	Unknown	0.040	−0.0047	0.0049	**0.004**
G01	0.6	29	0.322	Grazed	Large	Unknown	0.051	−0.0051	0.0053	0.11
G13	0.3	41	0.092	Grazed	Large	Unknown	0.014	−0.0010	0.0011	0.363
G16	1.4	29	0.904	Grazed	Large	Unknown	0.018	−0.0038	0.0038	**0.01**
G20	1.8	30	0.556	Grazed	Large	Unknown	0.006	−0.0001	0.0001	0.477
G21	0.3	30	0.895	Grazed	Large	Unknown	0.034	−0.0064	0.0066	**0.001**
G23	2.8	28	0.905	Grazed	Large	Unknown	0.065	−0.0104	0.0112	**0.001**
G26	0.6	38	1.586	Grazed	Large	Unknown	0.026	−0.0039	0.0040	**0.015**
G28	0.3	30	0.790	Grazed	Large	Unknown	0.002	0.0000	0.0000	0.535
G29	3.3	30	1.447	Grazed	Large	Unknown	−0.007	0.0039	−0.0039	0.808
G30	0.3	30	1.098	Grazed	Large	Unknown	0.036	−0.0079	0.0082	**0.005**
G31	0.7	30	1.411	Grazed	Large	Unknown	−0.002	0.0015	−0.0015	0.786
G37	0.3	27	1.322	Grazed	Large	Unknown	−0.011	0.0028	−0.0028	0.831
G40	0.3	31	0.716	Grazed	Large	Unknown	0.003	0.0004	−0.0004	0.593
G45	5.2	31	0.646	Grazed	Large	Unknown	0.009	−0.0001	−0.0001	0.523
G46	0.3	30	1.193	Grazed	Large	Unknown	0.002	−0.0004	0.0004	0.432
G47	2.0	28	1.426	Grazed	Large	Unknown	−0.001	0.0007	−0.0007	0.589
G48	0.3	29	1.340	Grazed	Large	Unknown	0.028	−0.0048	0.0050	0.056
G49	0.5	30	1.269	Grazed	Large	Unknown	0.019	−0.0014	0.0014	0.343
G50	0.3	28	1.030	Grazed	Large	Unknown	0.085	−0.0235	0.0257	**0.001**
G5a	0.8	27	1.036	Grazed	Large	Unknown	0.047	−0.0060	0.0063	0.163
Gzim	0.3	30	0.211	Grazed	Large	Unknown	0.017	−0.0026	0.0027	**0.041**
N03	0.3	29	0.302	Grazed	Large	Unknown	0.044	−0.0104	0.0109	**0.001**
E03	0.3	15	0.941	Ungrazed	Small	Unknown	0.091	−0.0166	0.0182	**0.001**
E04	0.3	30	0.421	Ungrazed	Small	Unknown	0.052	−0.0179	0.0189	**0.005**
E09	0.3	32	1.338	Ungrazed	Small	Unknown	0.047	−0.0083	0.0087	**0.001**
Nroth	0.4	14	0.573	Ungrazed	Small	Unknown	0.106	−0.0362	0.0404	**0.001**
A37	0.3	31	1.205	Ungrazed	Large	Colonized	0.067	−0.0121	0.0130	**0.001**
N10	0.3	25	0.733	Ungrazed	Large	Unknown	0.030	−0.0103	0.0106	**0.001**

Abbreviations: F_1, kinship coefficient at ln(distance = 1 m); N, number of individuals genotyped. slope (b_F) of the regression kinship coefficient on ln(distance); Sp statistic reflecting the intensity of SGS; larger values of S_i indicates higher connectivity. Significant P-values ($\alpha = 0.05$) of the regression slopes in bold.

