Biodiversity and Conservation (2019) 28:3951–3969
https://doi.org/10.1007/s10531-019-01859-9

ORIGINAL PAPER

Plant populations of three threatened species experience rapid evolution under ex situ cultivation

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Received: 20 March 2019 / Revised: 9 September 2019 / Accepted: 12 September 2019 /

Abstract

Many botanic gardens keep ex situ collections of rare species to prevent their extinction and to enable their reintroduction into the wild. A potential problem with ex situ collections is that relaxed selection, genetic drift, novel selection and inbreeding may cause rapid loss of adaptation to natural conditions and therefore may hamper success of reintroductions. Here, we investigated whether cultivation in ex situ collections of three threatened species—*Trifolium spadiceum*, *Sisymbrium austriacum* and *Bromus gros*sus—influenced trait differentiation. Using plant material from the original source populations and from the ex situ collections, we compared germination characteristics, growth and phenology under different environmental treatments. *Trifolium spadiceum* showed reduced seed dormancy in the ex situ collection compared to the wild population, whereas germination temperature requirements changed for *S. austriacum*. *Trifolium spadiceum* also showed reduced seed viability in the ex situ collection compared to the wild population. All species showed differences in plant growth between the plants from nature and from the botanic garden. Additionally, *B. grossus* showed advanced flowering time in plants from the botanic garden. These differences may reflect reduced performance or changes in life-history strategies. We conclude that all three species have rapidly differentiated between wild and ex situ origins and that effects of relaxed selection, genetic drift, inbreeding depression and adaptation to cultivation conditions in the botanic garden may have played a role in population differentiation, which may be unfavourable for reintroduction into nature. To explore this further we suggest broader studies across more species, populations and gardens, involving common garden, reciprocal transplant and molecular studies.

Keywords Botanic gardens · Common garden experiment · Ex situ cultivation · Rapid evolution · Threatened plant species

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Communicated by Daniel Sanchez Mata.

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Introduction

Anthropogenic habitat loss and fragmentation (Brooks et al. 2002; Dirzo and Raven 2003; Balmford and Bond 2005; Haddad et al. 2015), land use change (Luoto et al. 2002; Helm et al. 2006) and climate change (Thuiller et al. 2005; Urban 2015) cause plant population declines and species extinctions. To reduce global extinction rates, in situ conservation is the preferred option, since it retains species in their natural habitat, allowing them to evolve and adapt as the environment changes and to retain their ecological and evolutionary effects on the ecosystem (Adams 2004; CBD 2010). However, the above-mentioned impacts have had strong effects on plant diversity over recent decades such that success of in situ conservation action is hampered. As an alternative, botanic gardens have become increasingly important for the conservation of threatened plant species through ex situ cultivation (Hawkins et al. 2008). The Global Strategy for Plant Conservation includes as one of its targets that a minimum of 75% of threatened plant species are held in ex situ collections (Sharrock 2012).

There is a well-justified concern that cultivation in small populations in botanic gardens may cause drastic evolutionary changes (Ensslin and Godefroid 2018). These include random loss of genetic diversity through genetic drift due to bottleneck effects during sampling and small population size under cultivation, as well as increased levels of inbreeding in small cultivation collections (Vitt and Havens 2004; Guerrant et al. 2010; Hoban and Schlarbaum 2014). For example Lauterbach et al. (2012) have shown that genetic diversity of three ex situ populations of *Silene otites* (Caryophyllaceae) was reduced compared to their three wild source populations and decreased with increasing time under cultivation in the botanic gardens. Likewise, declines in genetic diversity over time were found in *Cochlearia polonica* (Rucińska and Puchalski 2011) and *Cynoglossum officinale* (Ensslin et al. 2011). Such loss of genetic diversity may lead to loss of fitness (Pluess and Stöcklin 2004; Leimu et al. 2006) and to random trait changes that may be maladaptive (Jacquemyn et al. 2012). Even if the population size is substantial, the benign cultivation conditions may lead to relaxed selection on traits important for fitness under natural conditions (Lahti et al. 2009), allowing for genetic drift in such traits over the course of generations.

Besides the effects of random genetic drift, inbreeding and relaxed selection, local adaptation to cultivation conditions is another threat to the conservation of wild species in ex situ collections. The abiotic and biotic conditions in botanic gardens are controlled to various extents by local climate and garden management, and these conditions can act as agents of selection leading to adaptation of populations to their local environmental conditions (Kawecki and Ebert 2004; Blanquart et al. 2013). Adaptation of wild species to the local environmental conditions may simultaneously cause the loss of adaptations to their natural origins (Vitt and Havens 2004; Ensslin et al. 2011).

