RESEARCH ARTICLE

Surrounding landscape structures, rather than habitat age, drive genetic variation of typical calcareous grassland plant species

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Abstract

Context   Land use change reduced calcareous grasslands throughout Europe during the last decades. Subsequent fragmentation and habitat deterioration led, moreover, to a massive biodiversity decline. To counteract this alarming development, a clear understanding of genetic variation patterns, as fundamental level of biodiversity, becomes inevitable.

Objectives   The aim of our study was to identify the drivers of genetic variation in common calcareous grassland plant species. More specifically, we tested whether genetic diversity or differentiation of *Asperula cynanchica*, *Campanula rotundifolia*, and *Linum catharticum* depend on habitat age, landscape structure, habitat quality, and/or population size.

Methods   We investigated 912 individuals, 304 per study species, from 19 calcareous grasslands across the Swabian Alb in Baden-Württemberg (Germany) using AFLP analyses.

Results   We observed no significant influence of habitat age on genetic diversity and differentiation.

Habitat quality also had no impact on genetic diversity and population size only showed weak effects. However, genetic diversity strongly depended on landscape structure represented by distance to the nearest settlement, total area of surrounding calcareous grasslands, and their connectivity.

Conclusions   Migratory sheep herding is considered as main land use in calcareous grasslands on the Swabian Alb and thus, landscape structures in the study region may describe movement patterns of grazing livestock. In this study, genetic variation in calcareous grassland populations was strongly affected by surrounding landscape structures and subsequent grazing patterns. Therefore, we assume that moderate grazing intensities over the long term may increase levels of genetic diversity, whereas periods of overgrazing or abandonment could lower genetic diversity.

Keywords   AFLP · Calcareous grassland · Genetic variation · Landscape genetics · *Asperula cynanchica* · *Campanula rotundifolia* · *Linum catharticum*

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Introduction

Central European calcareous grasslands represent local biodiversity hotspots due to their long existence, habitat diversity, and species richness (Steffan-

Dewenter and Tscharntke 2002; Karlik and Poschlod 2009; Poschlod 2017). More precisely, they constitute valuable habitats for many specialised, rare, and endangered plant or insect species and are, therefore, considered as key areas for biodiversity conservation in agricultural landscapes (Raatikainen et al. 2009; Rosengren et al. 2013).

The shift from traditional to modern (animal) husbandry caused a drastic decline of calcareous grasslands during the last 150 years (WallisDeVries et al. 2002; Poschlod 2017). Due to abandonment and intensification more than 70% of the calcareous grasslands on the Swabian Alb in south-west Germany disappeared by the 1990s (Mattern et al. 1992; Steffan-Dewenter and Tscharntke 2002). Remnant calcareous grasslands are often highly fragmented and small in size. Populations in these habitat patches may consequently suffer from reduced probabilities of gene flow and increased genetic drift (Aguilar et al. 2008). Therefore, habitat loss affects not only biodiversity at the species level, but also the genetic variation of local plant populations (Ouborg et al. 2006). Following May (1994), genetic variation represents the most fundamental level of biodiversity. Levels of genetic variation are shaped by changing environmental conditions driving natural selection, adaptation, gene flow, genetic drift, and stochastic processes (McKay et al. 2005; Rosengren et al. 2013). To protect biodiversity fundamentally, we need to identify the key variables influencing genetic variation.

Calcareous grasslands are characterized by a diverse land use history as well as management continuity and could, therefore, be found either on historically old ('ancient') or historically young ('recent') sites. High levels of gene flow at the time of founding and afterwards may result in identical levels of genetic variation between sites of different age (Vandepitte et al. 2010). Nevertheless, the genetic variation of populations on recent sites seems to depend on both the number and origin of colonists (Wade and McCauley 1988) as well as the rate of gene flow and selection after colonization (Barrett et al. 2008). These populations may, therefore, show reduced genetic variation by bottlenecks and increased divergence among populations by selection (Wade and McCauley 1988; Dlugosch and Parker 2008). Due to potential past and present bottleneck, selection, or gene flow events, we would expect an impact of habitat age on the genetic variation of typical calcareous grassland species.

Past and present landscape structures provide valuable information about potential gene flow and further dispersal processes (Prentice et al. 2006; Purschke et al. 2012). The impact of both habitat size and area of surrounding habitats on biodiversity was analysed for many species groups and habitats, since MacArthur and Wilson (1967) established the theory of island biogeography. Hence, various studies reported that plant populations on small and isolated calcareous grasslands, with reduced gene flow, increased inbreeding as well as genetic drift, showed reduced seed set (Kéry et al. 2000), genetic erosion (Honnay et al. 2007), and finally higher extinction risks (Spielman et al. 2004). Besides habitat size, habitat connectivity and the kind of grazing management supply essential information about possible gene flow and seed dispersal in networks of (fragmented) habitat patches (Reitalu et al. 2010). Grazing, e.g. by sheep (typically for calcareous grasslands), ensures propagule dispersal over large distances and improves habitat quality by trampling and browsing (Fischer et al. 1996; Willerding and Poschlod 2002). Such highly connected sites are expected to show increased colonization and reduced extinction rates due to rescue effects (Brown and Kodric-Brown 1977). Thus, it can be hypothesized that surrounding landscape structures and resulting gene flow mechanisms are important determinants for genetic variation in highly diverse calcareous grasslands.