Estimates of genetic diversity

Average estimates of genetic diversity tended to be higher in large and small populations of grazed patches relative to large and small populations of ungrazed grasslands (except for H_o in ungrazed-small vs grazed-small), with the largest difference for mean allelic richness (Table 2). ANOVA showed that both sheep grazing and population size had a significant effect only on mean allelic richness, and without significant interaction between factors (two-way ANOVA: sheep grazing: $F_{1,44} = 7.09$, $P = 0.011$; population size: $F_{1,44} = 6.3$, $P = 0.016$; and interaction: $F_{1,44} = 1.28$, $P = 0.263$). No significant differences were observed for H_o (two-way ANOVA: sheep grazing: $F_{1,45} = 0.48$, $P = 0.492$; and population size: $F_{1,45} = 3.2$, $P = 0.081$) and neither for H_e (two-way ANOVA: sheep grazing: $F_{1,45} = 0.08$, $P = 0.772$; and population size: $F_{1,45} = 0.36$, $P = 0.546$). The F_{ST} index was highest for
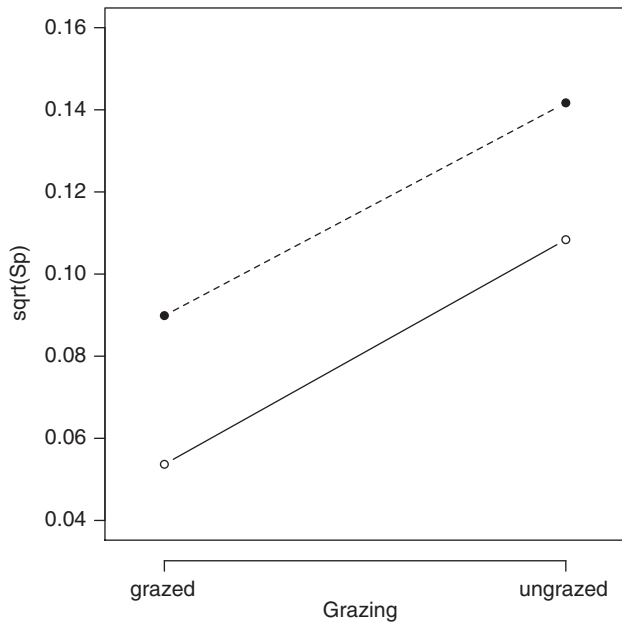
Figure 2 Interaction plot of the mean square-root transformed Sp statistic as a function of the factors grazing and population size in populations of *D. carthusianorum*. Both factors were statistically different and without an interaction. The dashed line indicates small population size ($F_1,45 = 4.74$, $P = 0.035$) and the full-line large population size ($F_1,45 = 4.28$, $P = 0.044$).

Table 2 Average values of the F_{ij} coefficient between pairs of individuals at the shortest distance class F_1 and Sp statistic in six treatment groups in *D. carthusiaromum* populations and with s.d.

Group	N	F_1 (s.d.)	Sp (s.d.)
Ungrazed-small	4	0.074 ± 0.029	0.022 ± 0.013
Ungrazed-large	2	0.049 ± 0.026	0.012 ± 0.002
Grazed-small	11	0.051 ± 0.033	0.015 ± 0.014
Grazed-large	32	0.028 ± 0.024	0.005 ± 0.006
Colonized	9	0.042 ± 0.028	0.08 ± 0.007
Pre-existing	7	0.058 ± 0.048	0.018 ± 0.02

small, ungrazed populations ($F_{ST} = 0.054$), whereas the lowest value was for large, grazed populations ($F_{ST} = 0.018$).

Colonized populations had similar values of H_o (0.53), H_e (0.59) and mean A_r (4.3) than pre-existing populations ($H_o = 0.54$, $H_e = 0.53$ and $A_r = 4.4$), and thus no significant differences were found (ANOVA: A_r: df = 2, $F = 0.98$, $P = 0.395$; H_o: df = 2, $F = 1.2$, $P = 0.326$; and H_e: df = 2, $F = 2.23$, $P = 0.139$). The F_{ST} was higher in colonized populations relative to pre-existing populations, but this difference was not large (Table 3).

