Directed dispersal by rotational shepherding supports landscape genetic connectivity in a calcareous grassland plant

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Abstract

Directed dispersal by animal vectors has been found to have large effects on the structure and dynamics of plant populations adapted to frugivory. Yet, empirical data are lacking on the potential of directed dispersal by rotational grazing of domestic animals to mediate gene flow across the landscape. Here, we investigated the potential effect of large-flock shepherding on landscape-scale genetic structure in the calcareous grassland plant *Dianthus carthusianorum*, whose seeds lack morphological adaptations to dispersal to animals or wind. We found a significant pattern of genetic structure differentiating population within grazed patches of three nonoverlapping shepherding systems and populations of ungrazed patches. Among ungrazed patches, we found a strong and significant effect of isolation by distance ($r = 0.56$). In contrast, genetic distance between grazed patches within the same herding system was unrelated to geographical distance but significantly related to distance along shepherding routes ($r = 0.44$). This latter effect of connectivity along shepherding routes suggests that gene flow is spatially restricted occurring mostly between adjacent populations. While this study used nuclear markers that integrate gene flow by pollen and seed, the significant difference in the genetic structure between ungrazed patches and patches connected by large-flock shepherding indicates the potential of directed seed dispersal by sheep across the landscape.

Keywords: calcareous grasslands, *Dianthus carthusianorum*, gene flow, Germany, landscape genetics, nuclear microsatellites, zoochory

Received 16 April 2013; revision received 5 December 2013; accepted 15 December 2013

Introduction

In plants, gene flow occurs through the dispersal of seeds and pollen. At the landscape scale, pollen flow is often expected to contribute to genetic connectivity more substantially than seed-mediated gene flow because pollen may travel longer distances and in larger numbers than seeds (Ellstrand 1992; Ennos 1994; McCauley 1997; Cruse-Sanders & Hamrick 2004; Petit

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et al. 2005). However, evidence from a growing number of studies suggests that seed-mediated gene flow might be as important as pollen-mediated gene flow for maintaining population genetic connectivity across the landscape (Cruse-Sanders & Hamrick 2004; Bacles *et al.* 2006; Jordano *et al.* 2007; Freeland *et al.* 2012).

Plants have a large variety of seed dispersal vectors that affect the distance, direction and destination where seeds are deposited away from the source (Schupp *et al.* 2011). For instance, wind turbulences can transport seeds over large distances (Tackenberg 2003; Soons *et al.* 2004), but rates of seed deposition in sites suitable for

seedling establishment are low because of the more stochastic process of wind dispersal (Nathan 2006). Seed dispersal by animal vectors (i.e. zoochory) is often nonrandom (Kollmann 2000; Spiegel & Nathan 2007), and some animal vectors can transport seeds in considerable numbers to sites of high probability of establishment relative to unsuitable sites (Howe & Smallwood 1982; Wenny 2001). This process known as directed dispersal (Wenny 2001) has been shown to influence the spatial structure of plant populations (Aukema & Del Rio 2002; Purves & Dushoff 2005; Briggs *et al.* 2009). However, less evidence has been collected on its effects on spatial patterns of genetic connectivity at the landscape scale. Most empirical studies have focused on species dispersed via ingestion by birds and mammals (i.e. endozoochory), especially trees and shrubs adapted to frugivory (Godoy & Jordano 2001; Jordano *et al.* 2007). A majority of these studies found that, even in the presence of extensive pollen flow, directed dispersal may generate strong spatial genetic structure by the aggregated deposition of genetically related seeds (Grivet *et al.* 2005; Garcia *et al.* 2007, 2009; Torimaru *et al.* 2007). The opposite pattern has also been found, where recurrent directed long-distance dispersal by frugivores over time homogenized spatial genetic variation (Karubian *et al.* 2010). These inconsistent results of different studies reflect the complexity of spatial patterns of gene flow resulting from the behaviour of animal vectors and their interaction with the landscape.

In the case of adaptation to frugivory, plants invest in the production of fruits that are attractive to their animal vectors, and animals deliberately consume these fruits (Lorts *et al.* 2008). In contrast, animals such as ungulates may inadvertently transport numerous seeds by attachment to their fur or hooves, or if seeds are ingested by chance during foraging (Janzen 1984). In agricultural landscapes, rotational grazing by domestic ungulates such as large flocks of horses, sheep or cattle may increase the chances of successful dispersal to suitable sites because animals move in a directed way between patches of the same habitat type, and seeds can remain attached to the animal fur for extended periods of time travelling long distances (Bruun & Poschlod 2006; Manzano & Malo 2006; Iravani *et al.* 2011). Calcareous grasslands in Central Europe are semi-natural and species-rich communities, which in many regions have traditionally been used for sheep grazing (Willems 1990). Abandonment of traditional shepherding in the early 20th century resulted in a severe loss of calcareous grasslands and species decline in remnant fragments (Poschlod & WallisDeVries 2002; WallisDeVries *et al.* 2002). Calcareous grasslands are nutrient poor, unfertilized and free of herbicides and pesticide application. They represent important refugia where many specialist plant species can establish within an otherwise intensively used agricultural landscape (Green 1972).

