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RESEARCH ARTICLE

A source of hidden diversity: soil seed bank and aboveground populations of a common herb contain similar levels of genetic variation

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ABSTRACT

- In many landscapes, successful re-establisment of plant populations depends on the presence of diaspores, either near or directly beneath sites to be restored. The soil seed bank is, therefore, an important part of ecosystem resilience and a vital pillar for regeneration of genetic diversity in many plant populations. However, regeneration from the soil seed bank and the siubsequent restoration can only be considered successful when genetic diversity of restored populations is not eroded nor genetic differentiation inflated.
- We compared genetic variation within and among soil seed bank and aboveground populations of *Origanum vulgare*, to test whether genetically variable populations can be restored from the soil seed bank. We explored levels of genetic diversity within aboveground populations and the corresponding soil seed banks. Furthermore, we assessed the extent to which the soil seed bank differs genetically from the aboveground population.
- Levels of genetic diversity were to generally similar in aboveground populations and the corresponding soil seed banks. Only levels of inbreeding were slightly higher in the lower layer of the soil seed bank compared to the aboveground populations, probably because of selection processes acting against homozygotes accumulating in the seed bank. Furthermore, significant genetic differentiation between the aboveground population and the corresponding seed banks was completely absent. Across all sites, genetic differentiation between the soil seed bank was similar to that between aboveground populations, probably due to the absence of severe climate conditions, strong bottlenecks or disturbance events.
- Our conclusions support the possibility of successful re-establishment of healthy, genetically variable plant populations after aboveground destruction or following soil re-allocation from persistent seed banks.

INTRODUCTION

Soil seed banks are one of the most remarkable characteristics of plants as they do not solely rely on seed dispersal in space but also in time by storing long-term dormant diaspores in the soil (Bakker *et al.* 1996b; Lamont & Enright 2000; von Blanckenhagen & Poschlod 2005). Through their soil seed banks, plants mitigate consequences of environmental and demographic stochasticity, which occur across a range of climate zones, habitats and life history types (Baskin & Baskin 2011). Especially for rare species or taxa in environments with harsh and highly unpredictable conditions, seed banks provide bet-hedging, one of the pivotal possibilities for reducing long-term risk (Zaghloul *et al.* 2013). Species occupying dynamic habitats with strong disturbance regimes, such as sites influenced by flood events or forest gaps, possess high-density seed banks, exceeding 10,000 seed·m^{-2} (Kiss *et al.* 2016; Poschlod & Rosbakh 2018). In calcareous grasslands, where conditions are relatively stable and potential disturbances are mainly related to grazing or mowing, a subset of species build persistent seed banks, but usually to densities not exceeding 5000 seed·m^{-2} (Bossuyt & Honnay 2008). In some cases, seed banks in calcareous grasslands can reach up to 8000 seed·m^{-2} (Poschlod & Jackel 1993; Karlík & Poschlod 2014).

Currently, the role of seed banks has become increasingly important in the context of ongoing climate change, which deepens fragmentation processes and fosters area loss of temperate European calcareous grasslands. Previous research revealed that small and fragmented populations are prone to extinction due to environmental and demographic stochasticity and possible genetic erosion (Andrén 1994; Young *et al.* 1996; Honnay *et al.* 2005). In addition, as a genetic consequence of fragmentation, common species seem to suffer more genetic erosion than rare species (Honnay & Jacquemyn 2007). Especially outcrossing species, depend on gene flow between populations to maintain genetic variation. The ongoing fragmentation and increasing distance between remaining populations make chances to exchange pollen less likely. Simultaneously with the fragmentation, decreasing population size leads to a further loss of genetic diversity (Honnay & Jacquemyn 2007).

However, long-lived seeds stored in soil seed banks may help to slow these processes as they face a lower fragmentation-

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caused extinction probability than species with short-lived seeds (Piessens *et al.* 2005). Stöcklin & Fischer (1999) found that in isolated grassland fragments, species possessing seeds with high longevity (>5 years) were less prone to extinction than taxa with low seed longevity. Tonsor *et al.* (1993) pointed out that soil seed banks might serve as a "genetic memory", conserving changes in the genetic constitution of populations, and retaining uncommon alleles over time. This will protect against unpredictable environmental dynamics and extreme conditions (Zaghloul *et al.* 2013), disturbances (Bosbach *et al.* 1982), or changes in breeding system with temporary or spatial variation in selfing rates (Schulz *et al.* 2018). Thus, seed banks buffer directional selection triggered by fluctuations in environmental conditions (Templeton & Levin 1979).

In the context of restoration, not only genetic diversity contained in surrounding populations (Iberl *et al.* 2022), but also the genetic diversity stored in soil seed banks may potentially contribute to restoration of genetically variable aboveground populations. For such an approach, the level of genetic diversity in the soil seed bank should, however, be at least as high as that of the present aboveground population (Honnay *et al.* 2008). Several studies have tested the ecological and evolutionary effects of long-lived diaspores stored in soils. Both in annuals (Lundemo *et al.* 2009; Falahati-Anbaran *et al.* 2011; Hanin *et al.* 2013) and perennials (Falahati-Anbaran *et al.* 2011), the soil seed bank can extend effective population size and outweigh a random loss of genetic variation caused by genetic drift (McCue & Holtsford 1998). However, previous findings on genetic diversity are inconsistent. Three situations have been reported: (1) higher levels of genetic diversity in the soil seed bank than in the aboveground population (McCue & Holtsford 1998; Morris *et al.* 2002); (2) no significant differences between soil seed bank and aboveground population (Mahy *et al.* 1999; Hanin *et al.* 2013; Plue *et al.* 2017); and (3) frequent detection of lower genetic diversity in seed banks than in aboveground populations (Tonsor *et al.* 1993; Cabin *et al.* 1998; Zaghloul *et al.* 2013; Schulz *et al.* 2018). A meta-analysis (Honnay *et al.* 2008) revealed no evidence that high levels of genetic diversity are stored in the soil seed bank. This was also confirmed by Mandak *et al.* (2012), who suggested that the role of the seed bank is rather to maintain than to accumulate genetic diversity.