The abandonment of migratory sheep farming and, in turn, lower grazing pressure on calcareous grasslands led to deteriorated habitat conditions in the last decades (Zulka et al. 2014). Biomass is not removed in the absence of grazing animals, which results in litter accumulation, eutrophication, and thus, increasing vegetation height with grasses dominating (Jacquemyn et al. 2011). The germination of calcareous grassland species depends on the availability of light and open soil (Grubb 1977). Moreover, thick litter layers acting as seed traps (Ruprecht and Szabó 2012) and high vegetation causing ground shadowing (Jensen and Gutekunst 2003) inhibit germination and establishment of these species. Therefore, an impact of the local vegetation structure, which is also an indicator for habitat quality, on genetic variation can be expected.

Despite intact habitat quality, habitat fragmentation could lead to isolated populations with decreased population size. Small populations are more sensitive to demographic and environmental changes due to the fixation of deleterious alleles by genetic drift (Young et al. 1996). These populations will show lower genetic variability, consequently increased levels of inbreeding (Van Treuren et al. 2005), and therefore, face a higher risk of extinction (Spielman et al. 2004; Ouborg et al. 2006). Many empirical studies observed a positive impact of population size on the genetic variation of calcareous grassland species (Leimu et al. 2006) and thus, we predict a positive association between population size and genetic variation.

Considering all these aspects, the aim of this study was to disentangle the relative impact of biotic and abiotic factors on the genetic variation of common calcareous grassland species. In changing environments, gene flow, migration, and/or dispersal potential of species may be represented by different levels of genetic variation (Holderegger et al. 2006). Thus, we asked the following questions: (i) Is genetic diversity influenced by habitat age? Are populations of different habitat age genetically differentiated? (ii) What is the impact of past and/or present landscape structure on genetic diversity? (iii) Is genetic diversity affected by the present habitat quality and/or population size?

Materials and methods

Study design

For our study, we selected 19 calcareous grasslands all over the Swabian Alb in south-west Germany (Fig. 1, Table S1). This region belongs to the largest Jurassic low mountain range in Central Europe (Park 2017). The climate is characterized by cool, humid westerly winds with an annual average temperature between 6.7 and 8.0 °C and an average precipitation from 750 to 1050 mm/year (Jooß 2014).

In order to study the impact of habitat age on genetic diversity and differentiation of common calcareous grassland species, we sampled populations on sites with different habitat age (Reitalu et al. 2010). Selected patches were currently calcareous grassland habitats with comparable habitat quality. We selected ten historically old patches ('ancient sites'), which are calcareous grasslands since before the 1820s, and nine

historically young patches ('recent sites'), which developed from arable fields during the 1900s (Fig. 1, Table S1). The habitat age was determined using historical cadastral maps from 1820 to 1850 as well as 1902 to 1914. Further, topographical maps from 1951 to 1953 and actual aerial photographs were examined using the software ArcGIS® 10.3.1 (Esri, Redlands, CA, USA) (Table S2).

At each site, we analysed genetic variation of three typical calcareous grassland species: *Asperula cynanchica* L., *Campanula rotundifolia* L. s. str., and *Linum catharticum* L. *Asperula cynanchica* (Rubiaceae; $2n = 22$, (44)) is flowering from June to September (Kühn et al. 2004). Main pollinators are insects, e.g. bees, bumblebees, wasps, bombylides, or syrphids, but occasionally *A. cynanchica* is self-pollinated (Kühn et al. 2004). The bell-shaped flowers of *C. rotundifolia* (Campanulaceae; $2n = 34$, 68) are mostly pollinated by bees between June and October (Kühn et al. 2004). The annual *L. catharticum* (Linaceae; $2n = 16$), which can sometimes live longer, flowers from May to July (Kühn et al. 2004). It is generally self-pollinated, but could also be insect-pollinated by bees, bumblebees, wasps, bombylides, or syrphids (Kühn et al. 2004). All three species can be dispersed ecto- and/or endozoochorously (Poschlod et al. 2003).