In the Southern Franconian Alb in Germany, restoration of calcareous grassland connectivity by re-introduction of rotational shepherding led to a significant increase in species richness of habitat specialist plants relative to patches that remained ungrazed (Wagner *et al.* 2013). Analysis of patch occupancy data showed that dispersal along shepherding routes and the diversity of microsites promoting establishment in focal patches were important predictors of patch occupancy for multiple species regardless of adaptations to zoochory (Rico *et al.* 2013). However, whether rotational shepherding connecting geographically fragmented calcareous grasslands also influences spatial genetic structure has not yet been shown.

In this study, we assess the effect of directed dispersal by large-flock rotational shepherding on landscape-scale patterns of genetic structure in the calcareous grassland herb *Dianthus carthusianorum*. Most calcareous grasslands in the study area are grazed by large flocks of sheep (approximately 400–800 ewes and their lambs) in three nonoverlapping rotational shepherding systems. If the effect of landscape connectivity by rotational shepherding on gene flow is considerable, we would expect that (i) landscape-scale spatial patterns of genetic structure are associated with shepherding systems. In addition and by comparing genetic distances of populations of grazed vs. ungrazed patches, we hypothesize that (ii) the genetic structure of ungrazed patches is explained by isolation by geographical distance (IBD) resulting from a lack of connectivity by shepherding, whereas (iii) for populations connected within the same shepherding system, population genetic structure should be explained by landscape connectivity along shepherding routes.

Material and methods

Study area and species

The study area is located in the Southern Franconian Alb near Weissenburg, Bavaria Germany, and has an extent of approximately 10 × 15 km. In the area, calcareous grasslands of the Gentiano-Koelerietum pyramidatae vegetation association (Oberdorfer 1978) declined from 970 ha in 1830–302 ha by 1989 due to abandonment of traditional shepherding (Dolek & Geyer 2002). In 1989, a conservation project was started to reconnect calcareous grassland patches that had been abandoned since at least 1960 ('previously abandoned patches', $n = 62$), with grassland patches that remained grazed over time in local systems ('core areas', $n = 34$). Since

approximately 1990, sheep flocks are herded in both directions following predefined routes in three nonoverlapping shepherding systems (Fig. 1). The grazing season lasts from April until early November. Of the 62 previously abandoned calcareous grasslands in the study area, 26 have been grazed three to five times per year since 1989 (typically for less than a day each time, depending on patch area), 13 were only grazed later in the season or only for a few years, and the remaining 23 grasslands were not included in any of the three systems and thus remained ungrazed (Wagner *et al.* 2013). As required by local conservation agencies, sheep flocks do not stay within a grassland patch for more than 0.5–1 days (except for the largest patches) to avoid excessive grazing on patches.
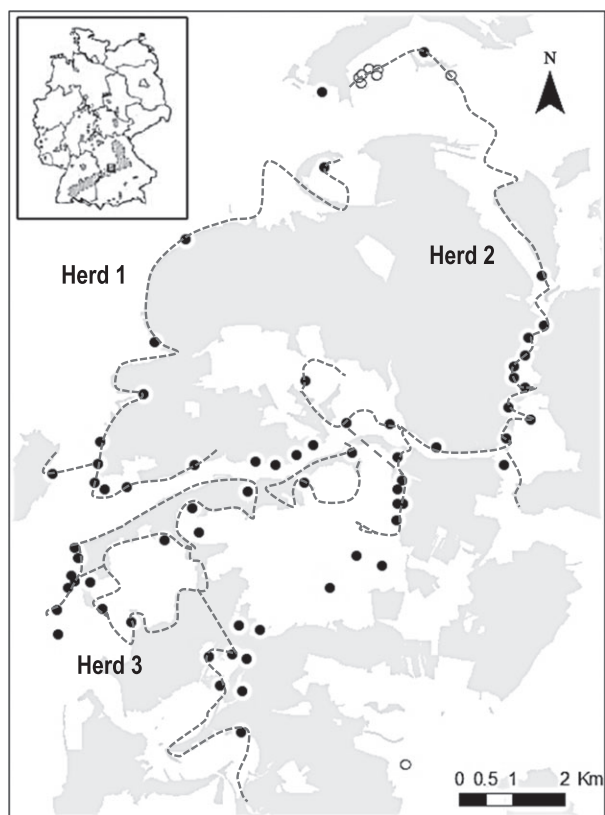

Fig. 1 Spatial distribution of calcareous grassland patches with occurrence of *Dianthus carthusianorum* in the study area in the Southern Franconia Alb, Germany (*n* = 64). Grey shaded areas in the background map show forested areas, which are largely restricted to the Upper Jurassic plateau. Closed circles indicate sampled populations, and open circles denote unsampled populations. Dashed lines denote shepherding routes for each of the three nonoverlapping shepherding systems. The inset map shows the distribution of calcareous grasslands (grey areas) in Germany and the location of the study area (map modified from Beinlich and Plachter 1995).

