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Research

Green infrastructure can promote plant functional connectivity in a grassland species around fragmented semi-natural grasslands in NW-Europe

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Species may benefit from green infrastructure, i.e. the network of natural and anthropogenic habitat remnants in human-dominated landscapes, if it helps isolated populations in remaining habitat patches to be functionally connected. The importance of green infrastructure is therefore increasingly emphasized in conservation policy to counter biodiversity loss. However, there is limited evidence, particularly in plants, that green infrastructure promotes functional connectivity, i.e. supports the colonization of habitat patches across a landscape. We applied landscape genetics to test whether the green infrastructure supports structural and functional connectivity in the grassland perennial *Galium verum*, in 35 landscapes in Belgium, Germany and Sweden. We used multivariate genetic clustering techniques, nestedness analyses and conditional inference trees to examine landscape-scale patterns in genetic diversity and structure of plant populations in the green infrastructure surrounding semi-natural grasslands. Inferred functional connectivity explained genetic variation better than structural connectivity, yielding positive effects on genetic variation. The road verge network, a major structural component of the green infrastructure and its functional connectivity, most effectively explained genetic diversity and composition in *G. verum*. *Galium verum* ramets occupying the surrounding landscape proved to be genetic subsets of focal grassland populations, shaping a nested landscape population genetic structure with focal grasslands, particularly ancient ones, harbouring unique genetic diversity. This nested pattern weakened as road network density increased, suggesting road verge networks enable high landscape occupancy by increased habitat availability and facilitates gene flow into the surrounding landscape. Our study proposes that green infrastructure can promote functional connectivity, providing that a plant species can survive outside of core habitat patches. As this often excludes habitat specialist

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species, conservation practice and policy should primarily focus on ancient, managed semi-natural grasslands. These grasslands both harbour unique genetic diversity and act as primary gene and propagule sources for the surrounding landscape, highlighting their conservation value.

Keywords: biodiversity conservation, functional connectivity, genetic diversity, green infrastructure, landscape ecology, semi-natural grassland

Introduction

The long-term survival of a plant species is dependent upon its ability to either adapt to environmental change or to migrate into suitable habitat (Berg et al. 2010). The latter is key to both sustaining viable meta-populations in patchy landscapes (Hanski et al. 1995, Gilbert-Norton et al. 2010) and tracking suitable climate (Cain et al. 1998, Lenoir et al. 2020). Many plant species migrate slowly at landscape, regional and continental scales (Ozinga et al. 2009, Lenoir et al. 2020), often further constrained by their degree of habitat specialisation (Verheyen et al. 2003). The consequences of this innate limitation have been exacerbated by the continuing loss, fragmentation and degradation of natural habitat. Many species now exist in increasingly isolated remaining habitat patches (Haddad et al. 2015) and as a result are at greater risk of local and regional extinction (Hodgson et al. 2011). The inability of numerous species, often those of conservation concern, to move through such low-connectivity landscapes, is considered a main cause of past, ongoing and future loss of biodiversity in response to global environmental changes (Haddad et al. 2015, Oliver et al. 2017).

Heavily altered, human-dominated landscapes are often veined by networks of remnant and anthropogenic, linear and patchy habitats, such as road verges (Auffret and Lindgren 2020), hedgerows (Vanneste et al. 2020), ditches (Meier et al. 2017), forest edges (Lindgren et al. 2018), power line corridors (Gardiner et al. 2018), small forests (Valdés et al. 2020) or midfield islets (Cousins and Eriksson 2002). These habitats can provide physical connections and stepping-stones between isolated habitat patches. Together, these networks are often referred to as green infrastructure, which might significantly extend the amount of available habitat in a landscape (Gardiner et al. 2018). The green infrastructure should also, at least in theory, increase the permeability of the hostile landscape matrix and enhance seed dispersal between remaining habitat patches (Gilbert-Norton et al. 2010, Hodgson et al. 2011, Auffret et al. 2017b, Thiele et al. 2018), helping to maintain functioning meta-populations by both lowering local extinction risk and enhancing colonisation success (Sullivan et al. 2011, Damschen et al. 2019). Therefore, policy makers increasingly recognise the importance of green infrastructure, placing it at the heart of biodiversity conservation management and policy (European Commission 2020).

The presence of green infrastructure can provide important structural landscape connectivity, but numerous environmental factors such as landscape management or matrix quality determine the efficacy of green infrastructure, particularly in plants (Baum et al. 2004, Aavik and Liira 2010, Jakobsson et al. 2018, Thiele et al. 2018, Auffret and Lindgren 2020). Hence, plant functional connectivity, i.e. effective dispersal and resulting colonisation and establishment of habitat patches across a landscape (Plue et al. 2019), is only partially driven by the physical amount and configuration of remaining habitat patches and the green infrastructure. Plant functional connectivity also depends upon how plant species and their pollen- and seed-vectors interact with the biotic and abiotic characteristics of both the green infrastructure connecting remaining habitat patches as well as the wider environment of the landscape (Auffret et al. 2017b). Functional connectivity is affected by 1) life-history traits influencing dispersal and recruitment success along the green infrastructure (Verheyen and Hermy 2001), 2) population dynamics affecting propagule pressure (Hufbauer et al. 2013), 3) the degree of habitat specialisation which imposes demands on habitat quality and management of recipient patches (Thomas et al. 2001) and 4) pollinator abundance and their use of the green infrastructure (Cranmer et al. 2012, Senapathi et al. 2017). Yet, these factors are in turn affected by landscape characteristics, its green infrastructure and remaining habitat patches (Baum et al. 2004, Gonthier et al. 2014, Zulka et al. 2014). These ecological interactions demonstrate the complexity involved in empirically evaluating how effectively the structural connectivity offered by the green infrastructure can promote plant functional connectivity in facilitating gene flow and seed dispersal between otherwise isolated habitat patches (Auffret et al. 2017b).

Landscape genetics may provide clarity, as this discipline combines spatial, environmental and population genetic data to understand how landscape variables such as connectivity may shape genetic diversity and meta-population structure (Manel et al. 2003, Storfer et al. 2007). However, landscape genetic studies attempting to infer functional connectivity or contrast it with structural connectivity are often limited to analysing discrete populations in discrete habitat patches (Murphy et al. 2010, Aavik et al. 2014, Favre-Bac et al. 2016). Landscape genetics is not limited to considering clearly defined, discrete populations (Manel et al. 2003). This is relevant because individuals or small populations which are scattered across small, remnant habitats in the landscape are potentially pivotal in sustaining gene-flow and mediating population genetic structure between established populations in core habitats. Nevertheless, the contribution of those individuals and populations occupying the green infrastructure to local and landscape-scale genetic diversity and population structure has received little attention so far (but see Van Rossum et al. 2004).