To analyse the impact of landscape structure on genetic diversity, we digitized historical cadastral maps (1820–1850) as well as actual topographical maps (2014–2018) in a circle with 3 km radius around the center of each study site (Table S2). As potential explanatory variables, we identified the area of each study site (AREA_S) and measured the past and present distance to the nearest settlement (DIST_1820; DIST_2018) (Table S3). Additionally, we calculated the past and present total area of surrounding calcareous grasslands per 3 km radius (AREA_1820; AREA_2018) as well as the past and present connectivity (CON_1820; CON_2018) per circle (Table S3). The connectivity was determined according to Hanski (1994) as $S_i = \sum_{j \neq i} \exp(-\alpha d_{ij}) A_j$ where S_i is the connectivity of the patch i, d_{ij} is the distance (km) between patches i and j, A_j is the area (ha) of the patch j, and α is the parameter of the exponential distribution setting the influence of distance on connectivity (Helm et al. 2006). Following Lindborg and Eriksson (2004) and Reitalu et al. (2010)
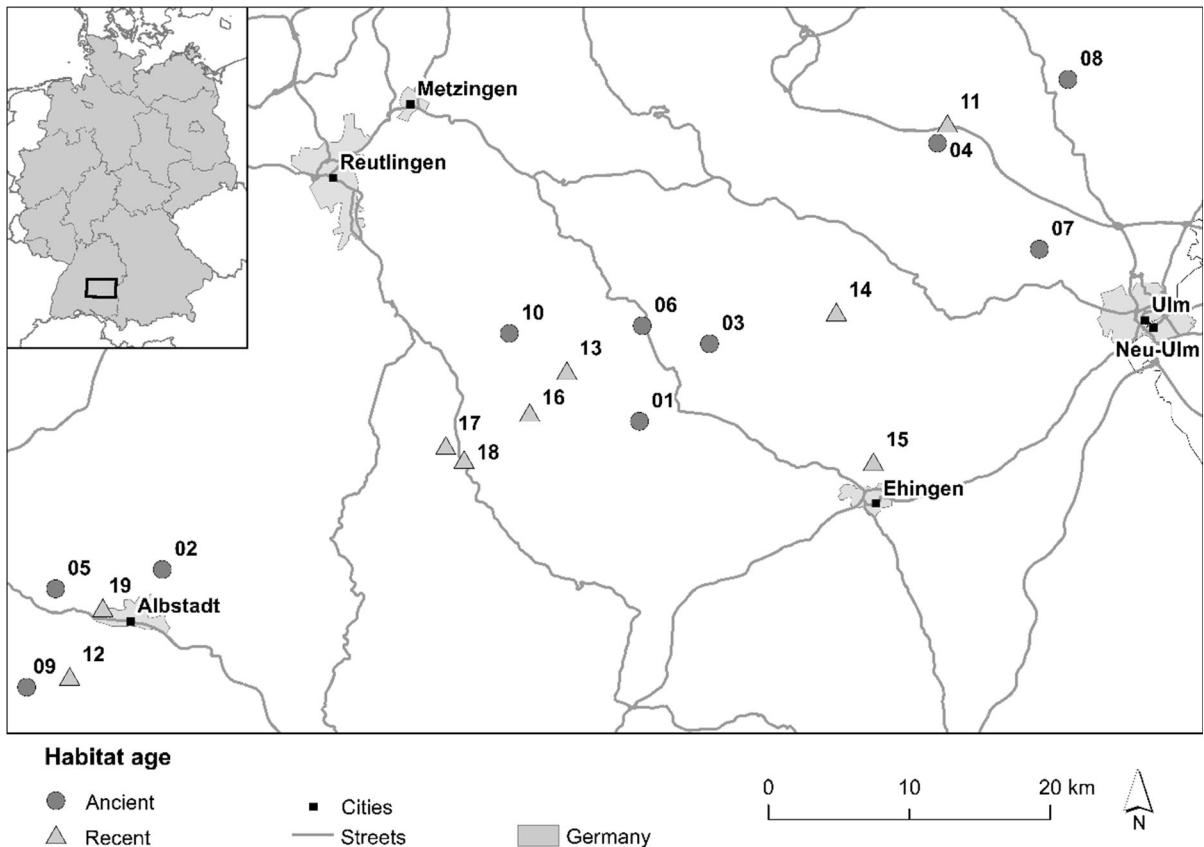
Fig. 1 Geographic position and habitat age of the analysed *A. cynanchica*, *C. rotundifolia*, and *L. catharticum* populations

α was set to one and not weighted by the dispersal abilities of the plant species in the community.

Data about the cover of vascular plants, mosses, litter, and open soil were incorporated per study site to investigate the influence of habitat quality on genetic diversity (Table S4). Furthermore, population size was determined by counting the number of individuals in 10–15 1 m^2 randomly placed plots in the field. The average number of individuals per square metre was then multiplied with AREA_S (Reisch et al. 2018) (Table S4). For those study sites, where no individual could be found in the 1 m^2 plots although plant material was collected, the total number of individuals was set from 0 to 1 before multiplying.

For molecular analyses we took leaf samples from 16 individuals per population and species to cover more than 90% of the total genetic diversity (Leipold et al. 2020).

Molecular analyses

DNA extraction was conducted following the CTAB protocol from Rogers and Bendich (1994) modified by Reisch (2007). DNA quality and concentration were determined with a spectrophotometer. All DNA samples were diluted to the same level of 7.8 ng DNA per μl H_2O. Genetic variation within populations was determined for 912 individuals using genome-wide genotyping with amplified fragment length polymorphisms (AFLP; Vos et al. 1995). The AFLP analyses were performed following the standardized protocol of Beckmann Coulter (Bylebyl et al. 2008; Reisch 2008). We screened 36 primer combinations per species to choose three appropriate primer combinations for the selective amplification (Table S5). An automated capillary electrophoresis machine (GeXP, Beckmann Coulter) was used to separate the fluorescence-labelled DNA fragments by capillary gel electrophoresis. Fragment data were analysed manually applying

the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses and thus, samples without clear banding pattern were repeated.