In our study, we analysed levels of genetic variation within and between the soil seed bank and corresponding aboveground populations of a common dry grassland species, *Origanum vulgare*. Unlike many previous studies, we also examined the vertical distribution of genetic variation, as we tested for potential differences between upper and lower soil layers. In the absence of disturbance regimes, deeply buried seeds were considered older than those near the surface (Grandin & Rydin 1998), and deeper soil layers were thought to accumulate seeds with higher longevity (Bekker *et al.* 1998). Moreover, the fraction of smaller seeds may fall deeper into soil fissures. Koch *et al.* (2003) found that increasingly dynamic habitats had less similarity between vegetation and soil, as well as reduced genetic homogeneity of seed bank populations in different soil layers. Consequently, we tested whether upper and lower layers of the soil seed bank differ from each other and from the aboveground population in terms of genetic variation. We analysed this in the context of restoration, since the genetic variation stored in the soil seed bank may help to buffer the detrimental effects of fragmentation (Poschlod *et al.* 1998). More specifically, we asked following questions:

1 How large is the genetic diversity maintained in the seed bank compared with the aboveground population?
2 How strong is genetic differentiation between aboveground populations and their corresponding seed banks and among the soil seed bank and aboveground populations from different study sites?
3 Can genetically variable populations of *O. vulgare* be restored from the soil seed bank, particularly after massive disturbance or local extinction?

MATERIAL AND METHODS

Study species

We selected *O. vulgare* (Lamiaceae), a perennial, long-lived, rhizomatous aromatic forb confined to dry grasslands, scrub, woodland fringe vegetation and thermophilous oak forests, but also found in highly disturbed alluvial meadows (Oberdorfer 2001; Van Looy *et al.*, 2009). This species has a long-term persistent soil seed bank and often produces high-density seed banks (Poschlod *et al.* 1991; Kleyer *et al.* 2008; Kiss *et al.* 2016). *O. vulgare* successfully colonizes restored calcareous grasslands (Helsen *et al.* 2013); is a gynodioecious species, predominantly outcrossing but with the possiblity of selfing (Klotz *et al.* 2002). It is pollinated by insects, mainly honeybees, bumblebees and hoverflies (Janovsky 2020). *O. vulgare* has dry fruits in clusters of four one-seeded nutlets. Seeds are small, oblong and egg-shaped, 0.9–1.3-mm long and smooth (Slavik 2000). Seeds are usually dispersed by gravity, wind and animals (Poschlod *et al.* 1998; Klotz *et al.* 2002). Germination takes place in spring in vegetation gaps.

Study area and sampling design

We selected five calcareous grasslands on the Franconian Jura in southeastern Germany (Table S1). The distance between study sites ranged from 0.2 to 17.0 km at 360–445 m a.s.l., and the climate is temperate-subcontinental. The selected grasslands are predominantly surrounded by woodland, scrub, hay production grasslands or arable fields. At each study site we established five sampling plots of 8 × 10 m containing *O. vulgare*. Each plot was divided in 80 subplots (10 rows × 8 columns) of 1 m^2. Using a chessboard design (Figure S1), we sampled plant and soil material in four subplots per row. Per subplot, two soil samples and leaf material from one individual were collected. During summer 2018, leaf material from 40 individuals per location (if available) was sampled, dried, and stored over silica gel until further processing in lab. Soil samples were extracted in March 2019 following the recommendations of Bakker *et al.* (1996a, 1996b). Sampling during late winter or early spring ensures winter stratification, providing more precise information on the persistent soil seed bank. In each subplot, we took two random soil samples using a soil corer (inner diameter 4 cm), yielding a sample area of $2 \times 12.56\ cm^2 = 25.12\ cm^2$, *ca.* 10-cm deep (*ca.* 0.25 L) per plot, according to the formula $\pi \times r^2$. In total, we sampled $0.25 \times 40 = 10$ L in each location. A sample volume of >6 L per site corresponds to the recommended sampling volume to

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capture species with persistent seed banks, including total seed banks (Hutchings 1986). Subsequently, we divided each core into two sections: 0–5 and 5–10 cm, if available. For each subplot, we separately pooled the core samples from both the upper and the lower layers in the cores. When seedlings emerged, we sampled one individual (if available) from both upper and lower soil layer from each subplot. In the soil populations, there were more samples in the upper than the lower soil layer.

Soil seed bank analysis

Before processing, soil samples were stored in plastic bags in a cool chamber at 4 °C. Soil was then washed through a 5-mm sieve cascade to remove stones and roots and 0.2-mm sieve to eliminate fine soil. This step is essential to reduce sample volume and improve conditions for seed germination (ter Heerdt *et al.* 1996). We did not detect any apparent differences in stoniness of the soil samples from different study sites. Soil samples were then spread in a thin layer *ca.* 3-mm deep into trays filled with sterilized horticultural substrate. To allow natural temperature fluctuations during day and night, and avoid disturbance by birds or mice, samples in the trays were cultivated in an unheated open cage greenhouse. The lowest temperature during this time period was −8 °C and the highest 38.9 °C (Source: www.wetterkontor.de). Samples were watered gently to ensure that seeds were not washed away. Cultivation took place from April 2019 to June 2020, until no new *O. vulgare* seedlings emerged. These seedlings were collected at juvenile stage with sufficient leaf tissue for molecular analyses. In total, 30 of these individuals per location were cultivated until flowering to determine percentage of female and hermaphrodite individuals of the gynodioecious species (total 150 individuals). Cultivation lasted from April 2019 to August 2020, until all plants had flowered. The aboveground populations contained, on average, 14.2% female plants (range: 7.7–21.4%), with a median of 15.2% female plants. The seed bank populations comprised, on average, 15.6% (range: 3.7–23.9%) with a median of 19% female plants. The difference between the two groups was not significant (Shapiro–Wilk test $P = 0.47$, paired t test $P = 0.72$).

