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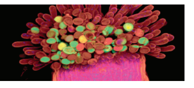

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RESEARCH PAPER

Rapid evolution in native plants cultivated for ecological restoration: not a general pattern

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ABSTRACT

- The growing number of restoration projects worldwide increases the demand for seed material of native species. To meet this demand, seeds are often produced through large-scale cultivation on specialised farms, using wild-collected seeds as the original sources. However, during cultivation, plants experience novel environmental conditions compared to those in natural populations, and there is a danger that the plants in cultivation are subject to unintended selection and lose their adaptation to natural habitats. Although the propagation methods are usually designed to maintain as much natural genetic diversity as possible, the effectiveness of these measures have never been tested.
- We obtained seed of five common grassland species from one of the largest native seed producers in Germany. For each species, the seeds were from multiple generations of seed production. We used AFLP markers and a common garden experiment to test for genetic and phenotypic changes during cultivation of these plants.
- The molecular markers detected significant evolutionary changes in three out of the five species and we found significant phenotypic changes in two species. The only species that showed substantial genetic and phenotypic changes was the short-lived and predominantly selfing *Medicago lupulina*, while in the other, mostly perennial and outcrossing species, the observed changes were mostly minor.
- Agricultural propagation of native seed material for restoration can cause evolutionary changes, at least in some species. We recommend caution, particularly in selfing and short-lived species, where evolution may be more rapid and effects may thus be more severe.

INTRODUCTION

Ecosystem restoration is now globally recognised as a key component in conservation programmes and essential to achieve long-term sustainability of our human-dominated planet (Aronson & Alexander 2013). In many cases, the critical first step of restoration projects is the re-establishment of native plant communities, with active planting or sowing as common restoration tools (McDonald *et al.* 2016). Both methods require large amounts of native seed that is either directly sown into a target locality or used in nurseries to produce planting material. The most common way of native seed production is direct seed collection from wild populations (Vander Mijnsbrugge *et al.* 2010; Kiehl *et al.* 2014). However, the demand for native seeds is currently so high that wild collection is unable to meet it (Menges *et al.* 2004; Meissen *et al.* 2015). Consequently, seed material of wild plants is increasingly propagated in seed orchards (Kiehl *et al.* 2014), and these farm-produced seeds are then used for restoration projects.

While agricultural propagation enables production of large amounts of seed, the propagation process itself may cause unintended selection or genetic drift and, consequently, change plant genetic diversity or reduce adaptation to the natural environment (Espeland *et al.* 2017). The first step in propagation of restoration material is collection of seeds from wild populations (Basey *et al.* 2015). By taking a relatively small sample from a large population, seed collection may cause genetic drift, and it may also involve unintended selection, for example, when seeds are collected only from a subset of plants that are large or flower at a specific time (Espeland *et al.* 2017). After the transfer from natural populations to farm propagation, plants face novel environmental conditions. Intraspecific competition replaces interspecific competition, stress is not as severe and heterogeneous as in nature because of fertilisation and watering, and there is usually pest control. Moreover, fitness is determined by the number of ripe seeds at the time of harvest, rather than, as in the wild, across the entire life cycle. These novel conditions will inevitably impose selection, and could affect plant genetic diversity and adaptation within a few generations (Husband & Campbell 2004; Espeland *et al.* 2017).

Based on our fundamental understanding of plant evolution, it is likely that plant material changes during cultivation (Espeland *et al.* 2017). However, experimental data are so far largely missing (but see Dyer *et al.* 2016). There is some evidence for

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phenotypic and genotypic changes in *ex-situ* collections of rare plants in botanical gardens. In these collections, population sizes are typically low, which makes them particularly susceptible to genetic drift (Ellstrand & Elam 1993), with both genetic and phenotypic consequences (Ensslin *et al.* 2011, 2015). In seed production for restoration, the situation is different because the cultivated populations are established from large initial field collections, and later generations usually grow in large populations containing several tens of thousands of individuals (Prasse *et al.* 2010). While genetic drift is likely small in such large populations, selection may significantly affect plant characteristics (Frankham *et al.* 2002). As the conditions during agricultural seed production strongly differ from those in *ex-situ* collection, we need to work directly with farm-propagated seed to evaluate the possible effects of cultivation on seed material.