Dianthus carthusianorum L. (Caryophyllaceae) is a perennial herbaceous habitat specialist of calcareous grasslands in the study area (Boehmer *et al.* 1990). The species is diploid, has a partially outcrossing mating system (Bloch *et al.* 2005) and has no persistent seed bank (Klotz *et al.* 2002). Flowering time is from May to October, and pollination is carried out by few specialized Lepidoptera species (Bloch *et al.* 2005). The species has no specialized adaptations to seed dispersal by wind or animals (Klotz *et al.* 2002). However, as release height is 30–35 cm and seed shed occurs throughout the main grazing period in the study area, seed dispersal is possible by attachment to the fur and hooves of sheep, or via ingestion and dung deposition. A previous analysis in *D. carthusianorum* in this study area suggested that both patch occupancy and genetic diversity are associated with rotational shepherding (Rico *et al.* 2013).

Sampling and microsatellite analysis

A total of 64 calcareous grassland patches with occurrences of *D. carthusianorum* (here considered as populations) were sampled, including core areas (*n* = 27), previously abandoned patches (*n* = 31) and grass verges along roads or forest edges (*n* = 6). This sample represented 100% of the known populations in the southern half of the study area, and 86% of the entire study area (Fig. 1). Where present, *D. carthusianorum* typically occurs in relatively low densities scattered throughout a patch. On the basis of previous plant species surveys of calcareous grasslands in our study system, we categorized the size of populations using four ordinal classes: 1 = 1–4 individuals, 2 = 5–39 individuals, 3 = 40–99 individuals and 4 = ≥100 individuals (Rico *et al.* 2013). In populations with <40 plants, we collected leaf material from all flowering individuals, whereas for larger populations, we sampled 30–40 flowering individuals evenly across each patch. Leaf tissue samples were immediately dried in silica gel.

Genomic DNA was extracted following the DNeasy 96 Plant kit protocol (QIAGEN). A total of 15 nuclear microsatellite loci for related *Dianthus* species were amplified (MS-DINMADSBOX, MS-DCDIA30, MS-DCAMCRBSY, MS-DINCARACC, DCA 221, DCD 224, DCB140; Smulders *et al.* 2000; DCB109, Smulders *et al.* 2003; CB018a, CB057a; CB004a, CB027a, CF003a, CB011a, CB020a; Kimura *et al.* 2009). Nuclear microsatellite amplifications were carried out with the QIAGEN multiplex kit as follows: 0.2–0.4 μL (5 μM) of each primer, 4.7 μL of master-mix and 5–10 ng of genomic DNA in a total reaction volume of 10 μL. PCR conditions followed those described in Smulders *et al.* (2003). We included a positive and negative control in each

96-well plate to check for contamination, and approximately 20% of the total sample sets were amplified twice to assess reliability. Fluorescent-labelled PCR products were run on an ABI 3730X automated sequencer (Applied Biosystems) with 500 LIZ size standard. Electrophenograms were analysed using GENMARKER v. 1.91 (Softgenetics) and with visual checking of all genotypes at least twice. A high proportion of nonamplifications (>40%) was detected for three microsatellite loci (DCA221, DCD224 and DCD140), which were discarded from analysis. We found <2% genotyping error for the remaining twelve microsatellites.

Departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for each population was tested using probability tests with 10 000 Markov chain iterations in GENEPOP v 3.4 (Raymond & Rousset 1995). Locus DC020a significantly deviated from HWE in 50 populations and was discarded from further analysis. Some loci combinations showed statistically significant LD in some populations, but there were no recurrent patterns across locus pairs. The genetic data set included 1613 individuals from 64 patches genotyped at eleven polymorphic microsatellite loci, but we discarded six populations from statistical analysis as those populations have less than five individuals.