Here, we apply landscape genetics to assess if and how landscape-scale green infrastructure and its characteristics may mediate functional connectivity. We focus on European semi-natural grasslands, which are high-priority habitats because of their exceptional species diversity (Öckinger and Smith 2007, Wilson et al. 2012) and the ecosystem services they provide (Billeter et al. 2008, Bengtsson et al. 2019), alongside their dramatic historical habitat loss and fragmentation (Cousins et al. 2015, Ridding et al. 2020) and the continued threat of land-use change (Watson et al. 2016). We assessed landscape-scale patterns of population genetic variation in the grassland plant species *Galium verum* (Family Rubiaceae), in the green infrastructure surrounding ancient and restored semi-natural grasslands across 35 fragmented grassland landscapes in Belgium, Germany and Sweden. Our focal species prefers semi-natural grassland habitat, but its life-history enables it to occupy the green infrastructure, helping us to investigate how population genetic structure and genetic diversity are influenced by green infrastructure. Specifically, we aim to assess how the green infrastructure and the degree to which a species occupies the green infrastructure explain landscape-scale patterns in population genetic structure and diversity. Finally, we hope to elucidate to what extent populations and individuals occupying the green infrastructure contribute towards total landscape-scale genetic diversity.

Material and methods

Study species

Galium verum L., lady's bedstraw, is an evergreen, perennial herb. This hemicryptophyte grows to be 15–100 cm in height and hypogeogenous rhizomes enable clonal growth. Although wind-pollination occurs (Hickey and King 1988), pollination occurs mainly through insects such as hoverflies, and small bee and fly species. The species is also self-compatible (Fitter and Peat 1994), and it regenerates by means of seeds and clonal growth. *G. verum* produces large numbers (up to 45 115 seeds per m^2 of *G. verum*; Grime et al. 1988) of small seeds (1.5 × 1.4 mm) which weigh between 35.9 and 64.9 µg. Seeds are dispersed by animal vectors, both through epi- and endozoochory (Fischer et al. 1996, Cosyns et al. 2005, Auffret and Plue 2014). Seeds are short-term persistent (Seed Longevity Index of 0.048), building a soil seed bank with seed densities between 40 and 2350 seeds per m^2. The species has a wide ecological amplitude in terms of soil conditions (pH 4–8) and uses a range of open habitats (dunes, rocky outcrops, wasteland, grasslands). *Galium verum* has a Eurasian distribution, with tetraploid cytotypes dominating the northern parts of its European distribution, including Belgium, Germany and Sweden (Kliphuis 1984).

Study area

We sampled *G. verum* in 12 fragmented grassland landscapes in each of three regions in Europe: Viroin (Belgium), Franconian Jura (Germany) and Södermanland (Sweden). Each region has seen a strong parallel decline in semi-natural grasslands due to habitat conversion over the past 150 years, resulting in significant reductions in structural and functional connectivity in all sampled landscapes (Kimberley et al. 2021). Remaining grasslands are embedded within a landscape matrix dominated by either arable fields, forests or both. The 12 landscapes per region were each centred around a large semi-natural grassland (hereafter focal grassland). Six of these were restored, mostly from sites where grassland management was abandoned, and the other six focal grasslands were ancient, i.e. had a long history of continuous grassland management. Their management generally entailed rotational grazing or shepherding via mobile herds of domestic livestock. The surrounding landscapes, which defined the unit of replication for genetic sampling, displayed gradients of habitat availability across each region, i.e. the sum of all landscape elements which may provide suitable habitat for semi-natural grassland plants (mean of 28.90 ha, range 6.72–62.26). A total of 35 landscapes were sampled as 11 out of 12 Belgian landscapes contained *G. verum* in the focal grassland.

Characteristics of the landscape and its green infrastructure

First, we digitized land use cover in a circular landscape (r = 1600 m, a distance relevant for seed dispersal in most plant species (Bullock et al. 2017), centre = centroid of the focal grassland) surrounding each focal grassland, based on recent colour aerial orthophotographs (Belgium: 2015; Germany and Sweden: 2017). During digitisation, we identified various land cover classes as well as the landscape elements such as road verges, hedgerows, complex forest borders and small grassland remnants which comprised the putative green infrastructure. In line with our aim to identify whether structural or inferred functional connectivity best explained population genetic patterns, the digitized maps were used to calculate 1) *structural connectivity* as a metric encompassing the total habitat amount of both semi-natural grasslands and the surrounding green infrastructure and their Euclidean distances to the focal grassland whereas 2) *inferred functional connectivity* was estimated through least-cost distance calculations. The latter was based on grassland habitat and landscape resistance maps reflecting the different ability of habitats to support grassland plant species and movement of their dispersal vectors (for details on how (1) and (2) were calculated, see Supporting information). To understand which characteristics of the landscape and its green infrastructure may be more important for shaping genetic patterns, we also calculated 3) the amount of semi-natural grassland (ha, *grassland habitat*), 4) the amount of road infrastructure (proxy for the amount of road verge offering additional habitat in which *G. verum* can thrive (Dunnett et al. 1998); m, *road infrastructure*) and 5) forest cover (sum of deciduous and coniferous forest as proxy for the inverse of landscape openness; ha, *forest cover*). For further details on the digitisation process, see Kimberley et al. (2021). For descriptive information and statistics on landscape characteristics and connectivity metrics, see Supporting information.

Sampling

In each focal grassland, we collected leaf material from up to 30 individual ramets of *G. verum*, evenly spread across the focal grassland (mean area of 1.41 ± 1.14 ha). A categorical estimate of *G. verum* population size was made: 1) < 50; 2) 50–100; 3) 100–250; 4) 250–500; 5) 500–1000; 6) > 1000 ramets (defined as individual members of a clonal plant species); hereafter referred to as *focal population size*). To representatively sample the green infrastructure in the surrounding landscape, we deployed a dual sampling strategy (Fig. 1). First, we sampled 10 *G. verum* ramets in the road verges of each of two roads attached to the focal grassland at exponentially increasing distances away from the focal patch (ramets located between 0 and 1901 m, measured along the road). Second, we selected ten green infrastructure elements which supported *G. verum* populations, located at increasing distances away from the focal grassland (ramets located between 44 and 1414 m). Two random *G. verum* ramets were sampled per green infrastructure element. This sampling design thus translated into a maximum of 40 individual ramets of *G. verum* sampled throughout the landscape surrounding the focal grassland. The resulting maximum of 70 individual ramets sampled per landscape will hereafter be referred to as the *landscape population*, while the total number of sampled ramets is referred to as the *landscape population size*. This sampling intensity is sufficient to return accurate estimates of allele frequencies and genetic diversity when using microsatellite markers (25–30 sampled individuals; Pruett and Winker 2008, Hale et al. 2012). All sampled individual ramets were at least 1m apart, and preferably more, to avoid sampling clones. All leaf material was dried and stored in silica gel.