Here, we used a multi-species approach to assess the effects of agricultural seed propagation on the genetic diversity and phenotypic variation of five common wild species. For each species, we worked with seeds from two to four different generations of cultivation, obtained from a large seed-producing company. This material allowed us to track changes of plant traits and genetic variability from generation to generation. We employed molecular markers and a common garden experiment to: (i) quantify changes in genetic diversity among generations under cultivation; (ii) test whether the cultivation generations were genetically differentiated; and (iii) test whether changes in molecular markers are accompanied by changes in phenotypes.

MATERIAL AND METHODS

Seed material

We worked with seeds from a company that produces certified regional seeds of wild plants, Saaten Zeller (Eichenbühl-Guggenberg, Germany). In general, the seeds of F_0 populations (Fig. 1) originate from multiple large populations (>1,000 individuals), located at least 1 km apart, within a given region. In each population, seeds are collected from at least 50 individuals to ensure high genetic variability. The offspring of these wild-collected seeds are carefully grown in horticultural settings and seeds are sometimes manually harvested to achieve a high yield of the F_1 seed. The seeds or seedlings of F_1 and subsequent generations are then sown or planted directly into agricultural fields, where the plants are grown on a large scale, and the produced seeds are machine-harvested and used for commercial purposes. The certificate rules allow propagating the seeds for up to five generations. All seeds are stored in a dedicated facility where temperature and humidity conditions are optimised to maintain germinability.

For our study, we obtained seeds of multiple cultivated generations of five species, with different numbers of source populations and cultivation generations for each: *Achillea millefolium* L. (F_1, F_2; one source population), *Centaurea cyanus* L. (F_0, F_1; one source population), *Galium album* Mill. (F_1, F_2, F_4; four source populations), *Medicago lupulina* L. (F_1, F_2, F_3, F_4; one source population) and *Plantago lanceolata* L. (F_1, F_2, F_3; four source populations). Originally, we also obtained seeds from the wild collections (F_0) of *Galium* and *Plantago*, but unfortunately our molecular analysis revealed that these seeds were only part of the original source populations (see Appendix S1), and they therefore did not allow a meaningful comparison. Our experiment thus started with the first cultivated generation, F_1, for most of the species. All studied species are self-incompatible outcrossers, except for the predominantly selfing *Medicago* (mean selfing rate 96%; Yan *et al.* 2009). *Centaurea* is an annual, *Medicago* annual or biennial, all other species are perennials.

Molecular markers

For the molecular analyses, we collected leaf material from all experimental and backup plants (see below), for a total of 11 to 22 samples per generation (mean 16.6). The samples were dried at 50 °C before the analysis. We performed an Amplified Fragment Length Polymorphism (AFLP) analysis following the protocol of Schulz *et al.* (2014). We used AFLP as it is well suited to assess genome-wide population differentiation across multiple species (see below and Appendix S1), although the limited number of markers and their dominance limits the ability to detect changes. For each species, we screened 16 primer combinations and selected four of them (seven in *Plantago*) for the final analysis (Table S1). Genotyping and marker selection followed Durka *et al.* (2017). Depending on the species, the number of AFLP markers ranged between 116 and 294, and the overall error rates varied between 0.62% and 2.0%, based on replicated analysis of three to 37 samples per species (Table S1).

To analyse the AFLP data, we used a band-based approach. Unless otherwise stated, all data analyses were done in R 3.1.2 (R Development Core Team 2015). Genetic variation within generations was quantified as mean band richness across loci using AFLPDiv (Coart *et al.* 2005). Band richness (Br) is a measure of genetic diversity for dominant markers that corrects for differences in sample size by rarefaction. To compare band richness between generations, we used ANOVA and a Tukey HSD test. To assess whether any bands were lost or gained, we checked whether bands were private or shared between generations and visualised the numbers in Venn diagrams. To test whether the observed number of private or shared bands differed from random expectations, we performed randomisations by assigning individuals 1,000 times at random to generations. Empirical values were considered to significantly differ from random expectation when they were outside the 95% percentile of the randomisations. Genetic population structure was assessed and quantified in two steps. First, we used principal coordinates analysis (PCoA) to illustrate Euclidean genetic distances between individuals. Second, we used analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) to quantify overall and pair-wise genotypic differentiation (F_{ST}) between generations, as implemented in GenAlex 6.5 (Peakall & Smouse 2012), testing for significance with 999 permutations.