DISCUSSION

We tested, within an empirical landscape system, the effects of rotational sheep grazing on the strength of SGS across multiple populations of a habitat specialist plant of calcareous grasslands. Our results showed that *D. carthusianorum* populations under rotational sheep grazing showed significantly weaker SGS than populations of ungrazed grasslands. Moreover, our results showed that recently colonized populations of grazed grasslands did not show stronger patterns of SGS nor lower genetic diversity than pre-existing

populations of grazed grasslands. Together these results suggest that rotational sheep grazing not only increases landscape-scale genetic connectivity of *D. carthusianorum* among patches (Rico et al., 2014b) but also plays an important role in reducing SGS in previously abandoned calcareous grasslands, likely by increasing the overlap of seed shadows. However, small plant populations showed significantly stronger SGS relative to large populations, and this effect was independent of grazing.

SGS and grazing effects

Our results are consistent with previous empirical evidence showing that species dispersed by zoochory (but mostly frugivores) exhibit weak SGS, which might be explained by effective seed dispersal over larger distances (Degen et al., 2001; Hamrick and Trapnell, 2011; Wang et al., 2011). Rico et al. (2014b) showed that rotational sheep grazing is an important determinant of spatial genetic structure at the landscape scale in *D. carthusianorum*, which was associated with long-distance seed dispersal among populations connected through the grazing route. Here, we demonstrated that rotational sheep grazing can as well influence spatial patterns of fine-scale genetic structure, even for a species lacking morphological adaptations to zoochory.

Few empirical studies have investigated the effects of grazing on genetic diversity and genetic structure at local spatial scales. For *Veronica chamaedrys*, Kloss et al. (2011) found that genetic structure was influenced by land use type, where populations in grazed grasslands showed lower genetic structure compared with mown pastures and meadows, but no effects on genetic diversity. For *Veratrum album*, Kleijn and Steinger (2002) also did not find any effect of grazing on genetic diversity within populations, but found that grazing increased the spatial aggregation of related genotypes relative to the strength of SGS of plant populations in meadows. The significant pattern of SGS in *V. album* for grazed grasslands was related to an enhancement of clonal growth by grazing and lower seedling recruitment (Kleijn and Steinger, 2002). In contrast to these studies, our results for *D. carthusianorum* indicate that landscape management by rotational sheep grazing influenced both genetic diversity and fine-scale genetic structure in small and large plant populations of previously abandoned grasslands. Formation of SGS in *D. carthusianorum* cannot be explained by vegetative propagation, as duplicated genotypes were practically absent (approximately <10 individuals). Moreover, the Sp values in *D. carthusianorum* fall within the range of values expected for outcrossing species: observed values ranged from 0.005 to 0.022, similar to Sp values expected for outcrossing species (Sp: 0.013), relative to higher values expected for mixed mating systems (Sp: 0.037; Vekemans and Hardy, 2004).