Microsatellite analysis

Total DNA was extracted from 10 to 20 mg silica-dried leaf tissue of 1926 individual samples following the NucleoSpin Plant II protocol (Macherey-Nagel, Germany). DNA samples were subsequently processed and genotyped using nine polymorphic microsatellites: four newly developed SSRs for *G. verum* (Supporting information), four SSRs specific for *G. trifidum* and one SSR specific for *G. catalinense* (Szczecińska et al. 2012, McGlaughlin et al. 2009), both of which successfully co-amplified in *G. verum*. Fourteen individuals (0.7%) were excluded, as they had missing data at four loci or more. Another 29 clonal individuals, spread evenly across populations and countries were equally removed, leaving 1883 individual samples. For full methodological details, see Supporting information. All individuals were assumed to be tetraploid during genotyping, and this was tested by flow cytometry on

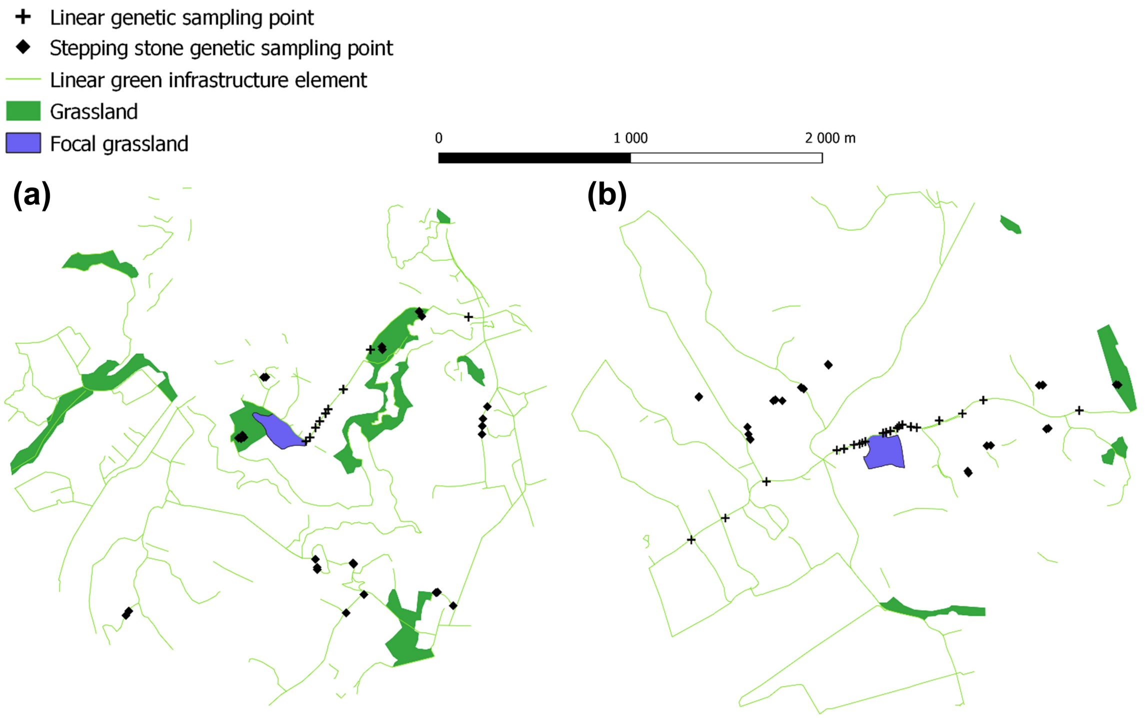

Figure 1. Landscape genetic sampling design in the green infrastructure (limited to visualize semi-natural grasslands (green) and road verges for clarity) surrounding the focal semi-natural grassland (purple) in two landscapes in Sweden ((a) Järna, 59.08N, 17.56E and (b) Lundby, 58.92N, 17.02E). A maximum of 70 *Galium verum* ramets were sampled per landscape for genetic analysis: 30 in the focal grassland, 20 at exponentially increasing distances along, if available, two road verges physically attached to the focal grassland (10 per road; +) and another 20 at increasing distances in various green infrastructure elements physically unattached to the focal grassland (stepping stones such as road verges, semi-natural grassland, forest edge, midfield islets; 2 per stepping stone; ■).

20 randomly chosen ramets per region, which confirmed tetraploidy in all 60 individuals (Supporting information).

Data preparation

Given the species is an autotetraploid (Soltis et al. 2007), population genetic structure and diversity were calculated using SPAGeDI (ver. 1.5, Hardy and Vekemans 2002) and the *adegenet* and *vegan* R-package (Jombart 2008, Oksanen et al. 2017). For each landscape, we calculated the following measures of genetic diversity for the focal grassland and landscape population: allelic richness (A), rarified allelic richness (A_R, allelic richness compared at the size of the smallest population) and gametic heterozygosity (H_S, i.e. diploid H_E extended for polyploids; Moody et al. 1993). For descriptive statistics on genetic diversity in focal grassland and landscape populations, see Supporting information.

Data analysis

Population genetic structure

To investigate how landscape characteristics, green infrastructure and focal grasslands affected landscape-scale genetic population structure, we took a two-step approach. First, we quantified the genetic similarity between *G. verum* individuals from the focal population and *G. verum* individuals sampled in the surrounding green infrastructure. We then applied a multivariate discriminant analysis of principal components on the individuals × allele presence/absence matrix per landscape (DAPC; Jombart et al. 2008), a suitable clustering algorithm for polyploid datasets where assumptions on the presence of a Hardy–Weinberg equilibrium may be violated (Dufresne et al. 2014). DAPC identifies genetically similar individuals by partitioning genetic variation into groups that maximise between-group and minimise within-group differentiation, enabling probabilistic membership assignment to each group. A priori, we defined two potential genetic clusters per landscape; the focal grassland population and the group of *G. verum* ramets sampled in the surrounding landscape elements which constitute the green infrastructure. The membership assignment procedure within DAPC then allowed us to test the extent to which of these hypothesised groups can reliably be discriminated based on their genetic similarity (for more technical background on the assignment procedure, see Jombart et al. 2008). To avoid DAPC overfitting the number of PCA axes included, the DAPC was optimized via a 90% cross-validation and the DAPC was re-run with the optimal PCA and linear discriminant (i.e. linear combinations of alleles which best separate the clusters) solution. Per landscape, the summed Euclidean distance between the centers of the two genetic clusters in the multivariate DAPC space was used as a summary statistic (further referred to as DAPC statistic), representing the compositional genetic similarity between *G. verum* ramets in the focal populations and those in the surrounding green infrastructure.