The reproducibility of the AFLP analyses was tested by calculating the genotyping error rate (Bonin et al. 2004). Therefore, 10% of all analysed samples were replicated twice and the percentage of fragments with differences between original and replicate was evaluated. The genotyping error rates of *A. cynanchica*, *C. rotundifolia,* and *L. catharticum* were 2.6%, 4.2%, and 2.5% respectively.

Statistical analyses

Binary (0/1) matrices were created applying Bionumerics 4.6. Using this matrices, genetic diversity within each population was calculated as Nei's gene diversity (GD) H = $1 - \sum (p_i)^2$, with p_i representing the allele frequency, in PopGene 32 (Yeh et al. 1997). We calculated a Kruskal–Wallis test with a post hoc Dunn's test and a Bonferroni p-adjustment in R (R Core Team 2017) to compare Nei's gene diversity on species level and to test the dependence of Nei's gene diversity on habitat age.

Hierarchical analyses of molecular variance, AMOVA, based on pairwise Euclidian distances among samples, were conducted applying the software GenAlEx 6.41 (Peakall and Smouse 2006). Thus, the genetic variation within and among populations as well as among populations of different habitat age was analysed.

Mantel tests with 999 permutations were calculated using GenAlEx 6.41 (Peakall and Smouse 2006) to test the correlation between geographic and genetic distances (Φ_{PT} values calculated in the AMOVA) among populations (Mantel 1967).

We then built a starting model with the full set of scaled and centred explanatory variables (except for habitat age) to analyse the relationship between genetic diversity and potential explanatory variables. More specifically, we formulated multiple linear regression models for each species in R (R Core Team 2017) to describe the variation of Nei's gene diversity related to (i) habitat age, (ii) area of each study site, (iii) past and (iv) present total area of surrounding calcareous grasslands, (v) past and (vi) present distance to the nearest settlement, and (vii) past and (viii) present

connectivity, which were described above. Further data about the coverage of (ix) vascular plants, (x) mosses, (xi) litter, and (xii) open soil, as well as the (xiii) population size of each species were included per study site. We then ranked all potential linear models according to AICc values (Akaike Information Criterion corrected for small sample sizes) to detect the models with the highest information content (Burnham and Anderson 2002). Differences between past and present landscape variables were tested by calculating Wilcoxon–Mann–Whitney tests.

Correlation tests (Pearson correlation coefficients) were conducted to show potential collinearity between the explanatory variables (ii–xiii) and to avoid, therefore, false interpretation of the linear regression models (Table S7).

Results

AFLP analyses resulted in 148, 151, and 146 fragments for *A. cynanchica*, *C. rotundifolia*, and *L. catharticum*. 69.59%, 68.49%, and 44.81% of these fragments were polymorphic. No identical genotypes were detected.

Nei's gene diversity of *A. cynanchica* populations ranged between 0.21 and 0.30 ($GD_{mean} = 0.27$) (Table 1). *Campanula rotundifolia* populations showed with 0.24 a lower mean Nei's gene diversity than *A. cynanchica* (p = 0.054) (Fig. 2a). It ranged from 0.22 to 0.26 (Table 1). *Linum catharticum* populations indicated a significantly lower mean Nei's gene diversity ($GD_{mean} = 0.16$) than *A. cynanchica* (p < 0.001) and *C. rotundifolia* (p < 0.001) (Fig. 2a). The lowest value was 0.13 and the highest 0.19 (Table 1). AMOVAs (Table 2) indicated only weak levels of differentiation among populations of *A. cynanchica* ($\Phi_{PT} = 0.072$), *C. rotundifolia* ($\Phi_{PT} = 0.048$), and *L. catharticum* ($\Phi_{PT} = 0.078$).

Moreover, AMOVAs revealed no differentiation among populations on ancient and recent grasslands (Table 2) Furthermore, genetic diversity did not differ significantly among populations on ancient and recent sites (Fig. 2b).

Mantel tests revealed significant correlations between pairwise genetic and geographic distances for *A. cynanchica* (r = 0.41; p = 0.001) (Fig. 3a) and *C. rotundifolia* (r = 0.37; p = 0.001) (Fig. 3b), but not for *L. catharticum* (r = 0.06; p = 0.263) (Fig. 3c).