Environmental differences between the botanic garden and the original habitat may be climate factors such as temperature, precipitation and solar irradiation which can usually only be partly controlled in botanic gardens through heating/cooling, watering and shading, respectively. Additionally, soil nutrients, competition and pathogens can be partly controlled by fertilisation, weeding and the prevention or eradication of pathogens with chemicals or natural enemies, respectively. Another important recurring observation is that a smaller proportion of seeds from botanic gardens is dormant compared to seeds from nature, possibly due to unintentional selection of early germinating seeds. This was shown in a greenhouse experiment with *Cynoglossum officinale* (Ensslin et al. 2011) and was also the major finding in a study comparing cultivated and natural populations of 72 species

(Ensslin et al. 2017). Finally, ex situ collections of threatened species are often genetically isolated and, although guidelines mention the need for immigration (Havens 2004) some collections are not being restocked with plants from other (wild or cultivated) populations, which enhances local adaptation to the garden environment (Maunder 1992; Maunder et al. 2001).

Evolution—whether in nature or under cultivation—can be rapid, especially when selection pressure is strong. Many examples document evolutionary change through both natural and anthropogenic factors. For example, a wild *Brassica rapa* population advanced its flowering phenology within only 7 years in response to strong selection by drought (Franks et al. 2007). Similarly, *Poa annua* evolved different meta-populations with various life history traits on a golf court through different tending techniques within only 25 years (Till-Bottraud et al. 1990). Phenotypic trait changes were also observed in two out of five common European grassland species after only one generation of cultivation for restoration purposes (Nagel et al. 2019).

Loss of genetic diversity and changes in other phenotypic traits such as dormancy, as found in the above studies, could lead to reduced fitness when plant material from ex situ collections would be reintroduced into their original habitats (Guerrant et al. 2010; Ensslin et al. 2011, 2015). Unfortunately, studies on rapid evolutionary changes of plant populations in ex situ cultivation, especially of threatened species, are still scarce.

Here we use a common garden approach to investigate phenotypic differentiation between ex situ collections and their wild origins in three threatened plant species: *Bromus grossus* (Poaceae), *Sisymbrium austriacum* (Brassicaceae) and *Trifolium spadiceum* (Fabaceae). The species are being cultivated in the Botanic Garden of the University of Tübingen and were originally sampled from the Swabian Jura. All species are on the Red List of the German state of Baden-Württemberg and have been in cultivation between 2 and 7 years (Table 1). To test for evolutionary changes, we performed germination and greenhouse experiments and applied ecologically relevant treatments, which represent key differences between the original habitat and the botanic garden. We investigated germination rate, seed dormancy and viability, shoot length, rosette diameter, above-ground biomass and flowering time. We asked the following specific questions: (1) Do seeds and plants from ex situ and wild origins show genetic differentiation in the investigated phenotypic traits? (2) Do seed and plant responses to experimental treatments suggest adaptation to their collection sites (nature vs botanic garden)?

Materials and methods

Study species and sites

The three study species (Table 1) were selected based on the following criteria: Firstly, the species should be endangered and cultivated ex situ in the botanic garden of Tübingen. Secondly, the exact origin of the cultivated material must be known. Thirdly, the study species should have a short life cycle, because evolutionary processes are likely faster—and therefore more pronounced—in short-lived species, and because short-lived species allow analysis of flowering time and lifetime fitness within the duration of a relatively short experiment. To set up the initial ex situ collections seeds from ca. 20 individuals per species were collected and collectors tried to equalize family sizes before mixing the seeds (B. Junginger, pers. comm.). Since initial wild seed collection, no restocking with wild seeds

Table 1 Study species including information on the plant family, their life cycle, location, estimated population size, the year of sampling for cultivation, the estimated number of generations under cultivation, the *Red List* status of these species in Baden-Württemberg (federal state in Germany) and the main causes for the threatened status