Common garden experiment

To test for phenotypic differences among cultivation generations, we conducted a common garden experiment. We sowed seeds of each species and generation into seeding trays filled with germination substrate, watered and placed them in a greenhouse. For *Plantago*, we stratified the sown seeds for 20 days in a cold room at 4 °C prior to the germination. When

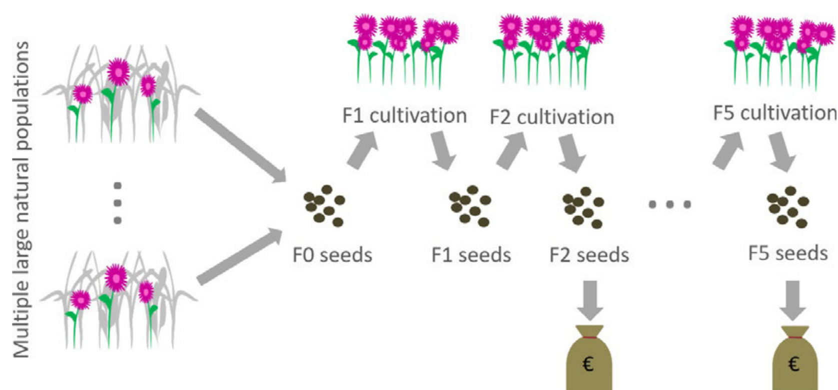

Fig. 1. Schematic of the propagation of wild plant seed for restoration purposes. The original seeds are collected from multiple large natural populations (>1000 individuals) within a region, from at least 50 plants within each population. The wild-collected seed (F_0) is mixed to ensure high genetic diversity and used for growing an F_1 generation on specialised farms. The F_1 seeds are then used to establish large-scale monoculture cultivation of the F_2 generation. The F_2 seeds are sold for restoration purposes, and a small part is used for re-establishing the next monoculture (F_3 generation). The cultivation is repeated until the F_5 generation. After that, the plantation must be re-established from a new wild seed collection.

the seedlings had developed their first true leaves, we transplanted 14 randomly selected plants per species and generation to 0.5-l pots filled with standard potting soil and placed them in a greenhouse in a fully randomised design. We watered the plants as necessary.

Four weeks after sowing (for *Plantago*: after transfer to the greenhouse) and approximately 2 weeks after transplantation, we recorded early growth for all plants and measured, depending on the species, plant height, leaf number or leaf size (Table S2). Throughout the experiment, we daily recorded the onset of flowering. We terminated the experiment after 90 days, when many of the plants had started to wither. We measured plant height, total number of inflorescences, branching, number and size of leaves, plus other species-specific traits (Table S2). In addition, we harvested the aboveground biomass of each plant. In species where this was relevant, we divided the biomass into reproductive and vegetative parts (Table S2), dried all biomass samples at 60 °C for 48 h and weighed them.

To test for differences in phenotypes between cultivation generations, we used linear or generalised linear models with Poisson, quasi-Poisson or binomial error distributions in R, where generation was tested as a fixed factor (Table S2). If the generation effect was significant, we used Tukey tests in the *multcomp* R package to compare individual generations within species (Hothorn *et al.* 2008).

Table 1. Genetic variation, measured as band richness (rarefaction sample size = 11) within each studied generation, and pair-wise population differentiation from AMOVA (F_{ST}), with significant ($P < 0.05$) values in bold.