Table 1 Number (n) of investigated individuals per population (No.) and mean Nei's gene diversity within populations of *A. cynanchica*, *C. rotundifolia*, and *L. catharticum*

No.	n	Nei's gene diversity		
		A. cynanchica	*C. rotundifolia*	*L. catharticum*
01	16	0.288	0.246	0.163
02	16	0.296	0.243	0.179
03	16	0.256	0.218	0.172
04	16	0.290	0.257	0.154
05	16	0.285	0.254	0.187
06	16	0.261	0.227	0.177
07	16	0.272	0.229	0.141
08	16	0.275	0.227	0.154
09	16	0.267	0.239	0.147
10	16	0.228	0.240	0.142
11	16	0.288	0.254	0.166
12	16	0.304	0.229	0.174
13	16	0.270	0.243	0.139
14	16	0.260	0.254	0.187
15	16	0.243	0.231	0.131
16	16	0.240	0.236	0.128
17	16	0.248	0.258	0.133
18	16	0.210	0.253	0.150
19	16	0.280	0.224	0.130
Mean		0.266	0.240	0.155
SD		± 0.006	± 0.003	± 0.005

Our study indicated a significant decline of the total area of calcareous grasslands, their connectivity, and their distance to the nearest settlement between the 1820s and 2018 (Table S6).

The AICc model selection generated significant linear models for all studied species (Table 3a–c). Nei's gene diversity of *A. cynanchica* populations was positively associated with present connectivity (p < 0.001). Nevertheless, past total area of surrounding calcareous grasslands (p = 0.019), present distance to the nearest settlement (p < 0.001), and the species' population size (p < 0.001) displayed a negative impact (Table 3a). Genetic diversity of *C. rotundifolia* populations increased with rising past distance to the nearest settlement (p = 0.022) (Table 3b), while Nei's gene diversity of *L. catharticum* populations was positively linked to present total area of surrounding calcareous grasslands (p = 0.021) (Table 3c).
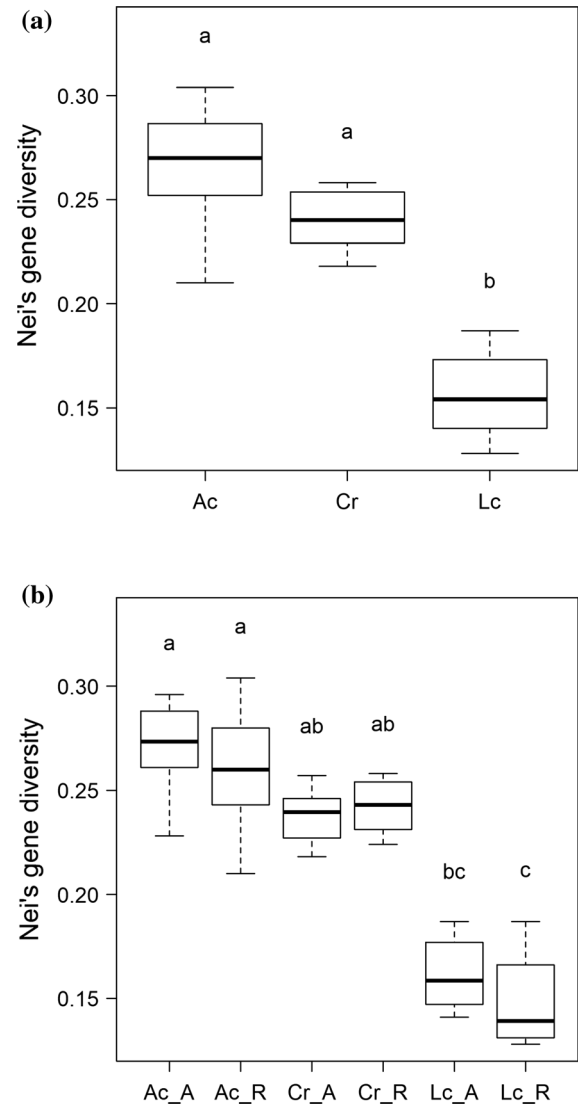
Fig. 2 Nei's gene diversity of *A. cynanchica* (Ac), *C. rotundifolia* (Cr) and *L. catharticum* (Lc) (**a**) and Nei's gene diversity per habitat age (A: ancient; R: recent) and species (**b**). The results of the Kruskal–Wallis test are indicated by the letters above the boxplots

Discussion

Genetic variation

Mean genetic diversity of our study species *A. cynanchica*, *C. rotundifolia*, and *L. catharticum* complied with the genetic diversity previously reported for common grassland species (Reisch and Bernhardt-Römermann 2014). Generally, genetic diversity

Table 2 Genetic variation per species among populations with different habitat age, among and within studied populations detected by AMOVA

Species	AMOVA	df	SS	MS	Est. Var.	%	Φ_{PT}
A. cynanchica	Among habitat age	1	34.15	34.15	0.00	0	
	Among populations	17	698.70	41.10	1.45	7	0.072***
	Within populations	285	5115.19	17.95	17.95	93	
C. rotundifolia	Among habitat age	1	32.26	32.26	0.00	0	
	Among populations	17	589.23	34.66	0.97	5	0.048***
	Within populations	285	5453.25	19.13	19.13	95	
L. catharticum	Among habitat age	1	19.09	19.09	0.00	0	
	Among populations	17	449.13	26.42	0.97	8	0.078***
	Within populations	285	3116.63	10.94	10.94	92	

Levels of significance are based on 999 iteration steps; Signif. code: ***$p \leq 0.001$

df degree of freedom, *SS* sum of squares, *MS* mean squares, *Est. Var.* estimated variation, % proportion of genetic variation, Φ_{PT} indicator for genetic differentiation among populations

depends on a species' pollination and mating system (Schoen and Brown 1991). Therefore, the insect pollinated, outcrossing species *A. cynanchica* and *C. rotundifolia* revealed significantly higher genetic diversity levels than *L. catharticum*, which is mostly self-pollinated (Kühn et al. 2004).