Analysis of landscape-scale patterns of genetic structure

We performed principal component analysis (PCA) of population allele frequencies to assess patterns of population genetic structure at the landscape scale as constrained by shepherding systems using the function dudi.pca of the library ade4 (Thioulouse & Dray 2007) in R (R development core team 2013). The first three PCA axes containing the largest variance components were retained for an interclass PCA among the four groups as defined above. The interclass PCA, which rotates the selected PCA axes to maximize the correlation with the predefined group structure, was applied using the function bca of ade4 (Thioulouse & Dray 2007). We used the interclass PCA scores to test whether populations from the three shepherding systems and ungrazed patches significantly differed from each other. We performed then pairwise comparisons with Tukey's HSD on the three interclass PCA scores and with a family-wise significance level of alpha = 0.05. Alternatively, we applied a spatial principal component analysis (sPCA) to assess the presence of global and/or local spatial genetic structure. sPCA is a multivariate method that uses population allele frequencies to identify genetic structure taking into account the spatial autocorrelation between sampling locations by constructing a connection network. Similar to Moran's I, large positive sPCA eigenvalues indicate global genetic structure (higher similarity between neighbours), while large negative eigenvalues indicate local genetic structure (higher differentiation between neighbours; Jombart 2008). We implemented the k-nearest neighbour algorithm to construct the connection network by increasing the number of neighbours until all patches were connected in a single network (neighbours = 8). Presence of significant global and local structures was assessed by randomization tests as implemented in the R library adegenet (Jombart 2008). Significant global and local eigenvalue scores were plotted in the geographical space to visually interpret resultant patterns of genetic structure. Furthermore, we carried out two separate analyses of molecular variance (AMOVA) in ARLEQUIN v. 3.5 (Excoffier et al. 2005), first partitioning the genetic variance between grazed vs. ungrazed patches and then among the three shepherding systems omitting ungrazed patches. Significance testing was carried out with 1000 permutations.

Additionally, spatial patterns of genetic structure were investigated using TESS v. 2.3 (Chen et al. 2007), which incorporates the spatial locations of individuals by constructing a neighbourhood network to assess spatial patterns of genetic structure. We performed 40 runs with the admixture model (CAR), using the default spatial interaction parameter $\psi = 0.6$, burn-in lengths of 10 000 and 5000 sweeps for of each k ranging from 2 to 10. We plotted values of the deviance information criterion (DIC) averaged for each run from k 2–10 to select the optimum number of clusters k as suggested by Chen et al. (2007). Mean membership scores per patch for the selected number of clusters were plotted as pie charts on a map using the R library plotrix (Lemon 2006) to visually assess the spatial genetic structure.

Isolation by geographical distance and connectivity by shepherding

To assess whether spatial patterns of genetic structure were explained by directed dispersal along shepherding routes or by isolation by geographical distance (IBD), we performed Mantel and partial Mantel correlations between the population genetic and two ecological distance matrices: interpatch geographical distances (Euclidean distances in km between UTM coordinates) and distance along shepherding routes. The distance along shepherding routes was calculated as the number of patches that sheep traverse along the route from patch i to path j (Rico et al. 2012). The matrix of genetic distances among populations was calculated using Cavalli-Sforza and Edwards' (1967) chord distance (Dc), which has been shown to be appropriate for microsatellite data

(Takezaki & Nei 1996). Dc was calculated using the function *dist.genpop* in the R library *adegenet* (Jombart 2008). We performed Mantel correlations to test the effect of IBD and shepherding connectivity on population genetic distances (Dc) as follows: (i) IBD across all population pairs in the study system ($n = 59$), (ii) IBD among populations pairs in ungrazed patches ($n = 12$), (iii) IBD among populations pairs within each herding system separately and (iv) effects of shepherding distances along herding routes for each individual herding system ($n = 3$ herds). Additionally, (v) to overcome the limitations of sample size for testing the effect of IBD and shepherding distances, we pooled the genetic data across all three herding systems, while restricting the population pair comparisons within the same system. To partial out the effect of geographical distance from the distance along shepherding routes in each system, we performed a partial Mantel test controlling for geographical distance among patches. Significance of Mantel correlation coefficients was tested by permuting observations 1000 times.

To complement the Mantel tests, which application in landscape genetics to assess the significance association between distance matrices has been questioned (see Legendre & Fortin 2010; Guillot & Rousset 2013), we performed an alternative analysis using multiple linear mixed models (LMM). LMM incorporates site effects and account for nonindependence of observations in regressions on distance matrices (Clarke *et al.* 2002). The LMM model included as fixed effects the two distances matrices of Euclidean and shepherding distances, and as random effect a matrix indicating which patches belong to each shepherding system or to the group of ungrazed patches. We used the function *lme* of the *nlme* R package (Pinheiro *et al.* 2013) to obtain parameter estimation by restricted maximum likelihood (REML), and significance testing was carried out by permuting observations within each shepherding system and the group of ungrazed patches 1000 times. R^2-coefficients based on the likelihood ratio for mixed models (LRT) were estimated with the package *lmmfit* in R (Maj 2013).