Microsatellite analyses

For molecular analyses, we used leaf material from aboveground populations and germinated individuals from both layers of the soil seed bank. Genetic variation in the aboveground population and in upper and the lower soil layers of the soil seed bank was determined using microsatellites. We extracted nuclear DNA from silica gel-dried leaf material according to the CTAB protocol, after Rogers & Bendich (1994) and adapted by Reisch *et al.* (2007). The extracted DNA was diluted with water to 7.8 ng·µl^{-1} then used for microsatellite analysis.

In total we examined 382 samples using nine microsatellite loci developed for *O. vulgare* by Novak *et al.* (2008) (OR 10, 12–14, 27, 40, 44, 64, 77). PCR was carried out in two multiplexes of four and five microsatellites, in 10 µl reactions containing 3.2 µl template DNA (7.8 ng·µl^{-1}), 0.8 µl H_2O, 0.5 µl forward primer (10 µM) multiplex (Biomers, Ulm, Germany), 0.5 µl reverse primer (10 µM) multiplex (Beckmann/vwr), 5 µl 2× Master Mix S (Biomers). The thermal cycling profile of Novak *et al.* (2008) was employed, beginning with denaturation at 95 °C for 15 min., followed by 35 cycles of 95 °C for 60 s, 59 °C for 60 s, 72 °C for 2 min, and a final elongation step of 72 °C for 9 min. After DNA amplification, 1 µl PCR product was added to 24.8 µl Sample loading solution and 0.2 µl CEQ Size Standard 400 (both AbSciex Germa, Darmstadt). Amplified DNA fragments were sized using capillary gel electrophoresis on an automated capillary electrophoresis machine (GeXP; Beckmann Coulter) and scored with Bionumerics (Applied Maths NV, Sint Martens Latem, Belgium), version 7.6.

Statistical analyses

Microsatellite data were checked for scoring errors caused by stutter bands, null alleles or large allele dropout using MICRO-CHECKER (van Oosterhout *et al.* 2004). Allele frequencies were computed at nine loci for each sample site and cohort, *i.e.*, aboveground population and upper and lower soil layers. There was a homozygote excess in 26% of the populations for locus OR12, and 13% for locus OR14, which possibly indicates the presence of null alleles. Therefore, we performed statistical analyses with and without these loci. As results calculated using nine, eight and seven loci were similar, we included all nine loci in the analyses.

In a first step, we used all samples from the vegetation and the seed bank to perform analyses of genetic diversity. However, since we distinguished two soil layer depths in the seed bank, thus gaining three cohorts (aboveground vegetation, upper and lower soil layers), we additionally performed an analysis with an equal sample size of ten samples per each cohort. The limiting factor for this analysis was the lower number of available plants from the lower soil layer. Therefore, samples from the vegetation and upper soil layer were randomly chosen from all available samples. For AMOVA, Principal Coordinates Analysis (PCoA), NeighborNet, and Structure Bayesian cluster analysis we used the total available number of samples from the aboveground and the seed bank populations, including both soil layers.

We employed Microsatellite Analyser (MSA; Dieringer & Schlötterer 2003) to convert microsatellite data into appropriate formats for analyses. Further, we used MSA to compute a chord distance matrix, after Cavalli-Sforza & Edwards (1967), applied to construct a NeighborNet diagram with the SplitsTree4 software (Huson & Bryant 2006). Genepop on the Web software (Rousset 2008), option 5, was employed to calculate an inbreeding index (Fis). Three indices of genetic diversity were calculated using GenAlEx 6.503 (Peakall & Smouse 2006): mean number of alleles per locus (N_a), expected heterozygosity (H_e), and Shannon's Information Index (SI; results in Supplementary data). We compared genetic diversity, computed for the seed bank and the vegetation, using paired Student's t-tests. All tests and figures related to the genetic diversity indices were calculated in R4.0.5, package stats (R Core Team 2014), using packages car, psych, Lattice, and DescTools.

Overall genetic differentiation was analysed based on Fst using analysis of molecular variance (AMOVA) implemented in GenAlEx 6.503 (Peakall & Smouse 2006). We conducted a three-level hierarchical analysis to infer differences between the three groups, *i.e.*, aboveground, upper and lower seed bank populations of *O. vulgare*, pooled across all locations. We further inferred differentiation between the vegetation and its

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corresponding seed bank populations for each location using two-way AMOVA. Additionally, we separately assessed differentiation between aboveground and seed bank populations across all five study locations. We tested for significance of the detected genetic differentiation based on 999 permutations.

We further performed PCoA to infer patterns of genetic similarity between individuals, based on co-dominant genotypic distances (GenAlEx 6; Peakall & Smouse 2006). We used two approaches: first, we inferred genetic similarities between the three cohorts within each separate study location. We then analysed each of the three cohorts separately, using samples from all locations belonging to the identical cohort. This created three separate diagrams for the vegetation, upper soil and lower soil layers (diagram of the second approach in Appendix S1).