Weak levels of differentiation and comparatively low Φ_{PT} values among populations led to the assumption that the spatial distance among populations (< 100 km) still seems to allow sufficient gene flow (Neel 2008), since seed dispersal by animals may exceed distances of 100 km or more (Fischer et al. 1996; Manzano and Malo 2006).

Habitat age

Our study revealed similar levels of genetic diversity concerning habitat age. Following Rosengren et al. (2013) the genetic diversity of recent sites may be increased if they are connected to continuously grazed ancient sites with a diverse gene pool. Therefore, sufficient gene flow at the time of founding and

afterwards might reduce the effects of habitat age (Vandepitte et al. 2010).

Furthermore, we observed no significant differentiation among populations on ancient and recent grassland sites. Genetic differentiation is often described as a direct function of dispersal (Oostermeijer et al. 1996). More than 50% of a local species pool could be transported by one sheep during a vegetation period (Fischer et al. 1996). Thus, especially dispersal by sheep is thought to have a detectable effect on the genetic variation of grazed calcareous grassland populations (Willerding and Poschlod 2002; Rico et al. 2014a, b). The suggested dispersal rate of 660,000 diaspores per 400-head sheep flock (Willerding and Poschlod 2002) results in a substantial gene flow over long time periods and large distances (Fischer et al. 1996; Poschlod et al. 1998; Poschlod 2017). Furthermore, hayseed of populations on ancient sites was used to establish calcareous grasslands artificially on abandoned arable fields until the 20th century (Poschlod and WallisDeVries 2002). According to the migrant pool model (Wade and McCauley 1988), genetic divergence could only occur
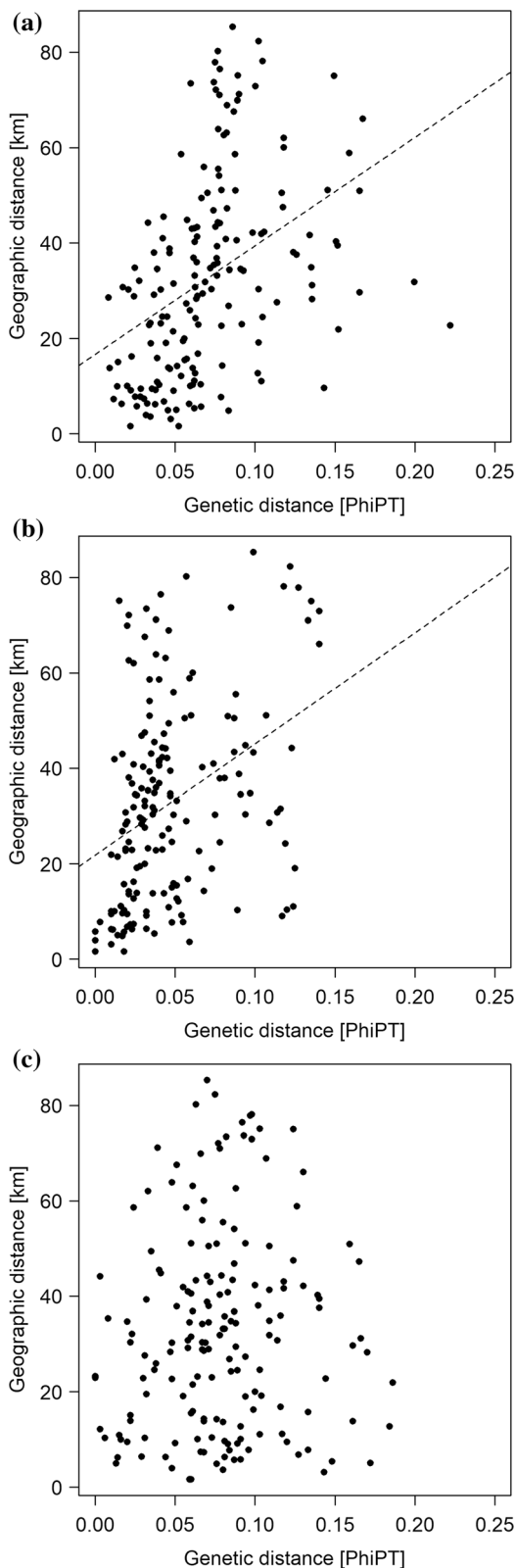
◀**Fig. 3** Correlation of genetic distance (Φ_{PT}) and geographic distance (km) (Manteltest) between the studied populations of *A. cynanchica* (**a**; r = 0.41; p = 0.001), *C. rotundifolia* (**b**; r = 0.37; p = 0.001), and *L. catharticum* (**c**; r = 0.06; p = 0.263)

if the number of colonists is less than twice the number of migrants.