Because habitat fragmentation is expected to reduce rates of gene flow between populations and thus increase drift and inbreeding in small populations (Young *et al.* 1996), we evaluated whether inbreeding F_{IS} differed between grazed and ungrazed patches and population size. In the absence of exact census data, we classified population size based on data from the previous species survey in two categories: small (classes 1 and 2, <40 individuals, $n = 19$) and large populations (classes 3 and 4, ≥40 individuals, $n = 39$). F_{IS} inbreeding coefficients estimated in ARLEQUIN v. 3.5 (Excoffier *et al.* 2005) were tested for significant differences between

groups with a two-way ANOVA, using type II sums of squares to account for unbalanced design.

Results

Landscape-scale patterns of genetic structure

We found population genetic structure among the three nonoverlapping shepherding systems, as revealed by the interclass PCA that factored in the three nonoverlapping herding systems and a fourth group containing all ungrazed patches (Fig. 2). The first interclass PCA axis significantly distinguished herd-2 populations from those grazed by herds 1 and 3 (Tukey's HSD: $P = 0.0001$; Table S1, Supporting information), the second interclass PCA axis significantly distinguished herd-3 from herd-1 populations (Tukey-HSD: $P = 0.0001$; Table S1, Supporting information), and the third axis significantly distinguished ungrazed populations from those in the three herding systems (Fig. 2; Tukey-HSD: $P = 0.0001$; Table S1, Supporting information). Randomization test of the sPCA indicated significant global genetic structure ($P = 0.01$), but no significant local genetic structure ($P = 0.78$). The map of scores corresponding to the first global eigenvalue, which had the largest variance and spatial autocorrelation, showed differentiation between patches in the east and west of the study area (white vs. black squares; Fig. 3), with the largest differentiation (big squares corresponding to large scores) between populations in the far east (herd 2) and far west (herds 1 and 3) of the study area (Fig. 3). This pattern is consistent with a cline-type genetic structure. According to the AMOVA results, the proportion of genetic variance explained between grazed and ungrazed patches (0.05%) was statistically no significant. In contrast, the AMOVA that contrasted only the three shepherding systems was significant, although the percentage of the genetic variance explained between the three groups was small (0.7%), and most of the genetic variance was found within populations (97.5%, Table 1).

Similar to the results from the sPCA, spatial clustering analysis in TESS also identified two main genetic clusters separating populations within patches in the east (herd 2) and the west (herd 1 and 3) of the study area, which suggests a cline-type genetic structure (Fig. S1, Supporting information). According to the DIC plot, DIC values started to plateau at $k = 4$, with the lowest value at $k = 8$ suggesting the presence of eight clusters (Fig. S2, Supporting information). However, when looking at the hierarchical development of clusters from $k = 2$ to 10, the two main east–west clusters were consistently detected (Fig. S3, Supporting information).
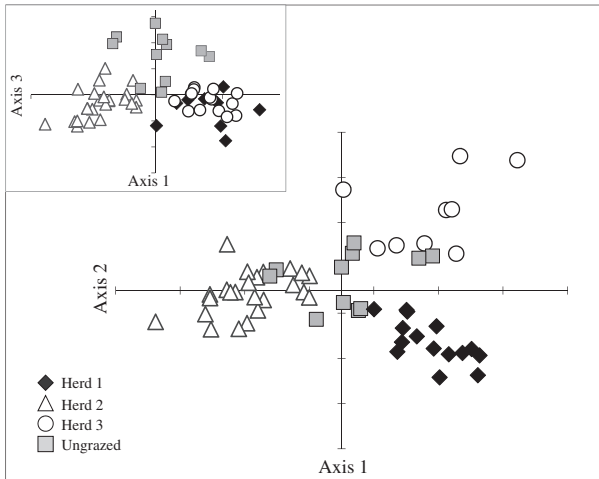
Fig. 2 Ordination of the first two interclass PCA axes based on population allele frequencies constrained by three nonoverlapping shepherding systems and the group of ungrazed patches (n = 59): PCA axis 1: 4.8%; PCA axis 2: 2.4%; PCA axis 3: 1.7%). Inset shows ordination of the first and third interclass PCA axes.
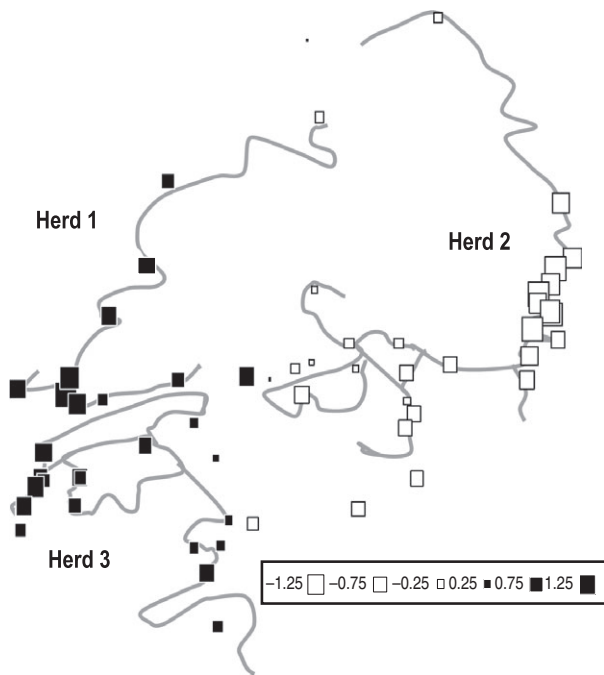
Table 1 Results from analyses of molecular variance (AMOVA) in *Dianthus carthusianorum*: genetic variance partition between (a) grazed and ungrazed patches, and (b) among the three shepherding systems