Using Bayesian cluster analysis implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000), we inferred a general genetic pattern for the whole dataset. We applied an admixture ancestor model without prior population information, assuming correlated allele frequencies. Individuals were clustered with the Bayesian Monte Carlo Markov Chain (MCMC) method using burn-in lengths of 100,000 and 100,000 MCMC sampling repeats. This program estimates probability of genotypes being distributed into 'K' number of clusters. The maximum number of clusters (K) were specified according to the number of populations +1. We considerd two cohorts (vegetation and soil) in each of the five study locations to be a population, *i.e.*, we tested a model using 10 + 1 = 11 K. Thirty independent runs for each value of K were carried out (Falush *et al.* 2003). For all individuals, a membership coefficient for every cluster was calculated. Group assignment was an *ad hoc* quantity procedure calculating ΔK *ad-hoc* statistics based on the rate of change in the log probability of data between successive K values (Evanno *et al.* 2005). We determined the best estimate of K according to the model that gave the highest value of ΔK and identical results for the multiple runs. We summarized the results using the programs Structure Harvester 0.6.34 (Earl & vonHoldt 2012) and Cluster Markov Packager Across K (Kopelman *et al.* 2015). The population membership in genetic clusters was visualized using ArcGIS 10.8. We additionally separately ran the STRUCTURE analysis for each study location including the three cohorts, *i.e.* aboveground and soil populations with partitioned soil layers. In this case, the highest possible value of K was set at 4. We otherwise used the same analysis settings as described above. For each location, we included two possible solutions for K, reflecting (1) the K-value corresponding to the highest probability of the data based on the Evanno approach (Evanno *et al.* 2005) and (2) a solution based on consistency of all 30 runs for each value of K (Kopelman *et al.* 2015).

A consensus NeighborNet graph was constructed with the software SplitsTree4 (Huson & Bryant 2006), based on the Cavalli-Sforza distances calculated using Microsatellite Analyser (Appendix S1). We further carried out an autocorrelation analysis (Appendix S1).

RESULTS

Soil seed bank

Seed distribution of *O. vulgare* in the soil was patchy. In 18.5% of the experimental 1 × 1 m subplots, no individuals of *O. vulgare* emerged from the soil samples. The number of seeds from the random soil sample was, on average, 4.55 per sampling area of 25.12 cm^2 in each experimental subplot. After correction for 1 m^2, the seed bank of *O. vulgare* contained, on average, 1,809 seeds·m^2 *O. vulgare* and this varied between locations (Figure S2b). The entire soil seed bank density (*i.e.*, including all emerged seedlings, irrespective of species) was, on average, 10,354 seeds·m^{-2}, and varied between locations (Figure S2a). In the upper soil layer, there were 736 individuals of *O. vulgare*, with an average 147.2 per study location; seed bank density was, on average, 1,465 seeds·m^{-2}. In the lower soil layer, we counted 173 individuals, on average, 34.6 per study location, and a seed bank density of 344 seeds·m^{-2}. The difference between the two soil layers in seed bank density was significant ($P = 0.02$).

Genetic diversity

We analysed 382 plants, 189 individuals from the standing vegetation, 144 seedlings from the upper and 49 from the lower soil layers. In total, we found six rare alleles (alleles with a frequency <0.035). There was an equal number of rare alleles (3) in both the aboveground populations and the upper soil layer (Table S2). However, there were no significant differences in genetic diversity between aboveground and seed bank populations, except for the inbreeding index (Fis). For all available samples, the mean number of alleles per locus (Na) in the aboveground populations varied between 2.89–3.67, average 3.42. In the seed bank (all available samples from pooled layers), the mean number of alleles per locus varied between 2.56–3.89, average 3.40. Expected heterozygosity (He) in the aboveground populations varied between 0.36–0.57, mean 0.47; in the seed bank it varied between 0.32–0.56, mean 0.47. Finally, inbreeding index (Fis) in the aboveground populations varied between −0.0007 to +0.06, average 0.01. In soil the values of Fis ranged between 0.05–0.14, mean 0.11. Here, the inbreeding index was slightly and significantly higher in the seed bank than in the aboveground population ($P = 0.04$; Table 1).

We additionally compared genetic diversity in the two soil layers (upper, lower) and the aboveground population. There was a significant difference between the aboveground population and the lower soil layer (inbreeding index, $P = 0.02$). In the aboveground population, the inbreeding index (Fis) ranged between −0.19 to 0.08, mean: −0.05. In the upper soil layers, Fis varied between −0.05 to 0.15, mean: 0.05. In the lower soil layers, Fis ranged between −0.02 to +0.38, mean: 0.21. The mean number of alleles per locus (Na) was slightly higher in both seed bank layers, and this difference was marginally significant ($P = 0.059$). In the aboveground population, Na ranged between 2.22–2.78, mean: 2.49. In the upper soil layer, Na varied between 2.67–3.11, mean: 3.00. In the lower soil layer, Na ranged between 2.67–3.22, mean: 3.04. No other indices of genetic diversity differed significantly (see Table S3).