Nestedness, here defined as the deterministic loss of alleles in response to an ecological gradient (Ulrich and Almeida-Neto 2012), was subsequently quantified as a second metric of landscape population genetic structure. Quantifying nestedness per landscape allowed us to investigate whether *G. verum* ramets in the surrounding green infrastructure were a less genetically diverse and deterministic, compositional subset of the focal populations. We first calculated the degree of nestedness in each landscape, by re-ordering the original ramets × alleles presence–absence matrix into a maximally packed matrix per landscape, that is rows and columns are ordered so that nestedness is maximised. This packed matrix hence shows *G. verum* ramets with more alleles at the top of the matrix and more frequently recurrent alleles at the left side. Based on the maximally packed matrix, a nestedness temperature is calculated, using the NODF metric, i.e. Nestedness based on Overlap and Decreasing Fill in the packed matrix (Almeida-Neto et al. 2008). This NODF metric incorporates information on both genetic diversity and compositional turn-over between individual ramets and the properties which affect the community of alleles in an individual ramet, as such providing a landscape-scale estimate of the extent to which genetically poor ramets exist as subsets of genetically rich ramets rather than containing a smaller, distinct set of alleles, based on individual-scale genetic variation. The unique synthetical properties of this metric storing information on compositional directionality enable the likely ecological processes driving the compositional nested structure of the population genetic data to be unravelled in a way that is not possible without using the concept of nestedness. Further modelling of this NODF metric across landscapes thus indeed allows the ecological gradient underpinning the degree of observed nestedness patterns to be identified. Specifically, we directly assessed whether green infrastructure populations are a nested subset of the focal grassland population, or vice-versa, and hence we aimed to uncover tentative evidence on the direction of gene-flow between the focal patch and the surrounding landscape. Moreover, relating the NODF metric to variation in landscape composition can even help understand which landscape characteristics may mediate the extent of gene-flow outward from the focal grasslands into the green infrastructure, or vice-versa. For details as to which predictor variables were used to address the questions, see the paragraph *Genetic diversity* below.

The DAPC (log-transformed) and NODF statistics were then used as response variables in general linear models (GLM) which aimed to investigate the effects of wider landscape, green infrastructure and focal grassland characteristics on population genetic structure and degree of nestedness. The tested predictor variables were: 1) *Structural connectivity* and 2) *Inferred functional connectivity* (least-cost distances, Supporting information; square-root transformed for normality); and the components of these connectivity metrics, which include 3) *Grassland habitat* (ha), 4) *Road infrastructure* (m) and 5) *Forest cover* (ha); and 6) *Restored* (whether the focal grassland was restored or ancient) and 7) *Landscape presence of G. verum* (an independent, proportional metric ranging between 0 and 1 quantifying how many segments of the surrounding landscape were occupied; Hooftman et al. 2021).

The modelling strategy aimed to identify which individual components of connectivity capture most genetic variation, and whether observed patterns are best reflected by simple structural connectivity or inferred functional connectivity. First, we constructed a baseline GLM with the non-collinear, fixed effects of (6) *Restored* and (7) *Landscape presence of G. verum* only, which captures variation not directly related to explicit landscape characteristics. In the NODF model, the difference between landscape-scale allelic richness and focal grassland allelic richness ($\Delta A = A_{\text{Landscape}} - A_{\text{Focal}}$) was also added to the baseline model. This predictor was included to establish the direction of nestedness (and tentatively gene-flow), i.e. if the population of *G. verum* individuals in the landscape was a nested compositional subset of the focal grassland population, or vice-versa. We used uncorrected allelic richness to calculate ΔA as the nestedness analysis incorporates absolute differences in allelic richness between the surrounding landscape and the focal grassland (Almeida-Neto et al. 2008). To test the importance of connectivity measures and their components in affecting population genetic structure, our modelling approach involved adding a single predictor variable into the baseline model on a one-by-one basis, to overcome multicollinearity between predictor variables (1–5) (Supporting information). Likelihood ratio tests compared the baseline model with the model which included one added predictor variable to evaluate the significance of adding that individual predictor to the model (Zuur et al. 2009). Each model (i.e. the baseline model including one metric describing a single aspect of the green infrastructure) was then reduced to the most parsimonious model via stepwise backward model selection (function *stepAIC*, *MASS* R package). Dependent and predictor variables in the final model were scaled (function *scale*, base R functions) so that model estimates per predictor can be read as standardized effect sizes (SES), rendering them comparable between predictors.

Genetic diversity

Population size affects genetic diversity (Young et al. 1996), yet population size itself may be influenced by landscape context (Wiegand et al. 2005). We thus first tested how both focal and landscape population size of *G. verum* were affected by landscape characteristics. Next, we investigated how characteristics of the landscape, its green infrastructure and the focal grasslands affected genetic diversity of the focal grassland population (A_{Focal} and $H_{\text{E-Focal}}$) and the landscape population ($A_{\text{Landscape}}$ and $H_{\text{E-Landscape}}$). For both population size and genetic diversity measures, we used the GLM procedure described above. Since we also aimed to identify the extent of additional landscape-scale genetic diversity present in *G. verum* ramets occupying the green infrastructure beyond that present within the focal grassland populations, focal grassland genetic diversity was also added to the baseline model for the models on landscape-scale genetic diversity. We used rarefied allelic richness of the focal patch ($A_{\text{R-Focal}}$) to compensate for differences in numbers of sampled individuals among focal grasslands (range 11–30). We did not test rarefied allelic richness at the landscape-scale ($A_{\text{R-Landscape}}$), as rarefaction will likely obliterate patterns in genetic diversity which would be attributable to variation in landscape-scale population sizes, which is of interest to this study.

Hierarchical interactions between landscape characteristics in driving genetic variation