Species	Family	Life cycle	Location	Estimated population size	Coordinates (Gauß-Krüger)	Year of sampling for cultivation	Estimated number of generations	*Red List* status (Breuning and Demuth 1999)	Causes for threatened status
Trifolium spadiceum	Fabaceae	Annual-biennial	*Irndorfer Hardt*	> 100	3495xxx/5328xxx	2013	2	2 (threatened)	Drainage of former wetland (Aichele and Schwegler 2000)
Sisymbrium austriacum	Brassicaceae	Annual-biennial	*Hohler Fels*	> 50	3501xxx/5325xxx	2008	7	V (warning)	Increased tourism (hiking) (Brandes 2012)
Bromus grossus	Poaceae	Annual	*Salmendinger Kapelle*	7	3505xxx/5356xxx	2009	5	2 (threatened)	Increased land use intensity (Käsermann and Moser 1999)

Coordinates have been censored to protect the natural population took place and no obvious deviations in population sizes between initial sampling and resampling for this study were observed (B. Junginger, pers. comm.).

The focal population of *Trifolium spadiceum* (n > 100) is located in the *Irndorfer Hardt*, a nature reserve characterised as a meadow with a high degree of interspecific competition and exposed to cold winter temperatures. We sampled *Sisymbrium austriacum* at the foot of the cliff *Hohler Fels* (n > 50). This species is associated with the so-called *Balmen* vegetation, i.e. occurring below calcareous rock spurs, a habitat enriched with nutrients, especially phosphorus, due to runoff over the cliff (M. Koltzenburg, pers. comm.). We sampled *Bromus grossus*, a winter annual associated with winter grain cultivation, near *Salmendinger Kapelle* from a population on the edge of an agricultural field among winter wheat and other ruderal grasses and herbs. This population is very small, and we observed only seven individuals in 2016. Original sampling of seeds for the cultivation collection in the botanic garden was conducted in 2013 (*T. spadiceum*) 2008 (*S. austriacum*) and 2009 (*B. grossus*). Based on this we estimate that the ex situ populations in the botanic garden are in cultivation for two (*T. spadiceum*), seven (*S. austriacum*) and six (*B. grossus*) generations (Table 1).

In the Botanic Garden of the University of Tübingen seeds of *S. austriacum* and *T. spadiceum* have been germinated in a greenhouse every year. Afterwards, gardeners transferred the seedlings into beds in the garden without any competition, and with regular fertilisation and watering. *Bromus grossus* naturally germinates in fall, but in the botanic garden seeds have been sown yearly in spring since the start of cultivation. Thus, the environment that *B. grossus* experienced in the botanic garden differs remarkably from the site of origin, especially concerning the temperature and daylight regime during the seedling establishment phase. For all three species, seed sampling usually took place once per generation when the majority of plants had ripe seeds. Gardeners collected all ripe seeds from all plants and mixed them without equalizing family sizes.

Between June and August 2016, we collected seeds from the ex situ collections in the Botanic Garden of the University of Tübingen and from the natural populations of origin. We stored seeds belonging to the same plant in one bag and treated them as a unique seed family. In all cases, we restricted our sampling of natural populations to a limited number of plant individuals (and to only a portion of the seeds produced by each sampled *B. grossus* individual) to limit impact on the populations' natural dynamics. Seed collection from the botanic garden was restricted in other ways: We were able to sample seeds from twelve *T. spadiceum* individuals but only from four *S. austriacum* individuals due to herbivory damage of other individuals by rabbits and snails. Furthermore, the complete ex situ *B. grossus* population grown in 2016 died of fungal disease, so we used seeds collected from the ex situ collection in 2015 instead. This precluded the use of seed families for *B. grossus* because the botanic garden seed collection protocol does not demand keeping different seed families separate. Thus, the number of generations between initial sampling and resampling is estimated to be five for *B. grossus*. We dried all seeds at room temperature and stored at 7 °C in the dark until germination experiments started.

Germination experiment

For two species, *S. austriacum* and *T. spadiceum*, we performed germination experiments in growth cabinets (Percival E36L, Percival Scientific, Perry, Iowa) and for *B. grossus* an outdoor germination experiment in the Botanic Garden of the University of Tübingen. For the growth cabinets we placed seeds onto two layers of moist filter paper in Petri dishes

(Ø 50 mm). For *T. spadiceum* we used 12 seed families per origin (garden vs. nature) and for each seed family we had four replicate Petri dishes each with eight seeds, totaling 96 dishes and 640 seeds in the experiment. For *S. austriacum*, we had eight seed families from the natural population and only four from the botanic garden. For each seed family we included eight replicate Petri dishes each with ten seeds, totaling 96 dishes and 960 seeds in the experiment. We positioned one of four or two of eight replicates in each of the four growth cabinets.