Genetic differentiation and structure

The three-level AMOVA detected zero vertical differentiation between the groups of aboveground, upper and lower soil populations. Additionally, we performed a two-level AMOVA for each location, between the vegetation and its corresponding

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Table 1. Genetic diversity and characteristics within vegetation and seed bank populations of *Origanum vulgare*.

cohort	Ntot	%F	Na	He	Fis
All samples from the vegetation and seed bank with pooled layers					
Veg total	37.8 ± 3.19	14.2 ± 5.4	3.42 ± 0.33	0.47 ± 0.07	0.01 ± 0.05
Soil total	38.6 ± 8.99	15.6 ± 8.5	3.40 ± 0.51	0.47 ± 0.09	0.11 ± 0.04
t-test		*P* = 0.70	*P* = 0.90	*P* = 0.71	***P* = 0.04**
Ten samples per vegetation and seed bank divided into upper and lower layers					
Veg	10	–	2.49 ± 0.20	0.40 ± 0.11	−0.05 ± 0.11
Upp	10	–	3.00 ± 0.19	0.46 ± 0.03	0.05 ± 0.09
Low	10	–	3.04 ± 0.21	0.49 ± 0.05	0.21 ± 0.16
t test veg × upp		–	***P* = 0.06 w**	*P* = 0.69	*P* = 0.67
t test veg × low		–	***P* = 0.06 w**	*P* = 0.30	***P* = 0.02**
t test upp × low		–	*P* = 1.00 w	*P* = 1.00	*P* = 0.21

Cohort: membership of a particular group (veg: vegetation; upp: upper soil layer; low: lower soil layer); Ntot: total number of samples; %F: percentage female plants in populations (measured for vegetation and seed bank populations); Na: mean number of alleles per locus; He: expected heterozygosity; Fis: inbreeding index; w: pairwise Wilcoxon test. All results are mean ± SD across the five study locations; significant *P*-values are in bold.

seed bank populations from upper and lower soil layers. Overall, genetic differentiation was zero or not significant. Furthermore, we inferred horizontal genetic differentiation between the different study sites using two-way AMOVA, which detected moderate but significant levels of differentiation between the aboveground populations (Fst = 0.14; $P = 0.001$) and the seed bank populations (Fst = 0.13; $P = 0.001$) from different sites (Table 2).

Using PCoA, we constructed five diagrams related to specific study locations that included samples from all three cohorts. The first two axis explained 28.76% (Aichahof), 30.64% (Eitelberg), 30.69% (Grabenhof), 35.38% (Kühschlag), 40.47% (Undorf) of variation. Here, there was no apparent clustering according to cohort (Fig. 1). Further PCoA results were similar to results of the Bayesian cluster analysis, NeighborNet analysis and AMOVA (Figure S3).

In the Bayesian cluster analysis, individuals from the whole dataset were assigned to five groups, reflecting the five study locations. At each study location, the aboveground and seed bank populations were assigned to the same genetic cluster (Fig. 2). This was the most probable solution for K, since ΔK reached a high value (ΔK = 54.7) (Evanno *et al.* 2005). At the same time, outputs of all 30 iterations for this value of K were identical (Kopelman *et al.* 2015). Additionally, at this level of K, the mean logarithm of the probability of the data [LnP(X|K)] reached a plateau (Figure S4), *i.e.*, no additional information was gained from further increasing the number of clusters (Pritchard *et al.* 2000). Bayesian cluster analysis for each location separately, including all samples from the vegetation, upper and lower soil layers, partitioned according to the five study locations. These two approaches gave the same information, implying an absence of any apparent genetic structure between the three cohorts within each location (Figure S5).

The NeighborNet diagram corroborated findings of previous analyses and displayed five groups corresponding to the five study sites, each containing three populations corresponding to the three cohorts (Figure S6). The spatial autocorrelation revealed no significant structure within our experimental plots, neither in the aboveground vegetation nor in seed bank populations (Figure S7).

DISCUSSION

Genetic diversity

The fundamental prerequisite of a seed bank, suitable for the restoration of aboveground populations, is that the genetic diversity must be higher or as high as in the aboveground population (Honnay *et al.* 2008). Overall, genetic diversity of *O. vulgare* was comparable to a previous study (Helsen *et al.* 2013). Despite a relatively low seed bank density of *O. vulgare* in our study, levels of genetic diversity detected in the soil cohorts rival those in aboveground populations. Virtually all indices of genetic diversity calculated for the total number of samples in the vegetation and seed bank were similar or nearly equal. Only the inbreeding coefficient was slightly higher in the soil seed bank. The number of rare alleles in the aboveground populations and corresponding seed banks was identical. However, low-frequency alleles tended to be more frequent in the soil seed bank (compare Honnay *et al.* 2008).

The slightly higher level of inbreeding observed in the soil seed bank compared to the aboveground population means that in the seed bank the ratio of homozygotes was higher than in aboveground populations and, consequently, heterozygosity increases during the life cycle. This is in accordance with previous observations (Tonsor *et al.* 1993; Vitalis *et al.* 2004; Mandak *et al.* 2006; Honnay *et al.* 2008). There are three possible reasons for this pattern. First, biparental inbreeding might have caused elevated inbreeding levels relatively to random mating. However, this explanation is improbable as we detected low inbreeding values in the aboveground vegetation. Second, the potential consequences of the Wahlund effect may have led to differences (Wahlund 1928; Garnier-Géré & Chikhi 2013). The temporal – rather than spatial – Wahlund effect depends on the number of seasons represented in the seed bank as well as the among-season variance in offspring allele frequencies. However, this assumption cannot be measured without a long-term dataset. Third, there may be a heterozygote advantage (Charlesworth *et al.* 1990). Inbred seeds often fail to identify germination cues and, as a result, accumulate in the soil. Moreover, once germinated, due to lower fitness they are less likely

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Table 2. AMOVA for all *Origanum vulgare* populations.