Finally, we explored how the characteristics of the landscape, its green infrastructure and the focal grasslands hierarchically interact to determine genetic diversity and composition simultaneously. We again turned to the concept of nestedness to address possible hierarchical interactions. This time, however, we analysed the characteristics of a single maximally packed, 1883 ramets × 185 alleles presence–absence matrix, which encompassed all individual ramets from all 35 landscapes (Almeida-Neto et al. 2008). This maximally packed matrix is ordered such that individual ramets with more alleles are in the top rows of the matrix, whereas alleles with the highest incidence occupy the columns to the left. We analysed the rank-order of the ramets in this packed matrix (i.e. the row number of a ramet in the packed matrix; the ramet with most alleles has highest rank (1); and the ramet with least alleles the lowest rank (1883)) in relation to the characteristics of the landscape, its green infrastructure and the focal grasslands. The rank-order of each individual ramet captures both its genetic diversity and composition. Hence, understanding the ecological processes driving the position of individual ramets within this matrix, elucidates factors controlling the nested genetic structure of plant populations occupying fragmented landscapes. Moreover, the follow-up analysis described next, provides further insights into the hierarchical interactions among the driving mechanisms of population genetic structure and diversity (Ulrich et al. 2009). Based on the rank-order of the ramets, we constructed a conditional inference tree via non-parametric recursive binary partitioning (Hothorn et al. 2006). Such trees combine tree-structure based regression and conditional inference procedures, introducing formal statistical hypothesis testing via permutational procedures to both variable selection and stopping during binary partitioning (Hothorn et al. 2006). The output is a tree diagram with branches determined by statistically significant splitting rules and terminal nodes that contain the mean group response. Hence, the tree classification model identifies values for landscape and habitat characteristic predictors which effectively group individual ramets according to their similar contribution to unique genetic variation within the landscape (i.e. their position in the nestedness matrix). From this, we were able to determine the landscape context which is associated with a higher degree of unique genetic variation. Predictors included (1–7) listed above, and 8) *Focal grassland* (binary variable: 0 the ramet was sampled in the surrounding landscape; 1 in the focal grassland) and 9) *Semi-natural grassland* (binary variable: 0 the ramet was sampled in habitat elements forming the green infrastructure; 1 in any managed semi-natural grasslands in the landscape) as predictor variables to model the nested rank of a ramet as dependent variable. Modelling parameters included 1) min. split size of 100, i.e. min. 100 ramets have to be contained in a node to test for further splitting; 2) min. bucket size of 50, i.e. an end tree branch contains min. 50 ramets to be allowed to be split off; 4) test-type was set to ‘quad’, setting the asymptotic χ^2 distribution as the conditional distribution for calculation of p; 5) test-statistic was set to ‘Univariate’ and 6) level of significance (α) was set to 0.05.

Results

Population genetic structure

Neither structural nor inferred functional connectivity, nor any of its components, had an impact on landscape-scale population genetic structure of *G. verum*, as quantified via the DAPC metric. Only land-use history (restored versus ancient) of the focal grassland had a moderately strong effect on this variable (standardized effect size (SES) = −0.29, 8.33% deviance explained), albeit marginally significant at p = 0.098. The genetic compositional similarity between *G. verum* individuals in the focal grassland and individuals in the surrounding green infrastructure may well tend to be higher if the focal grassland had been restored.

However, distinct patterns in nestedness were uncovered within *G. verum*’s landscape-scale population genetic structure. While neither structural nor inferred functional connectivity affected the degree of nestedness in the landscape, the abundance of *G. verum* in the landscape (SES = 0.33, p = 0.04) as well as higher genetic diversity in the landscape surrounding the focal grassland (SES = −0.62, p < 0.001) both significantly lowered nestedness in the landscape, combining to explain 34% of model deviance. However, the most parsimonious model (75.56% deviance explained) only included a strong, negative effect of road infrastructure (SES = −0.79, p < 0.001; and a small effect of the difference in genetic diversity between the landscape and focal grassland, SES = −0.16, p = 0.12). Combined, increasing road densities thus strongly reduced nestedness in the landscape, most likely by supporting *G. verum* in the landscape and reducing the difference in genetic diversity and differentiation between the focal grassland and the surrounding landscape. For full model results, see Supporting information.

Genetic diversity

Galium verum’s population size in the focal patches increased significantly as both structural and inferred functional connectivity increased, although the latter had a markedly larger effect size and doubled the explained variation in population size (SES of 0.50 versus 0.40 and 34.9 versus 16.3 % Deviance Explained, respectively). When the connectivity metrics were decomposed into their components, we found that focal population size increased as both more road infrastructure and more semi-natural grassland was present (SES = 0.72, p < 0.001 and SES = 0.61, p < 0.001, respectively). In these models, increased landscape occupancy of *G. verum* additionally increased focal population size, capturing more of the population size variation than the connectivity metrics per se (up to 63.12% deviance explained). In contrast, neither connectivity metric nor their components affected *G. verum*’s landscape-scale population size (% explained deviance = 0.02–5.44%; p = 0.21–0.94). However, size of *G. verum* populations at the landscape-scale increased significantly and only with increasing abundance of *G. verum* in the landscape (p < 0.001; SES = 0.71).

In focal grasslands, allelic richness and gametic heterozygosity did not correlate with structural connectivity, yet both increased significantly as inferred functional connectivity increased (Fig. 2d), although model performance was comparatively low to moderate ($A_{\text{R-Focal}}$ SES = 0.39, p = 0.04; H_S SES = 0.40, p = 0.02; 11.91 and 14.51% deviance explained at p = 0.06 and p = 0.03, respectively). However, more road infrastructure led to a steep rise in both allelic richness and gametic heterozygosity (Fig. 2a), explaining much of the variation in both genetic diversity metrics ($A_{\text{R-Focal}}$: SES = 0.70, p < 0.001; H_S: SES = 0.54, p = 0.001; 48.67 and 28.94% deviance explained at p < 0.001 and p = 0.001, respectively). None of the remaining landscape or focal grassland characteristics such as *G. verum* landscape abundance, forest cover or land-use history significantly explained variation in focal grassland genetic diversity.

At the landscape scale, allelic richness increased significantly with increasing inferred functional connectivity (Fig. 2e; SES = 0.29, p = 0.04), yet it did not respond to changes in structural connectivity (SES = 0.24, p = 0.09). Moreover, the effect of inferred functional connectivity seemed due to increasing road density, as road infrastructure significantly increased allelic richness (Fig. 2b; SES = 0.35; p = 0.04). Both increasing allelic richness in the focal patch (SES = 0.36–0.62, p = 0.03 to <0.001) and the occupancy of *G. verum* in the landscape (SES = 0.36–0.49, p = 0.01 to < 0.001) also consistently, significantly and additively increased landscape-scale allelic richness, at larger effect sizes than any connectivity measure. While neither structural nor inferred functional connectivity, or any of their components, had any significant effect on landscape-scale gametic heterozygosity, this measure significantly increased only in response to increasing allelic richness in the focal grassland (SES = 0.70–0.76, p < 0.001), explaining ca 57% of the variation in gametic heterozygosity.