Groups	d.f.	Percentage of variation
(a) Grazed vs. ungrazed patches	1	0.05
Among patches within groups	57	2.5*
Within patches	3145	97.5
(b) Among herding systems	2	0.7*
Among patches within groups	43	1.85*
Within patches	2584	97.5

*$P < 0.05$.

Isolation by geographical distance (IBD) and directed dispersal by sheep

The global Mantel test across all populations pairs (n = 59) showed a weak but significant effect of IBD ($r = 0.18$, $P = 0.008$). The Mantel test for populations of ungrazed patches showed a much stronger and significant IBD effect ($r = 0.56$, $P = 0.02$; Fig. 4a). In contrast, at the level of individual herding systems, we found nonsignificant IBD for all three systems (herd 1: $r = 0.12$, $P = 0.32$, herd 2: $r = -0.11$, $P = 0.2$, herd 3: $r = 0.25$, $P = 0.09$) and a significant sheepherding distance effect for two herding systems (herd 2: $r = 0.45$, $P = 0.001$, $n = 23$; herd 3: $r = 0.43$, $P = 0.001$, $n = 15$), which also remained significant after partialling out the effect of geographical distance. Herd 1, which had the smallest sample size with $n = 9$ patches, showed a positive but nonsignificant association of sheepherding distance with genetic distances ($r = 0.09$, $P = 0.5$). The pooled data showed no significant association with geographical distances ($r = 0.15$, $P = 0.2$; Fig. 4b), whereas shepherding distances had a strong and significant effect ($r = 0.41$, $P = 0.001$). This latter association remained significant after partialling out the effect of IBD ($r = 0.44$, $P = 0.001$; Fig. 4c). The LMM model also suggested a significant positive association of population genetic distances with shepherding distances (β-coeff. = 0.002, $P = 0.018$) with an R^2 of 0.15. There was no significant association with Euclidean distances (β-coeff. = 0.003, $P = 0.18$), and no significant random effect (β-coeff. = 0.27, $P = 0.99$).

The two-way ANOVA showed that F_{IS} differed significantly between grazed and ungrazed patches (d.f. = 1, $F = 5.81$, $P = 0.02$), while population size was not significant (d.f. = 1, $F = 0.008$, $P = 0.92$). There was a marginally significant interaction between the two predictors (d.f. = 2, $F = 3.25$, $P = 0.07$). Pairwise comparisons indicated that small populations of ungrazed patches had significantly lower F_{IS} (mean = 0.05 ± 0.06, $n = 7$) than