We did not observe significant effects on F_1 values for grazing or population size, although both small and large populations of ungrazed grasslands showed higher F_1 values relative to grazed grasslands. Significant differences in Sp but not in F_1 (mean F_{ij} value at 1 m distance) between grazed and ungrazed patches, might indicate that patterns of SGS in *D. carthusianorum* are largely determined by seed dispersal, while pollen-mediated gene flow is likely less restricted (Sork, 2016). While all populations of ungrazed patches showed significant patterns of SGS, only 42% of populations in grazed patches showed significant SGS. There are two likely mechanisms by which grazing can reduce patterns of SGS: seed dispersal (Fischer et al., 1996; Manzano and Malo, 2010) and small-scale disturbances (Adler et al., 2001; Faust et al., 2011). For instance, local disturbances from grazing can reduce SGS by trampling and grubbing (Adler et al., 2001) creating thinning of vegetation that promotes recruitment (Chung et al., 2003). In the case of dispersal, sheep can increase the variance of
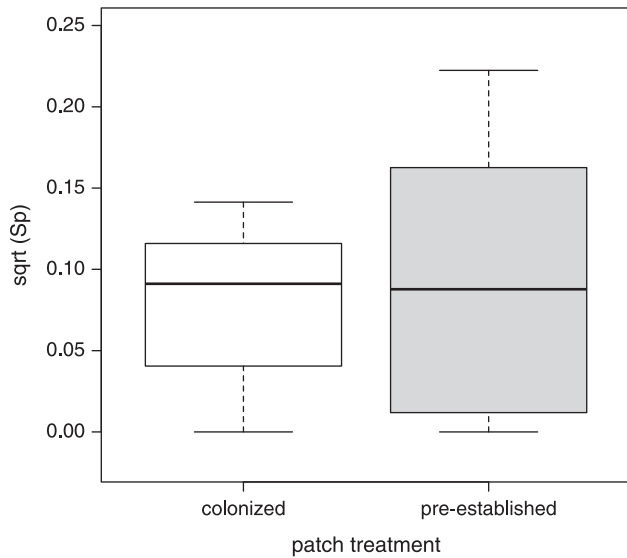
Figure 3 Boxplot of the strength of SGS quantified using the Sp statistics between colonized (mean Sp: 0.015) and pre-existing populations (mean Sp: 0.01) of grazed calcareous grasslands. There were no significant differences between groups ($F = 0.7$, $P = 0.416$).

Table 3 Summary of genetic diversity measures and fixation index of population differentiation (F_{ST}) in six treatment groups of *D. carthusianorum* populations

Group	H_o (s.d.)	H_e (s.d.)	A_r (s.d.)	F_{ST} (s.d.)
Ungrazed-small	0.53 ± 0.05	0.56 ± 0.04	4.11 ± 0.28	0.057 ± 0.02
Ungrazed-large	0.50 ± 0.003	0.56 ± 0.02	4.02 ± 0.08	0.032 ± 0.01
Grazed-small	0.52 ± 0.04	0.58 ± 0.02	4.21 ± 0.22	0.041 ± 0.00
Grazed-large	0.54 ± 0.02	0.59 ± 0.03	4.44 ± 0.23	0.018 ± 0.00
Colonized	0.53 ± 0.03	0.59 ± 0.02	4.29 ± 0.20	0.03 ± 0.004
Pre-existing	0.54 ± 0.04	0.59 ± 0.02	4.38 ± 0.31	0.04 ± 0.009

Abbreviations: A_r, allelic richness; H_o, observed heterozygosity; H_e, expected heterozygosity; N, sample size; s.d., standard deviation of the mean.

dispersal distances by attachment to the fur or hooves of sheep (Couvreur *et al.*, 2004). This will lead to a greater overlap of seed shadows from different plants within fragments compared with ungrazed grasslands (Hamrick and Trapnell, 2011).

SGS and population size effects

Population size is another important factor modifying SGS within populations. We observed that independent of grazing, small populations (<40 individuals) had significantly stronger SGS than large populations. For instance, within grazed grasslands, small populations exhibited stronger SGS and had higher proportions of cases (55%) of significant SGS than large grazed populations (40%). In small populations, which experience high rates of genetic drift, development of SGS can be intensified due to increased mating between relatives thus reducing N_e, which has been observed in small isolated populations in fragmented landscapes (for example, O'Connell *et al.*, 2006; Mimura and Aitken, 2007). Low plant density, which in turn could lead to a reduced overlap of seed shadows, is expected to influence the development of SGS (Hamrick and Trapnell, 2011). However, recent evidence showed that individual spatial aggregation instead of plant density is more relevant for the development of SGS

(Lara-Romero *et al.*, 2016). We do not have data on plant density to analyze any potential effects on SGS in small vs large populations. Here, we observed that small populations of grazed grasslands had significantly lower SGS and higher genetic diversity than small populations in ungrazed grasslands. Previous evidence showed that mean allelic richness in *D. carthusianorum* populations was positively correlated with patch connectivity through rotational shepherding, where ungrazed patches had lower allelic richness (Rico *et al.*, 2014b). The increase of seed mixing from varied sources facilitated by rotational sheep grazing can influence levels of genetic diversity within populations, and this effect could be expected more relevant for small populations than spatial isolation *per se*. Moreover, F_{ST} estimates of genetic differentiation were the highest among small, ungrazed grasslands. Overall our results suggest that gene flow was less restricted among grazed grasslands, which in turn would increase the effective size of populations (N_e).