source of molecular variation	df	SS	MS	%	F_{st}
All vegetation, upper and lower soil layer populations, pooled across all sites					
Between 3 groups: veg., upper and lower soil layers	2	5.5	2.7	0	
Between populations within the groups	12	240.8	20.1	14	**0.12*****
Within populations	749	1630.6	2.2	86	
Vegetation and corresponding seed bank populations, separately for each location					
Aichahof					
Between aboveground and seed bank population	1	3.2	3.2	1	0.006 n.s.
Within population	174	377.4	2.2	99	
Eitelberg					
Between aboveground and seed bank population	1	3.7	3.7	1	0.008 n.s.
Within population	150	347.9	2.3	99	
Grabenhof					
Between aboveground and seed bank population	1	2.2	2.2	0	0.00
Within population	164	347.8	2.1	100	
Kühschlag					
Between aboveground and seed bank population	1	1.5	1.5	0	0.00
Within population	154	394.9	2.6	100	
Undorf					
Between aboveground and seed bank population	1	0.8	0.8	0	0.00
Within population	112	176.0	1.6	100	
Vegetation and seed bank separately in 5 populations across sites					
Aboveground populations in 5 study sites					
Between aboveground populations	4	118.1	29.5	14	**0.14*****
Within populations	373	806.9	2.2	86	
Seed bank populations in 5 study sites					
Between seed bank populations	4	112.5	28.1	13	**0.13*****
Within populations	381	837.0	2.2	87	

df, degrees of freedom; SS, sum of squares; MS, mean squares; % the proportion of genetic variability; F_{st} the level of genetic differentiation. Levels of significance are based on 999 iteration steps and are indicated by asterisks (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). Significant values are given in bold.

to survive and establish as adult plants (Kalisz 1989; Tonsor *et al.* 1993). This factor is the most probable reason for the gradual decrease in homozygosity in subsequent life stages, from seeds to mature plants (Lesica & Allendorf 1992; Vitalis *et al.* 2004; Mandak *et al.* 2006).

We additionally analysed the effect of a mediating variable, soil sampling depth, on genetic differences between the seed bank and the aboveground population. Interestingly, there was no significant difference in inbreeding index between the vegetation and the upper soil layer. On the other hand, the difference between lower soil layer and aboveground population was significant. This makes sense since seeds in deeper soil layers were buried earlier than those in upper soil layers. Therefore, this is a more "ancient" genetic diversity, representing genetic diversity in the aboveground vegetation decades ago. In terms of homozygosity, this implies higher similarity between the vegetation and the upper soil layer. Since more inbred individuals are less likely to perceive germination cues, this increases their time and accumulation in the soil (Tonsor *et al.* 1993). Moreover, *O. vulgare* is a species with light-induced germination and this trait could additionally reinforce the observed pattern (Mašková & Poschlod 2021). Hence, higher inbreeding found in the seed bank would not necessarily devalue it as a source for restoration purposes. For this reason, the higher inbreeding index, especially in the lower layers of the seed bank, might jeopardize its beneficial role in restoration. Additionally, the post- and pre-germination selection would discriminate against homozygote individuals. Previous restoration results in *O. vulgare* confirm this perception. Most probably, not only the successful colonization process, but also the seed bank, prevent founder effects and genetic erosion, and thus contribute to a rapid build-up of genetic diversity in restored populations (Helsen *et al.* 2013).

In the analysis with partitioned soil layers, we found a marginally significant difference in the number of alleles per locus (Na), which was higher in both soil layers than in the vegetation. Previous observations using comparisons of seedlings or mature plants with seed banks, found allele frequencies had different patterns (Cabin 1996; Schulz *et al.* 2018). The meta-analysis of Honnay *et al.* (2008) suggested a certain accumulation of rare alleles in the soil; however, direct or indirect local selection could act as a filter on alleles present in the soil. This selection filter seems to prevent some seeds from germinating. Consequently, these alleles do not appear in aboveground populations and do not promote themselves in further generations (compare Cabin 1996). The relationship between the seed bank and the aboveground population in the *O. vulgare* populations was most probably shaped by post- or pre-germination selection. Therefore, our findings imply that the soil seed bank may contribute to maintain the evolutionary potential, especially in small and isolated populations, as previously suggested (Stöcklin & Fischer 1999; Piessens *et al.* 2005; Ayre *et al.* 2021).

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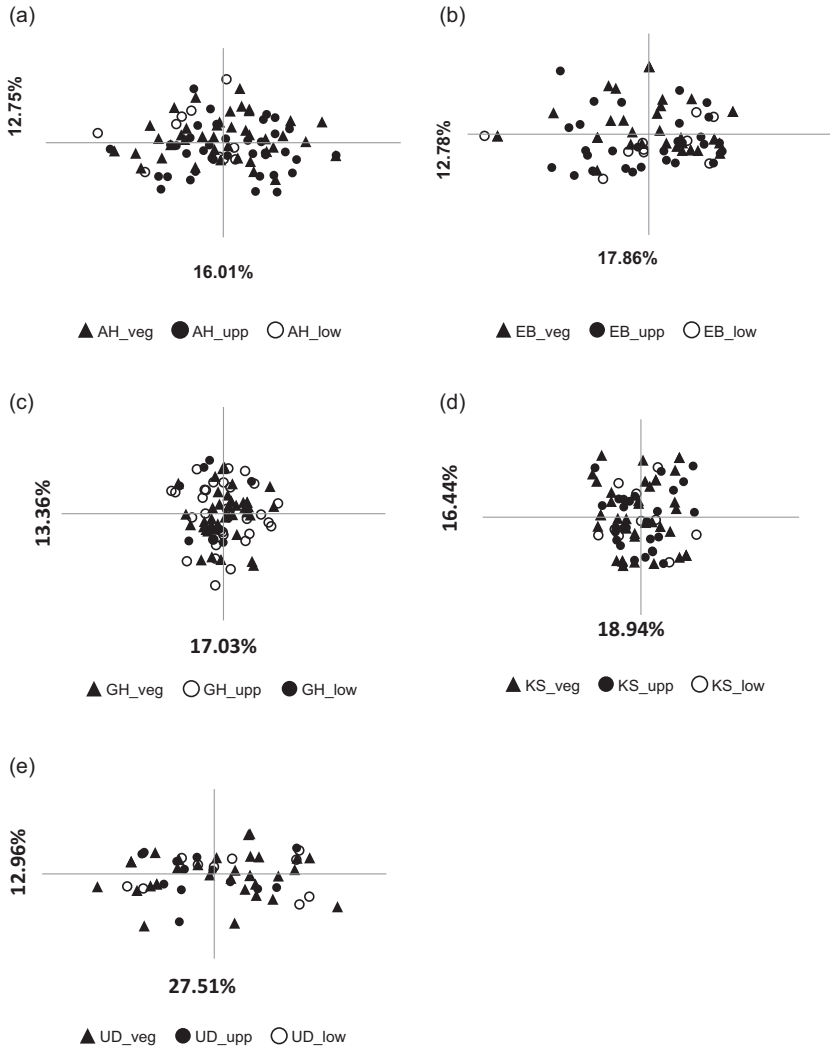