Fig. 3 Map of the first sPCA-positive eigenvalue scores of significant global genetic structure. Squares represent sampled populations within patches and the difference in colour denotes genetic differentiation (black vs. white). The size of the squares is proportional to the eigenvalue score such that larger squares denote higher dissimilarity. Light grey lines correspond to the three nonoverlapping herding routes connecting calcareous grasslands patches (n = 59).
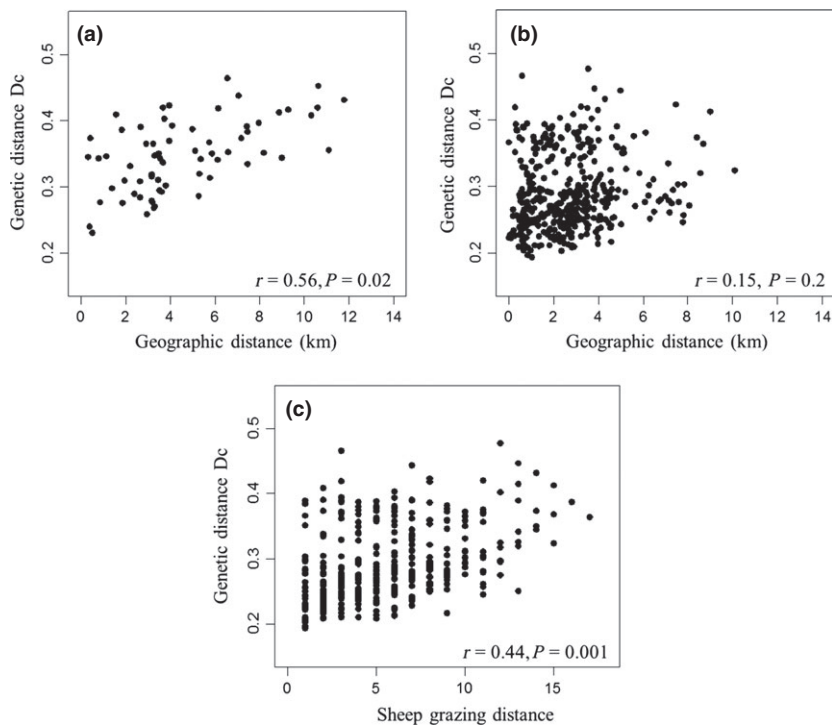
Fig. 4 Effects of isolation by geographical distance (IBD) and distance along shepherding routes on population genetic distances. (a) Effect of IBD for pairs of populations in ungrazed patches ($n = 12$); (b) Effect of IBD for pairs of populations in grazed patches within the same herding system ($n = 47$), and (c) Effect of shepherding connectivity for population pairs of grazed patches within the same herding system ($n = 47$). Mantel correlations and significant P-values are shown for each plot.

small populations of grazed patches (mean = 0.11 ± 0.05, $P = 0.02$, $n = 12$) and tended to have lower F_{IS} than big populations of grazed patches (mean = 0.1 ± 0.04, $P = 0.06$, $n = 34$). There were no significant differences between large populations of ungrazed patches (F_{IS} mean = 0.09 ± 0.03, $n = 5$) and large ($P = 0.9$) or small populations ($P = 0.8$) of grazed patches.

Discussion

Our results suggest that connectivity by rotational shepherding substantially influenced spatial genetic structure of populations in the calcareous grassland herb *D. carthusianorum*. Traditional land use by rotational shepherding is recognized to have a major role determining species richness of calcareous grasslands as habitat specialist plants are effectively dispersed by sheep among physically isolated grasslands (Fischer *et al.* 1996; Kahmen *et al.* 2002; Kuiters & Huiskes 2010; Reitalu *et al.* 2010; Rico *et al.* 2012; Wagner *et al.* 2013). However, there has been a lack of empirical data relating to the effect of sheep grazing on gene flow at the landscape scale. Our study showed that directed dispersal by grazing have the potential to shape spatial patterns of genetic structure. This result is especially important as *D. carthusianorum* lacks any seed morphological adaptations for zoochory (Klotz *et al.* 2002).

Only two empirical studies have investigated patterns of population genetic structure in association with shepherding practices in calcareous grassland plants. Willerding and Poschlod (2002) studied the grass *Bromus erectus*, but did not find a significant association of population genetic structure with shepherding routes. This lack of spatial genetic structure might be explained by the widespread distribution and abundance of this grass species in calcareous grasslands, as well as the high dispersability of *B. erectus* by sheep, which has been reported to be transported by sheep in high numbers (Fischer *et al.* 1996). Honnay *et al.* (2006) investigated the association of population genetic diversity and spatial genetic structure with temporal patterns of landscape connectivity (from 1850 to 1984) in the calcareous grassland plant *Anthyllis vulneraria*, but also did not find any effects. In contrast, we found a significant effect of IBD among all populations in the study system, but which was not significant when comparisons were restricted within each herding system, and instead population genetic distances of grazed patches were significantly associated with shepherding while controlling for the effect of geographical distance.

There are no specific data from observational or experimental studies reporting the presence of *D. carthusianorum* seeds to be attached to the fur of animals, but seeds of similar calcareous grassland plants lacking dispersal adaptation to zoochory (e.g. *Asperula cynanchica*; Fischer *et al.* 1996) had been found attached to the sheep fur and travelling long distances (Couvreur *et al.* 2004, 2005; Manzano & Malo 2006). Experimental studies on primary dispersal have documented that seeds of

most calcareous grassland herbs are dispersed over short distances (Diacon-Bolli *et al.* 2013), often within 1 m of the source plant (Coulson *et al.* 2001). Means of secondary dispersal will likely increase these primary dispersal distances (Nathan & Muller-Landau 2000). However, available evidence suggests that in the absence of rotational sheep grazing, colonization is unlikely (Poschlod *et al.* 1998; Maurer *et al.* 2003). Other possible vectors such as wild and domestic animals, humans and agricultural machinery may also transport seeds among grasslands, but they are likely to transport much fewer seeds than the herds of 400–800 ewes with their lambs moving in a directed way from one calcareous grassland patch to another. In the context of species conservation, a lack of connectivity by rotational sheep grazing may endanger the persistence of calcareous grassland specialist plants such as *D. carthusianorum* by reducing species' ability to colonize grasslands after local extinction (Soons *et al.* 2004; Rico *et al.* 2012) and increasing population genetic differentiation.