Anthropogenic land use allows comparatively high levels of gene flow (Neel 2008) and could, therefore, establish viable populations in a relatively short time by overcoming pronounced founder effects (Helsen et al. 2013). By this means, anthropogenic land use may have led to similar diversity levels as well as undetectable genetic differentiation among populations of different habitat age.

Landscape structure

During the last century, land use change caused a quantitative decline of semi-natural grasslands, especially of calcareous grasslands (Poschlod et al. 2005). More particularly, settlement expansion (Poschlod 2017) or abandonment of migratory sheep farming caused massive habitat loss (WallisDeVries et al. 2002). The present study revealed a significant decline of the total area of calcareous grasslands, their connectivity, and their distance to the nearest settlement between the 1820s and 2018 as well.

However, present connectivity appeared as the only positive explanatory variable for the genetic diversity of the analysed *A. cynanchica* populations. This result is corroborated by the findings of Raatikainen and Heikkinen (2009), although other studies revealed only an influence of the past connectivity on grassland species (Lindborg and Eriksson 2004; Helm et al. 2006). The model indicates the presence of a rescue effect (Brown and Kodric-Brown 1977). Thus, populations in small habitat patches could not only persist with a high probability (Helm et al. 2006), they even show increased genetic diversity if they are well connected.

In general, the distance to the nearest settlement and the area of surrounding calcareous grasslands may describe the movement patterns of livestock (Reitalu et al. 2010) because migratory sheep herding was the main land use in calcareous grasslands of the study region. Migratory sheep herding represents both an

Table 3 Linear models explaining genetic diversity of *A. cynanchica* (a), *C. rotundifolia* (b), and *L. catharticum* (c)

(a) *A. cynanchica*

		Estimate	Std. error	p-value
	(Intercept)	0.2664	0.00176	< 0.001***
Response variable	Explanatory variable			
Nei's gene diversity	CON_2018	0.0206	0.00219	< 0.001***
	DIST_2018	− 0.0114	0.00201	< 0.001***
	Population size	− 0.0052	0.00194	0.019*
	AREA_1820	− 0.0132	0.00212	< 0.001***

(b) *C. rotundifolia*

		Estimate	Std. error	p-value
	(Intercept)	0.2401	0.00253	< 0.001***
Response variable	Explanatory variable			
Nei's gene diversity	DIST_1820	0.0114	0.00450	0.022*

(c) *L. catharticum*

		Estimate	Std. error	p-value
	(Intercept)	0.1555	0.00398	< 0.001***
Response variable	Explanatory variable			
Nei's gene diversity	AREA_2018	0.0001	0.00005	0.021*

Signif. codes: ***$p \leq 0.001$; $0.001 < $**$p \leq 0.01$; $0.01 < $*$p \leq 0.05$

AREA_1820/AREA_2018, past and present total area of calcareous grasslands [ha]; DIST_1820/DIST_2018, past and present distances to the nearest settlement [km]; CON_2018, present connectivity

(a) Residual standard error: 0.007657 on 14 degrees of freedom; Multiple R-squared: 0.9243; Adjusted R-squared: 0.9027; F-statistic: 42.75 on 4 and 14 DF; p-value: 1.061e-07

(b) Residual standard error: 0.01102 on 17 degrees of freedom; Multiple R-squared: 0.2736; Adjusted R-squared: 0.2309; F-statistic: 6.404 on 1 and 17 DF; p-value: 0.02155

(c) Residual standard error: 0.01733 on 17 degrees of freedom; Multiple R-squared: 0.2771; Adjusted R-squared: 0.2345; F-statistic: 6.515 on 1 and 17 DF; p-value: 0.0206

important vector for seed dispersal (Fischer et al. 1996; Willerding and Poschlod 2002) and ecological disturbance by grazing and trampling (Olff and Ritchie 1998). Thus, overgrazing may lead to increased levels of gene flow and disturbance, while abandonment of migratory sheep herding is expected to reduce levels of gene flow as well as the probability of seedling establishment due to a denser litter and vegetation layer (Ruprecht and Szabó 2012). Intermediate levels of gene flow may reveal a positive impact on genetic diversity levels, while 'too low' and even 'too high' levels of gene flow may promote outbreeding depression and/or genetic 'swamping'

(Bradshaw 1984). Overgrazing and abandonment of migratory sheep herding may, therefore, decrease both species (Klimek et al. 2007) and genetic diversity.

Unexpectedly, *C. rotundifolia* populations showed a positive impact of past distance to the nearest settlement on Nei's gene diversity, resulting in decreased levels of genetic diversity around settlements. Although the relationship was not very strong, we assume that grasslands close to those settlements may reflect the impact of periodic overgrazing with increased levels of gene flow and disturbance during the 1820s.