	F_0	F_1	F_2	F_3	F_4
Genetic variation (band richness)					
Achillea		1.503	1.447		
Centaurea	1.873	1.894			
Galium		1.631	1.619	1.625	
Medicago		1.598	1.597	1.591	1.614
Plantago		1.667	1.639	1.691	
Genetic differentiation (F_{ST})					
Achillea					
F_2		**0.086**			
Centuarea					
F_1	0.000				
Galium					
F_2		0.014			
F_4		0.004	0.014		
Medicago					
F_2		**0.122**			
F_3		**0.128**	0.003		
F_4		**0.143**	**0.036**	**0.033**	
Plantago					
F_2		0.014			
F_3		0.000	0.004		

RESULTS

Molecular marker diversity and differentiation

The studied species differed in their levels of genetic variation (Table 1). Interestingly, *Medicago*, the only selfing species, had similar levels of variation as the outcrossers. For none of the species did the cultivation generations differ in their amount of overall genetic variation. However, in *Achillea* and *Galium* the F_1 generation harboured an excess number of private bands, indicating loss of alleles in later generations (Fig. 2).

There was significant genetic differentiation among generations in two out of the five species. In *Achillea*, the two studied generations were significantly differentiated ($F_{ST} = 0.086$, $P < 0.001$), which also is evident in the separation of generations along the first axis of the PCoA (Fig. 2). The differentiation was partly due to eight private bands in the F_1 generation. In *Medicago*, the overall differentiation was moderate ($F_{ST} = 0.081$, $P < 0.001$), with strongest differentiation between F_1 and F_2 generations (pair-wise $F_{ST} = 0.122$). This differentiation was not associated with a loss or gain of private bands (Fig. 2), so it must have resulted from changes in band frequencies. In *Centaurea*, no differentiation was observed, and the number of private bands did not differ from a random expectation. In *Galium*, the overall differentiation was low but significant ($F_{ST} = 0.010$, $P < 0.001$) and there was an excess number of private bands in the first generation, but all

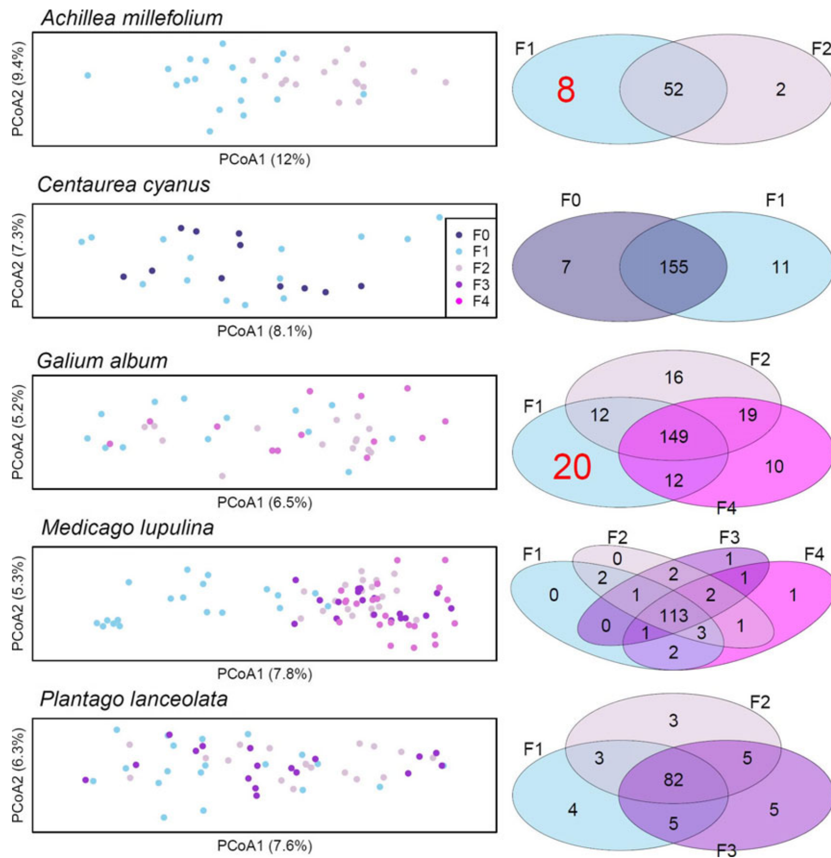

Fig. 2. Principal coordinates analyses and Venn diagrams summarising the AFLP marker results of different cultivation generations (F_0–F_4) of the five studied species. The legend shown in the PCoA plot of *C. cyanus* is valid for all species. The Venn diagrams show the numbers of bands that are private to one or shared by several generations. The large red numbers indicate cases where the numbers are higher than expected by chance ($P < 0.05$).