The spatial pattern suggesting a clinal differentiation between populations in the east and west of the study area, as shown by the sPCA and TESS, may, in part, reflect spatial genetic structure associated with shepherding practices dating back to at least 1830 (Dolek & Geyer 2002; Bender *et al.* 2005). During the 19th century, regional transhumance shepherding travelled annually along large strips of calcareous grasslands located on the slopes between the lower and upper Jurassic (western part of herding systems 1 and 3). These grasslands were linked regionally to other grassland areas to the west and further south of the study area. In contrast, calcareous grasslands on the main plateau were grazed locally in communal systems (Hornberger 1959; Jacobeit 1961).

In the study system, calcareous grasslands are embedded within a matrix composed of forest, intensive agriculture, meadows and settlements. Habitat fragmentation is expected to reduce population genetic diversity by drift and inbreeding (Young *et al.* 1996). Population genetic diversity in patches grazed by sheep exhibits higher levels of genetic diversity compared with ungrazed patches (Rico *et al.* 2013). Average inbreeding F_{IS} in *D. carthusianorum* was rather low ($F_{IS} = 0.095$), but contrary to our expectation, small ungrazed populations showed lowest F_{IS} coefficients, which were significantly lower than those of small populations of grazed patches. Although our classification of population size is not based on exact census data, which may affect our conclusions when estimates of F_{IS} between small vs. large populations are contrasted, the comparison of F_{IS} between small populations in grazed and ungrazed patches is not biased as all individuals were sampled in these patches (<40

plants). Moreover, evidence from meta-analysis in outcrossing plants showed that F_{IS} is often not associated with population size (Leimu *et al.* 2006). Inbreeding in plants, measured by F_{IS}, could be the result of biparental inbreeding or selfing (Mimura & Aitken 2007). As *D. carthusianorum* is partially outcrossing and insect-pollinated by specialized butterflies, gene flow by pollen would be expected to be substantial (Jennersten 1988; Bloch *et al.* 2005). However, sheep grazing may affect the occurrence of flowering plants through gap creation and biomass removal (Faust *et al.* 2011) and may thus indirectly affect the movement behaviour of pollinators. In addition, grazing influences the number of flowering individuals as well as their flowering period through flower consumption, which again affects plant reproduction rates (Reisch & Poschlod 2008; Kloss *et al.* 2011). It is thus possible that individual plants of *D. carthusianorum* in grazed patches are suffering from a partial lack of mating partners and that in consequence, grazed patches show a slight increase in F_{IS} values, even though patch connectivity by sheep on its own would lower genetic differentiation within connected patches and increase genetic diversity (Rico *et al.* 2013) for grazed patches. Our data based on nuclear markers do not allow direct estimation of the relative contributions of pollen- and seed-mediated gene flow. Quantifying the ratio of pollen- and seed-mediated gene flow (Ennos 1994; Bacles *et al.* 2006) would clarify how connectivity by rotational shepherding differentially affects rates of gene flow among populations.

To conclude, this study provides evidence on the influence of large-flock shepherding on landscape-scale patterns of genetic structure in calcareous grasslands. Our findings also show that seed-mediated gene flow can be substantial as found in other plant species dispersed by animal vectors (Bacles *et al.* 2006; NDiade-Bourobou *et al.* 2010; Freeland *et al.* 2012). Further research is needed to assess whether patterns of landscape-scale genetic structure associated with shepherding as found in *D. carthusianorum* are typical for other plant species of calcareous grasslands.

Acknowledgements

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Data accessibility

Neutral microsatellite data at eleven loci for 59 sampled patches in *Dianthus carthusianorum* are deposited on DRYAD doi:10.5061/dryad.jn1v7.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Pie charts of population membership scores for the two main genetic clusters identified by TESS. Light grey lines correspond to the three nonoverlapping herding routes connecting calcareous grasslands patches ($n = 59$).

Fig. S2 Plot of values of the deviance information criterion (DIC) averaged over ten run using TESS with admixture models varying K from 2 to 10.

Fig. S3 Bar plots of posterior estimates of cluster memberships for k_{max} 2–8.

Table S1 Multiple comparisons with Tukey's HSD of mean scores for the three interclass PCA axes accounting for three shepherding systems and populations in ungrazed patches.