Fig. 1. Principal coordinates analysis (PCoA) generated in GenAlEx based on the microsatellite data. Each diagram relates to one of the five study locations: (a) Aichahof, (b) Eitelberg; (c) Grabenhof; (d) Kühschlag; (e) Undorf. Triangles: individuals from the vegetation (veg), black circles: individuals from the upper soil layer (upp), and empty circles: individuals from the lower soil layer (low). Samples from different cohorts were fully admixed, without any detectable separation.

Genetic differentiation

Overall genetic differentiation among aboveground populations of *O. vulgare* was higher than previously reported for this species (Helsen *et al.* 2013), most probably due to the higher distances among the studied populations and lower grassland connectivity in our experiment. Common species, especially outcrossing species, are demonstrably more dependent on gene flow between populations to maintain genetic variation than selfing species, since the former require pollen exchange between populations (Honnay & Jacquemyn 2007). If populations shrink in size and number, connectivity becomes weaker. As a consequence, not only genetic diversity is prone to erosion, but also population genetic differentiation tends to increase (Leimu *et al.* 2006; Honnay & Jacquemyn 2007; Willi *et al.* 2007). However, persistent seed banks may help to mitigate the impact of habitat fragmentation and shield species from genetic drift and population genetic differentiation (Honnay *et al.* 2008) through beneficial allele supply from buried seeds, and enhanced effective population size (Del Castillo 1994; Stöcklin & Fischer 1999; Vitalis *et al.* 2004).

In our study, there was high similarity between the aboveground population and the underlying seed banks, including both soil layers. AMOVA analyses at all levels, as well as PCoA and Bayesian cluster analysis, detected an absence of any apparent vertical genetic differentiation, *i.e.*, between the aboveground population and its corresponding seed bank. This is in contrast to some previous observations (Cabin 1996; Mandak *et al.* 2006; Zaghloul *et al.* 2013). Not surprisingly, high levels of genetic differentiation were especially pronounced in environments found in extreme climate conditions. Apparently, strong bottlenecks acting on aboveground populations – both natural and human-induced – may reinforce this differentiation paradigm (Zaghloul *et al.* 2013). However, similar to Mahy *et al.* (1999) and partly to Schulz *et al.* (2018), we did not observe this pattern. Mahy *et al.* (1999) suggested that this might be, *inter alia*, the result of a low number of sexual

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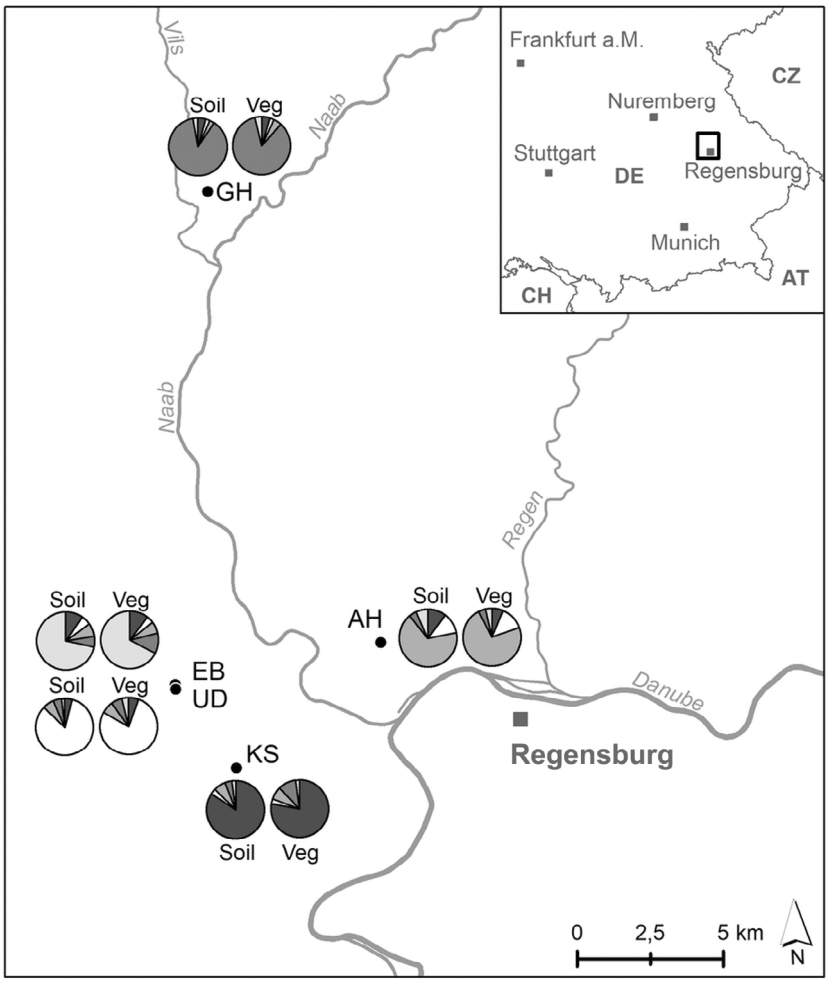