Hierarchical interactions between landscape characteristics in driving genetic variation

The conditional inference tree analysis split the 1883 *G. verum* ramets into eight distinct clusters (Fig. 3). The landscape occupancy of *G. verum*, inferred functional connectivity, location in or outside of a semi-natural grassland, restoration status, total semi-natural grassland habitat and road infrastructure together provided the highest explanatory power for the nested structure of *G. verum* ramets across the fragmented landscapes. The genetically most impoverished groups of ramets were those in low-occupancy landscapes (median rank of 1134 versus median rank of 897 in high-occupancy landscapes; p < 0.001),

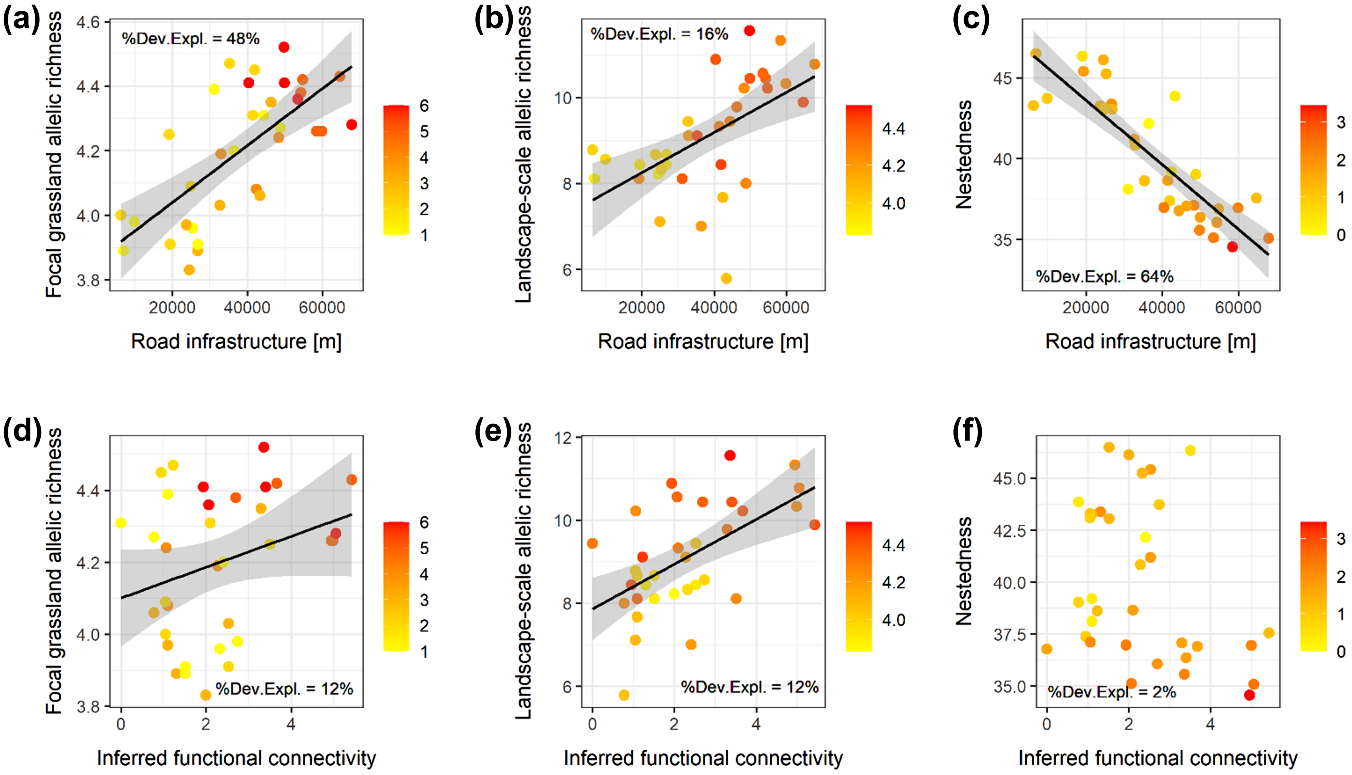

Figure 2. Impact of road infrastructure versus the impact of inferred functional connectivity on genetic variation of the grassland perennial *Galium verum* across 35 landscapes in NW-Europe, modelled via general linear models: (a and d) allelic richness in the focal grassland (color legend = population size), (b and e) landscape-scale allelic richness (color legend = allelic richness in the focal grassland) and (c and f) degree of nestedness of the landscape population genetic structure (NODF metric, Almeida-Neto et al. 2008; color legend = absolute difference in allelic richness between landscape and focal populations). Black regression lines identify significant relations at $p < 0.05$. %Dev. Expl. represents the amount of deviance explained by adding the predictor variable (road infrastructure or inferred functional connectivity) to the baseline model. For full analytical results, see Supporting information.

i.e. where *G. verum* occupied less than 70% of sampled landscape segments. In these landscapes, increasing inferred functional connectivity had a buffering effect, as the median rank of functionally connected landscape populations (median rank of 990) was significantly lower than that of landscape populations with less functional connectivity (median rank of 1211; $p < 0.001$). Even in high-occupancy landscapes, ramets sampled in surrounding habitat elements were a genetic subset of those ramets sampled within semi-natural grasslands (median rank of 910 versus 864; $p = 0.023$). *Galium verum* ramets in restored semi-natural grasslands were in turn compositional genetic subsets of ramets in ancient semi-natural grasslands (880 versus 716 as median rank, $p = 0.039$). Still, restored grasslands did benefit from increasing inferred functional connectivity ($p = 0.04$), as *G. verum* ramets in better functionally connected landscapes (median rank of 878) proved to be a genetic subset of those *G. verum* ramets occupying restored grasslands in less functionally connected landscapes (median rank of 1112). Moreover, the genetic composition of *G. verum* ramets growing in ancient semi-natural grasslands in high-occupancy landscapes further depended on the interaction with total amount of grassland habitat ($p = 0.029$) and road infrastructure ($p = 0.035$). *Galium verum* ramets surrounded by low amounts of grassland habitat (median rank = 949) were compositionally impoverished compared to ramets surrounded by larger amounts of grassland habitat (median rank = 662). Finally, the most genetically diverse *G. verum* ramet groups were found in ancient semi-natural grasslands in high-occupancy landscapes with large areas of remaining grassland and with a low road density (median rank of 519 versus 930 in high road density landscapes).

Discussion

Galium verum's population size increased with increasing habitat amount in the surrounding landscape, particularly road infrastructure and other semi-natural grasslands. Road verges are a habitat where *G. verum* can thrive (Dunnett et al. 1998), certainly when sufficiently old (Auffret and Lindgren 2020), wide (3–4 m; Aavik and Liira 2010) and properly managed (Jakobsson et al. 2018). This underlines the importance of habitat amount for local and landscape-scale population dynamics (Wiegand et al. 2005), given the weaker, albeit positive effects of structural and inferred functional connectivity (Watling et al. 2020). Both increasing inferred functional connectivity and increasing amount of road infrastructure also increased genetic diversity and gametic heterozygosity in