SGS and population history

In accordance with our expectations, we did not find differences in the strength of SGS and genetic diversity between recently colonized and pre-existing populations of grazed patches. Restoration of calcareous grasslands by rotational shepherding since 1989 promoted colonization by diverse plant species in numerous grasslands patches (Rico *et al.*, 2012). Before the implementation of the landscape management project, *D. carthusianorum* occurred in 44% of 62 previously abandoned patches and in more than 90% of the core areas (Boehmer *et al.*, 1990). An evaluation survey in 2009 showed that successful colonizations increased the species' occurrence to 82% in previously abandoned patches, whereas occurrence in ungrazed patches remained at 38%. Our results provide insights in the colonization process of previously abandoned grasslands after reintroduction of rotational sheepherding in the study area. Theoretical genetic models of colonization predict that genetic diversity will be low and genetic differentiation among recently colonized populations will be high if populations are founded by colonists from few sources (propagule pool model). On the other hand, high genetic diversity and low genetic differentiation will occur if colonists are from diverse seed sources (migrant pool model; Whitlock and McCauley, 1990; Pannell and Dorken, 2006). The lack of significant differences in SGS, values of genetic diversity and F_{ST} estimates of genetic differentiation between recently colonized and pre-existing populations in grazed grasslands, suggest that population founders likely came from varied sources. A migrant pool model of colonization likely associated with seed dispersal by sheep has also been reported for the calcareous grassland forb *Anthyllis vulneraria* (Helsen *et al.*, 2015). Empirical studies in expanding populations have found that the strength of SGS can decrease as populations mature due to seedling mortality during the establishment process, and by competition within individuals of the same cohort (Chung *et al.*, 2003; Tero *et al.*, 2005). However, the opposite has also been reported as the result of restricted seed dispersal and aggregation of juveniles around the mother plant (Chung *et al.*, 2011). Unfortunately, we were unable to test the effect of population size on population history because most of colonized populations were small, while many pre-existing populations were large.

Spatially restricted gene flow at the landscape and local scales in outcrossing plant species can negatively increase homozygosity, which in turn affects individual fitness and the viability of populations in the long run (Leblois *et al.*, 2006). These effects are predicted to become stronger as populations become smaller (Keller and Waller, 2002). Although we observed low levels of inbreeding in *D. carthusianorum*,

small populations exhibited higher inbreeding relative to large populations (Rico *et al.*, 2014a). Small populations should be important targets in conservation management as high rates of genetic drift can considerably compromise the adaptive potential of populations to persist under ongoing climatic changes (Eizaguirre and Baltazar-Soares, 2014). Our results have important implications for landscape management and restoration as we found a positive effect of rotational shepherding in terms of a significant reduction of SGS across plant populations in previously abandoned grasslands. Importantly, this effect is more relevant for small populations because a decrease of SGS would tend to increase N_e and in turn reduce the negative effects of genetic drift on levels of genetic diversity. Our study highlights the effectiveness of landscape management by rotational sheep grazing to modify spatial patterns of genetic structure at local scales within plant populations of restored grasslands. Further investigations should establish whether the observed effects of grazing on fine-scale SGS are consistent across a range of typical grassland species.

DATA ARCHIVING

Population and individual microsatellite genotype data available on Dryad doi:10.5061/dryad.jn1v7.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

YR: conception and design of research, field work, molecular genetic lab work, statistical analysis, main writing of paper and editing. HHW: conception and design of research, field work, statistical analysis and editing of manuscript.

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