Table 2. Summary of the genotypic and phenotypic changes observed across cultivation generations for each species.

species (# of generations)	genotypes: loss of alleles	genotypes: genetic differentiation	phenotypes number of traits that did not change	phenotypes number of traits that did change	affected traits
Achillea millefolium (2)	YES	YES	7	0	–
Centaurea cyanus (2)	NO	NO	7	0	–
Galium album (3)	YES	NO	6	3	Early growth
Medicago lupulina (4)	NO	YES	1	5	Phenology
Plantago lanceolata (3)	NO	NO	15	0	–

pair-wise differentiation values among generations were non-significant (Table 1). In *Plantago,* the overall differentiation among the three generations was very low ($F_{ST} = 0.003$, $P < 0.001$) and pair-wise comparisons were also not significant.

Phenotypic differentiation

Only in two out of the five studied species, *Galium* and *Medicago*, did we find significant changes in phenotype across the cultivated generations (Table 2). In *Galium*, plants from individual generations differed in their early growth: the F_2 generation plants were significantly smaller than F_1 and F_4 generation plants, and they had fewer and smaller leaf rosettes (Fig. 3, Table S2). These differences disappeared towards the end of the experiment. In *Medicago*, the phenotypic differences between generations were strongest, and they were largely related to phenology. Plants from the F_1 generation were taller but had lower biomass, and in contrast to the later generations, most of them flowered during the experiment and produced several flowers (Fig. 4, Table S2).

DISCUSSION

Wild plant seeds for restoration are often propagated in agricultural settings like crops. While this approach allows the production of large amounts of seed, the plants in cultivation face a novel environment and may be subject to both artificial and natural selection, which may affect the genetic and phenotypic composition of the cultivated populations. We used molecular markers plus a common garden experiment to test for genotypic and phenotypic changes in five plant species during multiple generations under cultivation. Although the molecular markers did not detect a decline of overall genetic variability, we found that in two species a significant proportion of alleles was lost during cultivation. Moreover, there was significant genetic differentiation among generations in two out of the five species, and this differentiation was driven either by shifts in allele frequency or by allele loss. In two species, the genetic changes were also accompanied by changes in phenotypes, with

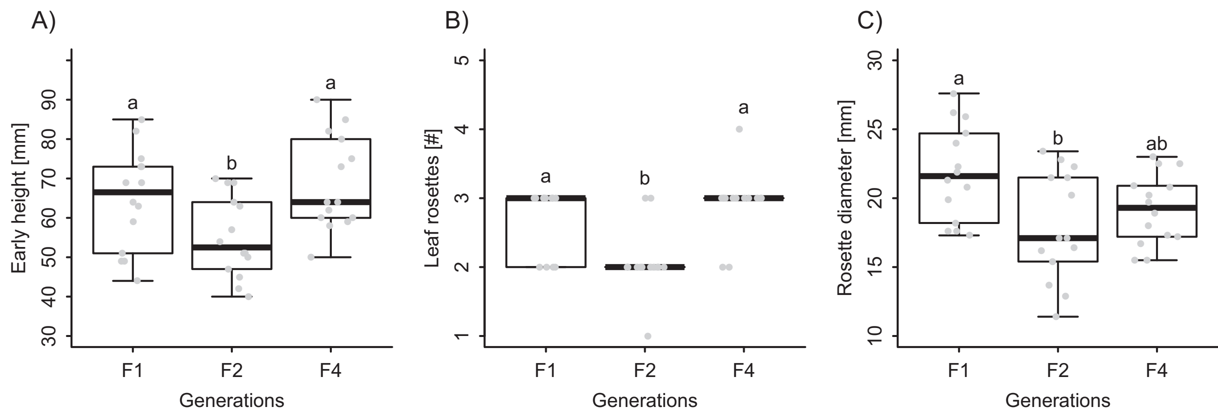

Fig. 3. Significant phenotypic variation in early growth among cultivation generations in *Galium album*. A: Height of the plants, B: Number of leaf rosettes, C: Diameter of the second rosette, all measured at the 28th day of experiment. Points represent individual observations and letters indicate significant differences at $P < 0.05$.