After 1 week of stratification in darkness at 5 °C (2–9 February 2017), we programmed the following conditions: light (day) with 70% (ca. 150 µmol cm^{-2} cm^{-1}) light from 6 am till 10 pm and no light (night) from 10 pm till 6 am. We set two growth cabinets at 25 °C during light and 15 °C during dark conditions; the other two cabinets at 15 °C during light and 5 °C during dark. During the experiment, we recorded the date of germination (i.e. root and/or shoot visible) for every seed. At the end of the experiment we checked non-germinated seeds as to whether they were dormant or unviable. We crushed the seeds with pressure between two fingers (imbibed seed crush test) and scored soft seeds as non-viable and hard seeds as viable and therefore dormant (Borza et al. 2007).

For each Petri dish, we derived the proportion of viable (as compared to total) and of dormant (as compared to all viable) seeds. We also calculated the mean germination time (MTG; Daws et al. 2005) per dish as follows:

$$MTG = \Sigma\left(\left(n_i * d_i\right)/N\right)$$

where n_i is the number of germinated seeds in a dish, d_i is the days until germination, and N is the total number of seeds.

For *B. grossus* we performed an outdoor germination experiment at the usual growing location of this cultivation collection in the Botanic Garden of the University of Tübingen. In a plant bed fertilised with horse dung, twelve plots (20 cm × 20 cm) each with 30 seeds (five rows with six seeds per row) were sown on 20 November 2016, i.e. six plots from both origins in checkerboard arrangement. We added a thin layer of sand after sowing and we installed a red metal wire ca. 30 cm above the plot to prevent seed herbivory by birds. We observed the location of every seed individually and marked germinated seeds with a coloured tooth pick, and scored the date of germination of each seed.

Greenhouse experiment

After germination, we transferred seedlings of the three species to a greenhouse at the University of Tübingen. We planted every seedling in a 9 cm × 9 cm × 9 cm pot with a sieved soil mixture of 1/3 local soil and 2/3 sand (Sand- und Kieswerk Rottenburg Matthäus Bischoff GmbH and Co. KG) with a layer of 1 cm germination compost on top. During the experiment we watered all pots regularly. To reduce insect damage, we used yellow sticky boards distributed randomly in the greenhouse. We adjusted the greenhouse temperature to 20 °C at daytime and 18 °C at night. We positioned the three species separate from each other and randomized pots within species every fortnight.

We applied treatments that simulate supposedly important environmental differences between the original habitats and the ex situ cultivations in the botanic garden. We used competition treatments for *T. spadiceum* and *B. grossus* and fertiliser treatments for *S. austriacum*. For *T. spadiceum*, we germinated seeds of *Senecio vulgaris* (Moench) Garcke (B and T World Seeds, Aigues-Vives, France), a strong competitor able to overshade *T. spadiceum*, in a growing chamber and transplanted one seedling randomly into half of the pots. For *B. grossus* we used *Poa annua* L. (Kiepenkerl, Münster, Germany) as strong underground competitor, sowing 2.5 mL of seeds into half of the pots. For *S. austriacum*, associated with the nutrient rich *Balmen* vegetation, we applied three nutrient levels: (1) without fertiliser, (2) with standard fertiliser, (3) with standard 7-5-6 NPK fertiliser (Grünpflanzendünger mit Spurenelementen, TOOM, Gebr. Mayer GmbH, Wahrenholz) and additional potassium-dihydrogen-phosphate (KH_2PO_4). Over a period of 10 weeks (6 March till 8 May), we added 11.5 mL fertiliser dissolved in water each week divided over 145 pots (treatments 2 and 3) resulting in a total application of 80 kg ha^{-1} a^{-1} nitrogen. For treatment 3 we added a total of 1.92 g KH_2PO_4 to increase the input of phosphorus from 25 kg ha^{-1} a^{-1} already in the fertiliser to a potentially toxic input of 100 kg ha^{-1} a^{-1}.

Since the used soil mixture was rather nutrient poor, we fertilised all pots of *T. spadiceum* and *B. grossus* as well. Every week during 4 weeks between 18 April until 9 May we added a total of 73 mL of the standard fertiliser dissolved in water to all pots ($n=344$). This supply equals an input of 80 kg ha^{-1} nitrogen and 25 kg ha^{-1} phosphorous.