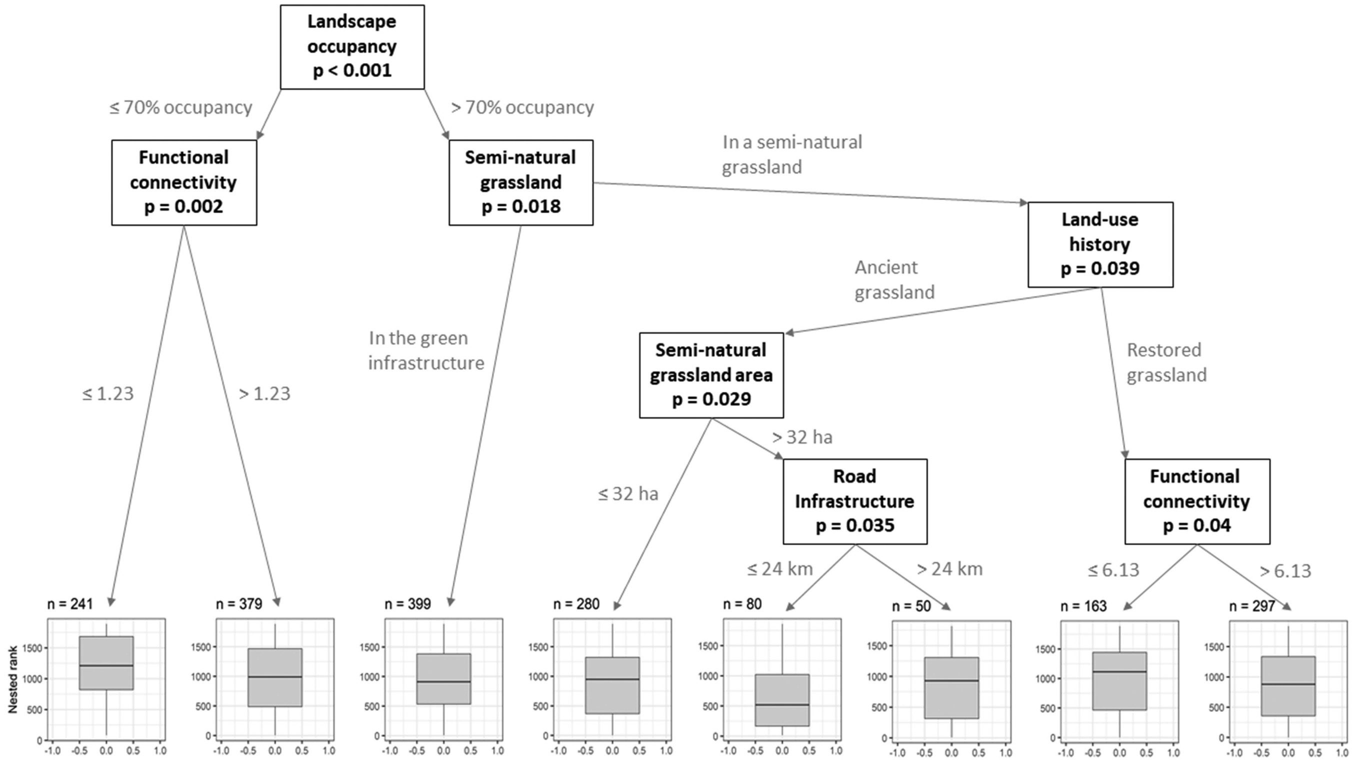

Figure 3. Conditional inference tree following recursive binary partitioning of 1883 *Galium verum* ramets according to their position in a maximally packed nested matrix (so-called rank), calculated using the NODF metric applied to the raw 1883 ramets × 185 alleles presence–absence matrix reflecting their genetic diversity and composition. The closer the ramets' rank is to one, the more genetically diversified the ramet is. The closer a ramets' rank is to 1883, the ramet is increasingly genetically impoverished whilst being of compositional subset of the individual ramets preceding it. The splitting rules presented along the tree branches are based on the best performing focal grassland or landscape characteristic (node) for splitting each node after permutational testing (p-value), that is the characteristic which maximizes homogeneity of the two nodes following a split. The boxplots (median, 25 and 75th percentile (box) and 1.5 × interquartile range (whiskers)) at the branch end present the mean position in the maximally packed matrix of the *G. verum* ramets of that branch.

G. verum focal populations. This effect could be attributed indirectly via the positive impact of inferred functional connectivity and road infrastructure on population sizes, given the latter's predictive power for genetic diversity (Young et al. 1996). Alternatively, this may suggest a degree of ongoing dispersal towards the focal grasslands, as we observed an added positive feedback on focal population sizes by an increasing landscape presence of *G. verum*, serving as a proxy for high propagule pressure. Hence, besides supporting *G. verum* populations in the green infrastructure, the road network in particular may act as functional corridors for pollen and seed dispersal (Tikka et al. 2001), facilitating meta-population dynamics (Suárez-Esteban et al. 2013).

The *G. verum* populations in the green infrastructure proved to be important for genetic diversity at the landscape-scale as well, with increasing population sizes translated into a parallel increase in landscape-scale genetic variation ($R_{Pearson}$ Allelic richness versus Landscape population size = 0.61, $p < 0.001$; Young et al. 1996), enhanced further by high road densities (Tikka et al. 2001, Suárez-Esteban et al. 2013). Inversely, if the green infrastructure is not occupied, habitat amount and connectivity become irrelevant to the species' landscape population size, and its level of genetic variation. Although trivial per se, this finding thus acknowledges the value of genetic variation contained within the green infrastructure, highlighting the vulnerability of more specialised grassland species absent from the green infrastructure and for which landscape-scale genetic diversity is therefore likely to be affected to a much greater degree by the loss and fragmentation of core grassland habitat. Still, even for *G. verum*, a species capable of effectively utilising non-core, green infrastructure habitats such as road verges, the focal grassland's genetic diversity proved at least or even more important for the landscape genetic diversity and gametic heterozygosity compared to the genetic diversity contained within the surrounding green infrastructure. Large semi-natural grasslands seem instrumental in maintaining high levels of landscape-scale genetic diversity. Indeed, populations in the green infrastructure consistently proved to be a compositional subset of the genetic composition of focal grasslands' *G. verum* populations. This nested pattern was strengthened by focal populations holding more of the landscape's genetic diversity, and by an increasing presence of *G. verum* in the landscape. The latter suggests that populations in the green infrastructure carry limited unique genetic variation, because they are likely smaller, with genetic drift eroding their genetic variation (Leimu et al. 2006). Interestingly, high road density alone strongly diminished the nested population structure (Fig. 2c). Large amounts of road verges may thus weaken nestedness, achieving this mechanistically by enabling both high landscape occupancy through increased habitat availability and by enabling gene flow from the focal grassland into the surrounding landscape along their resident *G. verum* populations. Road verges therefore may sustain both pollen and seed movement (Phillips et al. 2020). Pollen and seed flow along linear landscape features also significantly enhance plant reproductive success at receiving grasslands (Cranmer et al. 2012). This strengthens plant functional connectivity, bolstering population growth and size in isolated grasslands, helping them maintain genetically diverse plant populations (Fig. 2a, d).