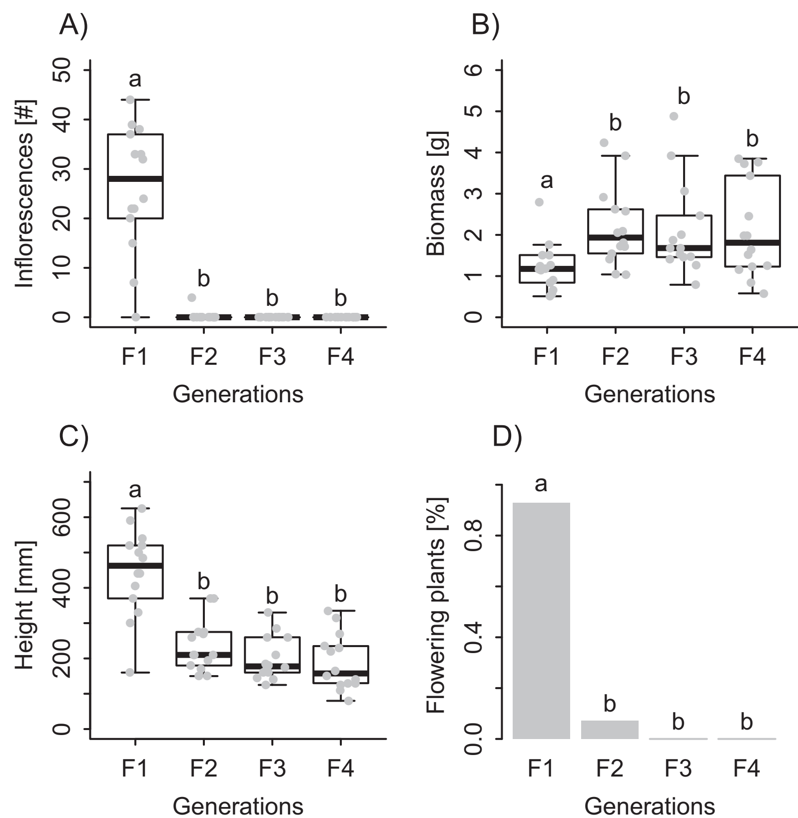

Fig. 4. Significant phenotypic variation among different cultivation generations of *Medicago lupulina*: A: Number of inflorescences, B: Aboveground biomass, C: Height of the plants, D: Proportion of flowering individuals in generation. All traits have been measure at the end of the experiment. Points represent individual observations and letters indicate significant differences at $P < 0.05$.

particularly strong effects on phenology in one of the species (Table 2).

Genetic diversity

Overall, genetic diversity did not decrease during cultivation, which indicates that genetic drift had little impact in the large-scale cultivation of these species. This contrasts with the frequently observed loss of genetic variation in *ex-situ* cultivation of rare species (Lauterbach *et al.* 2012). This difference is likely explained by the contrasting population sizes in the two approaches. While the rare plants in botanical gardens are usually kept in small effective population sizes and thus are sensitive to genetic drift, the plants cultivated for restoration purposes are grown in large populations where the effect of drift is much less severe (Frankham *et al.* 2002; Prasse *et al.* 2010). Alternatively, our study may have found no changes because we did not include the original F_0 seeds for most of the species. While Lauterbach *et al.* (2012) compared plants in *ex-situ* collections directly with plants from wild-collected seeds, we mostly studied different cultivated generations. If most changes happened during the first generation of cultivation, we could not detect them. However, we think that the latter scenario is less likely. The amount of wild-collected seed is

necessarily limited, and because seed producers wish to obtain as many F_1 seeds as possible, special care is usually taken with growing the F_1 plants, for example through manual planting and harvesting in a horticultural setting. Consequently, the loss of genetic variation between F_0 and F_1 is minimised and should be much lower than in later generations that are machine-sown and -harvested. Our argument is supported by the *Centaurea* data. This was the only species where we did include F_0 seed, and we did not find a loss of genetic variation.