We recorded the flowering date of *B. grossus* at 2-day intervals, which occurred between 23 May and 18 June 2017. Both *S. austriacum* and *T. spadiceum* did not flower during the 3 months of the experiment. Before harvesting the above-ground plant material on 6 June (*T. spadiceum*), 7 June (*S. austriacum*) and 18 June (*B. grossus*), we measured shoot length, i.e. the length of the longest shoot or ramet, for *T. spadiceum* and *B. grossus*, and the rosette diameter for *S. austriacum*. Furthermore, we counted leaves for *T. spadiceum* and *S. austriacum* and number of shoots for *B. grossus*. After drying at least 48 h at 60 °C we weighed the total aboveground biomass of each plant.

Data analysis

We analysed all traits from the germination and greenhouse experiment except flowering date in *B. grossus* using linear mixed-effects models with Satterthwaite approximation of degrees of freedom. For all models we included origin (botanic garden versus nature), treatments and their interaction as fixed factors, and seed family (or plot number in the outdoor experiment for *B. grossus*) as random factor. We used survival analysis with Cox' proportional hazards and linear mixed-effect model analyses to analyse flowering date of *B. grossus*. We assessed all model residuals visually for normality. For data analyses we used R (R Core Team 2017) and the packages lme4 (Bates et al. 2015), lmerTest (Kuznetsova 2016), survival (Therneau 2017) and coxme (Therneau 2015).

Results

Germination experiment

Effects of origin—With regard to origin effects using the above mentioned linear mixed-effect model, seeds of *T. spadiceum* from the botanic garden were only half as viable (Fig. 1a, $F_{1,22}=39.87$, $p<0.0001$) and fewer seeds were dormant (15% vs. 50%, Fig. 1c, $F_{1,21.5}=28.70$, $p<0.0001$) than seeds from nature. For *S. austriacum* there was no significant difference in viability between seeds from both origins (Fig. 1b, $F_{1,92}=2.85$, $p=0.09$), but the origin had a strong effect on dormancy; a higher fraction of seeds from the botanic garden was dormant compared to seeds from nature (Fig. 1d, $F_{1,10.1}=48.46$, $p<0.0001$). With regard to germination speed seeds from the botanic garden germinated

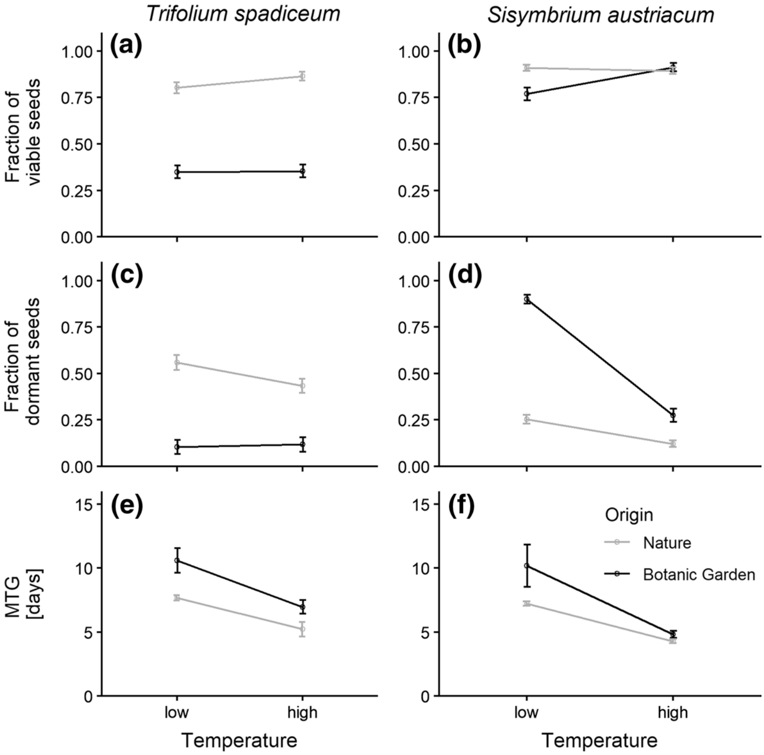

Fig. 1 The proportion of viable and dormant seeds and the mean time until germination (MTG) of *Trifolium spadiceum* and *Sisymbrium austriacum* from two different origins, nature vs botanic garden, germinated under two different temperature regimes. Bars show means and standard errors based on Petri dish means

significantly later than seeds from nature in both *T. spadiceum* (Fig. 1e, $F_{1,21.4} = 14.2$, $p = 0.001$) and *S. austriacum* (Fig. 1f, $F_{1,12.6} = 15.5$, $p = 0.002$) with a delay of ca. 2 days.