The landscape and its green infrastructure, particularly road infrastructure, leave a discernible imprint on population genetic diversity and structure (González et al. 2020). Moreover, focal grassland characteristics, more specifically its history, may potentially exert further control over the landscape's population genetic structure. If the focal grassland had been passively restored, *G. verum* ramets in the green infrastructure appeared more likely to be incorrectly assigned by the DAPC to the focal grassland population, or vice versa. This may suggest that the genetic similarity between ramets in the green infrastructure and the focal grassland is higher where the focal grassland had been restored. While this might advocate that the surrounding green infrastructure promotes natural colonization into restored grasslands, the results also seem to reaffirm the unique genetic diversity contained within ancient grasslands. Moreover, considering severe time-lags in recovery of genetic diversity (Reynolds et al. 2013), and the need for a diverse landscape-wide gene pool to sustain such recovery (Helsen et al. 2013, Aavik and Helm 2018), this suggests the conservation of ancient semi-natural grasslands should take precedence over restoration efforts, even if grassland restoration can lead to a rapid recovery of genetic diversity (Helsen et al. 2013, Lehmair et al. 2020).

The imposed hierarchical structure of the drivers of genetic composition among individual *G. verum* ramets endorsed population-based results. High species' occupancy of the green infrastructure was pivotal, likely positively affecting meta-population viability by facilitating spatial gene-flow. Increased functional connectivity – inferred as the integrated effect of grassland amount and road infrastructure – significantly buffers the loss of genetic variation in low-occupancy landscapes. Still, *G. verum* ramets were significantly less genetically diverse compared to their counterparts in high-occupancy landscapes, irrespective of further local and landscape characteristics. If too few individuals occupy the green infrastructure, the latter's potential to sustain effective gene flow seems impaired. Even in high-occupancy landscapes, *G. verum* ramets in green infrastructure elements are an impoverished, genetic subset of those in semi-natural grasslands, likely populating small, less genetically diverse populations. Still, as outlined before, this does not undermine their importance in reinforcing the green infrastructure's corridor function. Moreover, genetic variation in *G. verum* ramets continued to increase with the presence of semi-natural grasslands, both in terms of their amount (increasing propagule pressure; Hufbauer et al. 2013) and their land-use history. The latter underlines that *G. verum* ramets in ancient grasslands hold unique genetic diversity no longer present in restored grasslands. Increased functional connectivity does, however, temper this pattern, suggesting some recovery of genetic diversity in functionally well-connected, restored grasslands. On the other hand, *G. verum* ramets in poorly connected, restored grasslands in high-occupancy landscapes closely resemble impoverished *G. verum* ramets in poorly connected, low-occupancy landscapes in terms of genetic composition and diversity (Fig. 3). While this predicts little genetic recovery in these restored grasslands in spite of abundant *G. verum* in the surrounding landscapes, these observations clearly highlight the need for an integrated landscape approach to e.g. restoration site selection (Aavik and Helm 2018).

Our study firmly reinforces that structural connectivity may be an incomplete proxy for functional connectivity (Aavik et al. 2013, Auffret et al. 2017b), with the latter consistently outperforming structural connectivity in explanatory power. Still, structural connectivity provided by the integrated effect of grassland amount and the green infrastructure remains an important component of functional connectivity, with road infrastructure appearing to be a key part of the structural element of inferred functional connectivity in *G. verum*. Indeed, neither metric incorporating all green infrastructure elements, nor any other landscape characteristic explained genetic variation better, besides landscape occupancy. In consequence, the importance of the road infrastructure in *G. verum* is considerable, suggesting linear green infrastructure may fulfil an important functional role when occupied (cf. Holderegger and Di Guilio 2010). Nevertheless, caution remains warranted upon extrapolation towards management and policy recommendations. To be able to assess green infrastructure's contribution to plant functional connectivity, the focal species needed to be sufficiently common in the green infrastructure. This implies that our focal species likely possesses a suite of functional traits helping it survive in the green infrastructure (Dunnett et al. 1998, Hooftman et al. 2021). *Galium verum* is a clonal grassland perennial, tetraploid and self-compatible (Grime et al. 1988), and its vast seed production sustains spatial and temporal dispersal (Auffret and Cousins 2013, Plue and Cousins 2018). All these traits reduce susceptibility to genetic erosion (Hamrick and Godt 1996, Honnay and Jacquemyn 2007, Plue et al. 2018), lowering local extinction risk, as noted in species with similar trait syndromes (e.g. *Campanula rotundifolia*; Plue et al. 2017). As many grassland specialists possess quite contrasting trait syndromes, being diploid, short-lived, non-clonal and poor spatial and temporal dispersers (Saar et al. 2012, Auffret et al. 2017a, Plue et al. 2018), the green infrastructure's potential to sustain functional connectivity may well be lower for many grassland specialists (cf. Saura et al. 2014). Ultimately, low functional connectivity is therefore likely to lead to lower species and trait diversity in plant communities in the green infrastructure, and in remaining grasslands over time.

In conclusion, the protection and preservation of ancient semi-natural grasslands in human-dominated landscapes should be a top conservation priority, in both management and policy actions (Hodgson et al. 2011, Watson et al. 2016), as opposed to efforts prioritizing restoration and creating new green infrastructure. First, although green infrastructure occupancy may promote plant functional connectivity in a common grassland plant as *G. verum*, this may be less effective for many other grassland specialists, given their low occupancy rates, poor colonization capabilities and environmental requirements (Saar et al. 2012). Second, the further degradation of habitat quality in remaining grasslands and the green infrastructure needs to be reversed to prevent further biodiversity loss (Chase et al. 2020), requiring a challenging, time-demanding and sustained effort (Jakobsson et al. 2018, Vanneste et al. 2020). Third, even though genetic diversity seems to recover in some restored grasslands, ancient semi-natural grasslands still contained unique genetic diversity, supporting outward gene-flow into the surrounding landscape. Management and policy focus should therefore lie with the conservation and protection of remaining, ancient semi-natural grasslands, managed from an integrated landscape perspective (cf. DiLeo et al. 2017, Plue et al. 2019). This combination will be a critical first step towards insuring landscape genetic and species diversity and sustaining future landscape-wide recovery (Aavik and Helm 2018). Moreover, even those species present and benefitting from a functioning green infrastructure still depend upon remaining semi-natural grasslands. Without the latter, the green infrastructure will likely lose much of its functional importance to support plant functional connectivity and meta-population dynamics in degraded human-dominated landscapes.

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Data availability statement

The full dataset (Microsatellite genotype for all 1926 sampled *G. verum* ramets and all landscape characteristics, focal patch characteristics and population data, linked to each individual *Galium* ramet) is available from the Figshare Repository, <https://doi.org/10.6084/m9.figshare.20528868.v1> (Plue et al. 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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