Differentiation of generations under cultivation

While overall genetic diversity did not change, we observed genetic differentiation between generations in three out of the five species. Although in the other two species we found no changes, this absence of evidence does not necessarily mean that there were no genotypic changes, because AFLP produces a limited number of markers, and we cannot exclude that changes due to selection at individual loci not covered by AFLP remained undetected. The changes we detected were likely caused by selection or genetic drift during cultivation because the main axis of variation (PCA1 in Fig. 1) appears to be related to the length in cultivation in several species. Distinguishing further between the effects of selection *versus* drift is difficult. Although AFLP markers are generally considered neutral, and their changes are thus mostly attributed to drift, individual markers may nevertheless be linked to loci under selection (*e.g.* Michalski & Durka 2012), either directly or, more likely, because of hitchhiking of AFLP markers with genes under selection (Stefenon *et al.* 2008). Thus, it is possible that at least some of the AFLP marker differentiation reflects selection, especially since the observed marker changes were accompanied by phenotypic changes in some species.

The species with the strongest phenotypic changes during cultivation was the annual to biennial plant *Medicago lupulina*. In this species, the F_1 generation contained mostly small (Fig. 4B), short-lived plants with a maximum life span of 3 months, which entered senescence before the end of the experiment. The following generations, in contrast, contained only longer-living plants. This striking difference is most probably caused by the timing of harvesting on the farm. The short-lived plants quickly flower, produce few seeds and die during summer; the longer-living plants flower later, produce more biomass and consequently more seeds in autumn. In our study, these longer-lived plants did not even start to flower within the 90 days of the experiment. During the on-farm propagation, the farmer likely harvested seed in autumn, when the large, long-lived plants had produced their seeds. By that time, the short-lived plants had already shed their seed and consequently did not contribute to the F_2 generation.

Another possible reason for the rapid phenotypic and genotypic change of *M. lupulina* could be its breeding system. This species was the only predominantly selfing species in our study (Yan *et al.* 2009). All other species are outcrossing (Ehrendorfer *et al.* 1996; Klotz *et al.* 2002). In outcrossing species, gene flow takes place both through pollen and seed, and the plants that are not selected for their own traits can still contribute to the next generation *via* pollen. In predominantly selfing plants, the transfer of genes *via* pollen is rare, resulting in pure line selection, which accelerates both genotypic and phenotypic changes (Glémin & Ronfort 2013). The more rapid genetic and phenotypic changes in *Medicago* could thus be attributed to selfing, which maintains the short-lived and longer-lived plants separately. However, pure line selection should cause a reduction of overall genetic variation, which was not the case here. A possible explanation is that, even though the species is predominantly selfing, the level of outcrossing was still sufficient to maintain considerable intrapopulation genetic variation (Br = 1.6; Table 1; see also Yan *et al.* 2009). In contrast to other selfing plant species (Voss *et al.* 2012; Durka *et al.* 2013), each *Medicago* plant displayed a distinct multilocus genotype. Apparently, the markers were distributed across plants so that the selection on phenotype alleles did not eliminate other genetic variation, but only led to shifts in band frequency. This process was likely accelerated by the high level of selfing.

The second species with significant phenotypic differentiation was *Galium album*. Surprisingly, the observed differentiation did not increase with time under cultivation, as the most strongly differentiated generation was the F_2. The F_2 seeds germinated later than the two other generations (data not shown) and therefore the young plants were smaller. In mature plants, the differentiation was no longer apparent. There are several possible explanations for this difference in F_2 plants. Germination and early life stages are often affected by maternal effects (Roach & Wulff 1987), and it is possible that the difference in F_2 plants was caused by maternal effects, rather than by genetic changes. Alternatively, the relatively high performance of the first generation in cultivation could be due to a hybrid vigour effect resulting from the admixture of plants from different populations (Schröder & Prasse 2013). Since the degree of heterozygosity likely decreased in the F_2 plants, the hybrid vigour may have decreased in this generation. The subsequent increase in early performance in the F_4 generation may then be driven by selection. Another explanation for this pattern may be fluctuating selection over years due different weather or altered cultivation methods (Siepielski *et al.* 2009). However, the hybrid vigour and fluctuating selection scenarios appear less likely because, although the F_2 generation differed in phenotypes, molecular markers did not reveal any significant differentiation between the F_2 and F_4 generations.