Effect of temperature—Germination temperature had no effect on dormancy of *T. spadiceum* seeds (Fig. 1c, $F_{1,64.2} = 0.725$, $p = 0.40$) but it did influence the dormancy of *S. austriacum* seeds (Fig. 1d, $F_{1,81.1} = 232.23$, $p < 0.0001$). Furthermore the high temperature treatment led to an advanced germination in both species (Fig. 1ef, *T. spadiceum*: $F_{1,58.0} = 25.5$, $p < 0.0001$, *S. austriacum*: $F_{1,82.7} = 107.7$, $p < 0.0001$).

Interaction between origin and temperature—An interaction between origin and temperature occurred for seed viability, dormancy and speed of germination in *S. austriacum*. Seeds from the botanic garden were 14% less viable under colder conditions than under warm conditions whereas seeds from nature did not respond to the temperature treatment (Fig. 1b, $F_{1,92} = 4.87$, $p = 0.03$). Moreover, seeds from the botanic garden showed a larger drop of 63% in dormancy under high compared to low temperature whereas seeds from nature only dropped 12% (Fig. 1d, $F_{1,81.1} = 87.75$, $p < 0.0001$). An origin by temperature interaction indicated that *S. austriacum* seeds from the botanic garden were more strongly influenced by the temperature treatment than seeds from nature (Fig. 1f, $F_{1,82.7} = 9.0$, $p = 0.004$). Interactions for *T. spadiceum* were not observed.

In the outdoor germination experiment with *B. grossus* we found that seeds only germinated in spring and that there were no significant differences in germination behaviour between seeds from the botanic garden and seeds from nature.

Greenhouse experiment

Trifolium spadiceum—At the time of harvest, *T. spadiceum* plants from the botanic garden had significantly smaller shoot length than plants from nature (Table 2). The other traits did not show origin effects. Competition had negative effects on all plant traits (Fig. 2a–c), whereas the interaction between origin and competition was only significant for shoot length and indicated that plants from nature suffered relatively more from a competitive environment (Table 2, Fig. 2b).

Sisymbrium austriacum—The origin of *S. austriacum* affected the diameter of the rosettes (Fig. 2e) and number of leaves (Fig. 2f). Plants from the botanic garden grew to a diameter of 110%, but only had 83% number of leaves, compared to plants from nature. Fertilisation of *S. austriacum* had positive effects on plant growth: plants were larger, had more leaves and a higher biomass (Table 2, Fig. 2d–f). However, there were

Table 2 Results of the linear mixed-effects models for the measured plant traits in the greenhouse, including origin (nature and botanic garden) and the species-specific treatments as fixed factors and seed families for *T. spadiceum* and *S. austriacum* and plot number for *B. grossus* as random factors

	Shoot length or rosette diameter (cm)			Number of shoots or number of leaves			Aboveground biomass (g)		
	F value	d.F.	p value	F value	d.F.	p value	F value	d.F.	p value
Trifolium spadiceum									
Origin	19.85	1, 177	**<0.001**	0.89	1, 16.1	0.361	1.48	1, 14.9	0.242
Treatment (competition)	69.87	1, 177	**<0.001**	35.96	1, 158.1	**<0.001**	226.79	1, 157.1	**<0.001**
Origin × treatment	4.16	1, 177	**0.043**	3.35	1, 158.1	0.069	3.29	1, 157.1	0.071
Sisymbrium austriacum									
Origin	10.82	1, 9.5	**0.009**	33.12	1, 8.2	**<0.001**	0.56	1, 9.7	0.471
Treatment (fertiliser)	345.57	2, 199.0	**<0.001**	251.10	2, 198.4	**<0.001**	1010.31	2, 199.1	**<0.001**
Origin × treatment	1.32	2, 199.0	0.268	2.86	2, 198.4	0.060	1.91	2, 199.1	0.150
Bromus grossus									
Origin	6.94	1, 10.2	**0.025**	13.71	1, 10.2	**0.004**	2.20	1, 10.3	0.168
Treatment (competition)	10.87	1, 156.8	**0.001**	230.82	1, 157.4	**<0.001**	324.86	1, 157.5	**<0.001**
Origin × treatment	0.03	1, 156.8	0.874	9.00	1, 157.4	**0.003**	0.42	1, 157.5	0.516