Nevertheless, the linear model for *A. cynanchica* displayed a negative impact of present distance to the nearest settlement. Thus, the highest levels of genetic diversity occurred in populations near settlements in 2018. Despite nowadays ongoing decline of livestock grazing (Poschlod 2017), we suspect that grazing intensity and associated gene flow are still at an intermediate level around present settlements. Based on the model of Reitalu et al. (2010), an unimodal association between genetic diversity and the distance to the nearest settlement could be considered as possible explanation. The authors interpreted this result in terms of the intermediate disturbance hypothesis of Connell (1978) on a landscape scale. In calcareous grasslands, the landscape scale corresponds to the intensity of grazing and associated disturbance. Therefore, these results could also be explained in terms of a classic intermediate disturbance hypothesis (Connell 1978).

Unexpectedly, the linear model for *A. cynanchica* displayed a negative impact of the past total area of surrounding calcareous grasslands on genetic diversity. This idiosyncratic result is inconsistent with the generally accepted expectation that populations, which are embedded in a landscape matrix containing a large proportion of grasslands, are more likely to reveal high levels of genetic diversity (Rosengren et al. 2013). Therefore, we suggest that comparatively high levels of gene flow in the past may have led to a highly unified and impoverished gene pool. Moreover, *A. cynanchica* populations could have also been affected by periodic overgrazing with increased levels of disturbance during the 1820s. Thus, *A. cynanchica* still seems to suffer from a kind of 'over-connection' with comparatively high measures of gene flow and/or increased levels of disturbance by grazing animals during the 1820s.

Nei's gene diversity of *L. catharticum* populations was positively associated with the present total area of surrounding calcareous grasslands. On the one hand, small and isolated habitat fragments may show reduced fitness levels and finally extinction if they suffer from edge effects and the invasion of generalist species (Leimu et al. 2006). On the other hand, a large patch size and a high proportion of surrounding grasslands may increase the variability of the incoming gene flow (Prentice et al. 2006). Thus, the total area of surrounding grassland patches has not only a positive effect on species richness and presence

(Raatikainen et al. 2009), it could also increase the genetic diversity of species (Dahlström et al. 2006). Although the relationship was not very strong, levels of genetic diversity seem to come up with an intermediate gene flow level, since significantly lower values of the present total area of surrounding calcareous grasslands (compared to the past total area of surrounding calcareous grasslands) positively affected genetic diversity in *L. catharticum*. Therefore, high genetic diversity seems to depend on an intermediate size level of surrounding calcareous grasslands or rather gene flow.

However, following Jacquemyn et al. (2006), pollination as well as dispersal vectors determine gene flow over great geographic distances. The analysed species revealed different isolation by distance patterns, although all three species are dispersed ecto- and/or endozoochorously (Poschlod et al. 2003), e.g. by grazing sheep. The insect pollinated perennials, *A. cynanchica* and *C. rotundifolia*, showed lower gene flow over increasing distances (isolation by distance) because pollinating insects may rarely travel distances larger than 1 km (Kwak et al. 1998; Steffan-Dewenter and Tscharntke 2002). The mainly self-pollinated *L. catharticum* did, therefore, not reveal any isolation by distance.

Habitat quality and population size

The habitat quality showed no impact on the genetic diversity of the analysed calcareous grassland species. However, the linear model for *A. cynanchica* displayed an influence of the species' population size. Although correlations between population size and genetic diversity are generally positive (Leimu et al. 2006), the genetic diversity of *A. cynanchica* decreased with increasing population size. Grassland plant species with comparatively large population size, long life cycles, and slow intrinsic dynamics may occur as remnant populations in modern landscapes (Maurer et al. 2003). Additionally, Piqueray et al. (2011) observed that the present occurrence of species can be influenced by past habitat configuration. These species often show a time lag between habitat loss, fragmentation, and their consequences on genetic diversity (Helm et al. 2006). Various studies revealed a significant relationship between the genetic diversity and the linkage of the studied populations in the past landscape. Thus, they indicated a delayed response of

genetic diversity to habitat fragmentation (Honnay et al. 2007). The total area of calcareous grasslands as well as the connectivity of the study sites significantly decreased since the 1820s. Thus, especially the huge populations of *A. cynanchica* seem to suffer from a kind of extinction debt today. We, therefore, assume that the slow response of *A. cynanchica* populations to previous habitat loss events led to decreased genetic diversity levels although the present population size is high.

Conclusions

From our study it can be concluded that habitat age seems to have no impact on genetic variation within and among populations if a sufficient number of source populations is nearby and gene flow is high. Therefore, our results support the assumption that the populations of the study species have previously been or are still connected by gene flow.

However, our study revealed a significant impact of the surrounding landscape structure and related land use patterns. Thus, we suspect that moderate grazing intensities over a long period of time may lead to increased levels of genetic diversity by intermediate levels of gene flow, while periods of overgrazing or abandonment seem to result in genetically less variable plant populations.

Finally, neither habitat quality nor population size appeared as crucial variables for genetic diversity patterns in our study. These findings provide evidence that surrounding landscape patterns are more important to preserve the genetic variation of typical calcareous grassland species than local site conditions.

Data availability Datasets generated during this study are available from the authors upon request.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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