The effect of cultivation on the genetic structure and phenotypic traits of the other species was rather moderate and was species-specific. While in *Plantago* and *Centaurea* there was no change at all, we detected some loss of alleles and genetic differentiation between generations in *Achillea*. Much of these between-species differences can probably be attributed to the different cultivation methods for different species. Seeds of *Achillea* are harvested only once per season, which effectively selects only the plants that have ripe seeds at the time of harvest, whereas seeds of *Plantago* are harvested several times per season (seed producer, personal communication), which may relax the selection imposed by harvesting. *C. cyanus* is a special case. It is a weed of cereal fields and the F_0 seeds for cultivation were not collected from wild populations but obtained as threshing remains from cereal harvests. It is possible that the species is already well adapted to agricultural cultivation, and the main selection already took place before or during the initial harvesting of the F_0 seeds.

In summary, with exception of one species, we detected only moderate changes of genetic diversity and plant traits during cultivation. However, some caution is necessary when interpreting these results. First, as we mostly lacked the source

populations, we cannot assess potential effects in the first generation, which undergoes the strongest habitat change and where the strongest selection is expected (Espeland *et al.* 2017). Second, AFLP analysis of genetic structure provides only a limited number of markers, and we cannot exclude that some evolutionary changes remained undetected. Third, the experiment started with transplanting seedlings and ran for only 3 months. Consequently, we could not detect any differentiation in seedling emergence and establishment or in the later stages of the life cycle of the plants. Finally, we measured plant phenotypes under nearly optimal conditions, and possible loss of adaptation to stress may appear only when the plants are exposed to a given stress (Kawecki & Ebert 2004).

Implications for practice

The propagation of seeds for restoration is necessarily a trade-off between maintenance of natural characteristics and practical feasibility, and as such, it has drawbacks. After transfer to cultivation, plants undergo strong environmental change, and unintended selection is almost inevitable (Espeland *et al.* 2017). Environmental managers are aware of these risks, and to minimise the negative effects they recommend a number of measures, ranging from large population sizes during propagation to limiting the period of propagation to a maximum of five generations (Prasse *et al.* 2010; Espeland *et al.* 2017). Here, we show that despite all these efforts, the cultivation process can change phenotypic and genetic traits. However, the changes observed in our study were species-specific, and while the majority of species were little affected by cultivation, we detected a substantial change in one short-lived species. To obtain more general results and develop more comprehensive management guidelines, we need to test many more species (Bucharova *et al.* 2017), compare cultivated generations with wild-collected seed, and relate possible negative effects to species traits and cultivation methods. For the time being, we suggest that seed production for restoration should be accompanied by a rigorous genetic and phenotypic assessment that includes source populations and the different generations of seed production in order to assure high performance and adaptive potential of the restored populations.

ACKNOWLEDGEMENTS

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DATA ACCESSIBILITY

Data are available in DRYAD repository (http://datadryad.org/), https://doi.org/10.5061/dryad.vf46853

AUTHOR CONTRIBUTION

AB, OB and WD designed the study; RN performed the common garden experiment and analysed the phenotypic data; WD performed molecular marker analysis and analysed the genetic data; all the authors wrote the manuscript.

SUPPORTING INFORMATION

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

Appendix S1. Why we could not use the F_0 seeds of *Galium album* and *Plantago lanceolata*.

Table S1. Primer combinations used in the AFLP, number of markers, polymorphic markers, replicate samples and mean error rates across loci (total number of mismatches/(number of markers × number of replicate pairs)).

Table S2. List of all traits measured during the experiment in each species.

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