Fig. 2. Map of the studied *Origanum vulgare* populations from two cohorts defined as soil (Soil) and vegetation (Veg). Pie diagram slices are equivalent to population membership in five genetic groups inferred by Bayesian cluster analysis STRUCTURE (Pritchard *et al*. 2000). Group 1, white, Undorf (UD); group 2, light grey, Eitelberg (EB); group 3, grey, Aichahof (AH); group 4, dark grey, Grabenhof (GH); group 5, black, Kühschlag (KS).

generations since the time of population founding. In our study system, the most probable reason is the absence of strong bottlenecks, disturbances, and extreme climate conditions.

When comparing two soil depth layers, similar to Koch *et al.* (2003) for *Cardamine amara* in wet woodland, we found that the two soil cohorts did not substantially differ from each other. This is not surprising, as it most likely reflects continuous seed supply from the surface population over the years and over numerous generations. However, in the dynamic habitat of riverbanks and creeks, the two soil layers of *Cardamine amara* displayed clear genetic differences (Koch *et al.* 2003), most probably driven by recurring disturbances in upper soil layers caused by flooding events.

We also conducted a horizontal analysis of genetic differentiation, *i.e.*, between the aboveground populations at the study sites, and between the seed bank populations. The aboveground populations displayed a genetic differentiation of 14%, the seed bank populations of 13%. When analysed separately in the two soil layers, a previously described trend of lower differentiation in early life stages (*i.e.*, seed populations) was even slightly reversed (differentiation of 16% among populations from the lower soil layer). Similar patterns were observed in lower soil layers of wet meadows in *Cardamine amara* (Koch *et al.* 2003). This is in contrast to some previous observations suggesting that the partitioning genetic variation among populations increases with population age, *i.e.*, from soil seeds to mature plants (Cabin *et al.* 1998; Mandak *et al.* 2006; Zaghloul *et al.* 2013). A possible reason might be unequal severity of local selection pressures in aboveground vegetation. Thus, the persistent seed banks comprise and represent contributions of multiple generations of plants under different selection pressures in the face of environment fluctuations. The aboveground populations, in turn, have to cope with aboveground environmental conditions and usually become more differentiated than the underlying seed pools (McCue & Holtsford 1998; Zaghloul *et al.* 2013). Most probably, this is related to genotype-dependent local selection (Mandak *et al.* 2006; Honnay *et al.* 2008). However, we did not observe any apparent expression of this pattern. This might be a natural consequence of the lack of strong selection pressures in the vegetation of our study system.

In accordance with previous analyses, we detected no apparent spatial genetic structure within our study plots. This is possibly due to (i) high outcrossing rates; (ii) insect pollination in the study species within the populations, which contributed to a random distribution of alleles within the boundaries of the

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experimental plot; (iii) the low levels or absence of clonality; and (iv) the long-term persistent soil seed bank. Evidence from previous research suggests that clonality and/or a limited seed dispersal may lead to genetic correlation over short distances (Schnabel & Hamrick 1990; Reisch *et al.* 2007; Listl & Reisch 2012). In our study, most probably, seed and pollen dispersal exceeded the spatial scale of our experiment.

CONCLUSIONS

We found that soil seed banks contain levels of genetic variation comparable to the aboveground populations. Considering our observation in the context of the ongoing fragmentation of suitable habitats, we conclude that the genetic diversity stored in the soil seed bank may counteract detrimental effects of the fragmentation process, preventing random loss of alleles through genetic drift, and slowing the increase in genetic differentiation resulting from a lack of gene flow between fragmented populations. Therefore, our conclusions support the possibility of successful re-establishment of genetically variable plant populations after aboveground destruction or after soil re-allocation from persistent seed banks. The study sites are in a relatively constant habitat; therefore, it would be interesting to learn more about the seed bank of *O. vulgare* in contrasting environments under different selection regimes, *e.g.*, along a habitat gradient toward alluvial sites under a stronger regime of disturbance.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Location name, abbreviation and geographic position (Latitude N, Longitude E) for the five study sites of *O. vulgare*

Table S2. Rare alleles detected in the vegetation and the seed bank Names of locations are given using abbreviations of the study site names; cohorts are given as the vegetation (veg) and the upper soil layer (upp); further, locus names, allele size and allele frequencies are specified.

Table S3. Population name, abbreviation, cohort, number of samples and genetic characteristics of *Origanum vulgare* populations.

Figure S1. Design of plot with subplots applying a chessboard pattern. Numbers indicate subplots included in analyses, i.e. from which vegetation and soil samples were collected.

Figure S2. Seed bank density given as a number of seeds/m2, related to (a) all seedlings which emerged in course of our germination experiment, comprising the full scope of species; (b) all emerged seedlings of *Origanum vulgare*.

Figure S3. Principal coordinate analysis (PCoA) generated in GenAlEx based on the microsatellite data.

Figure S4. Mean posterior probability plot of STRUCTURE over 30 runs for each value of K=11 computed for the whole dataset.

Figure S5. Bayesian cluster analysis (STRUCTURE, Pritchard *et al.* 2000) for all individuals of *Origanum vulgare* from the three cohorts, vegetation (veg), upper (upp) and lower (low) soil layers, partitioned according to the five study locations.

Figure S6. NeighborNet diagram constructed using SplitsTree4 software (Huson & Bryant 2006).

Figure S7. Correlogram with the correlation coefficient r as a function of distance.

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