For degrees of freedom (d.F.) the first number indicates the degrees of freedom for the numerator and the second number for the denominator (using the Satterthwaite approximation)

All significant *p*-values (i.e. when smaller than 0.05) are given in bold

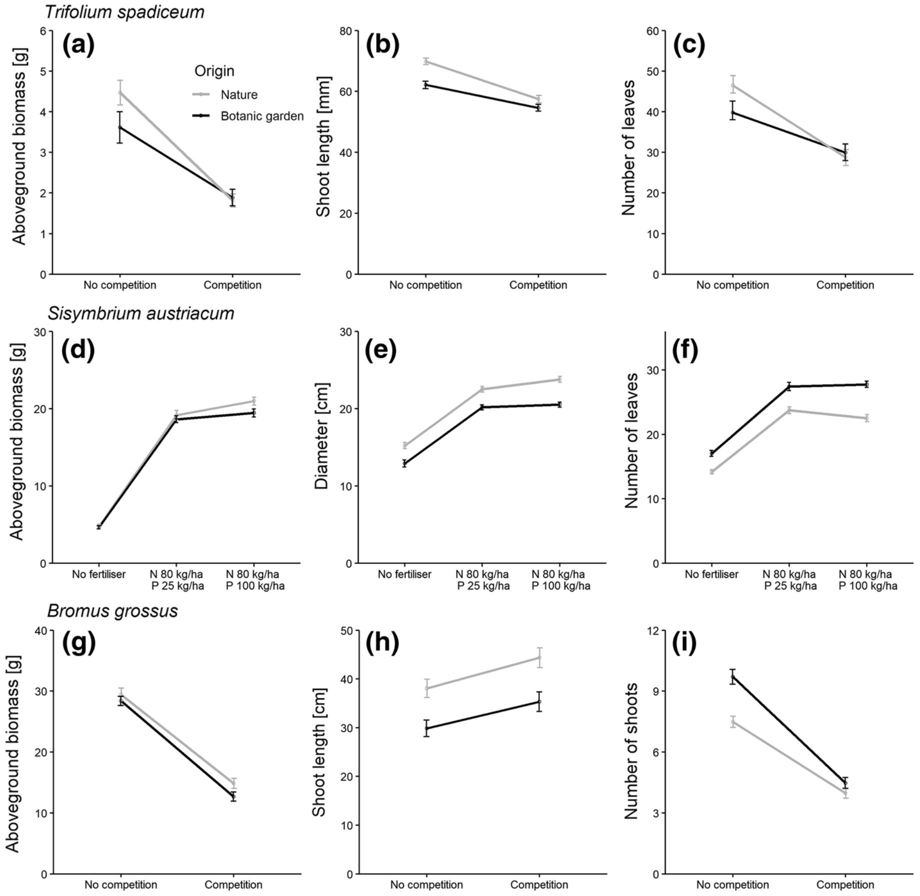

Fig. 2 Three different phenotypic traits per study species from two different origins, nature vs botanic garden, grown under different treatments in the greenhouse. Bars show means and standard errors

neither significant differences between the standard fertilisation and the fertilisation with phosphorus (Post-Hoc test, data not shown), nor an interaction between origin and fertilization (Table 2).

Bromus grossus—Plants from the botanic garden grew only to a height of 79% (Fig. 2h), but achieved 130% number of shoots (Fig. 2i), compared to plants from nature. Competition had a significant effect on all traits (Table 2, Fig. 2g–i). Plant height was positively affected, and all other traits were negatively affected, by competition (Fig. 2g–i). An origin by competition interaction for the number of shoots indicated that the negative response to competition was stronger in plants from the botanic garden than in plants from nature (Fig. 2i). Flowering time of the plants in the greenhouse was significantly influenced by origin (Fig. 3, $Chi_1^2 = 8.9$, $p = 0.003$) and competition (Fig. 3, $Chi_1^2 = 15.8$, $p < 0.0001$), but these factors did not interact. Plants from the botanic garden flowered on average 4 days later than plants from nature and plants growing under competition flowered 3 days earlier.

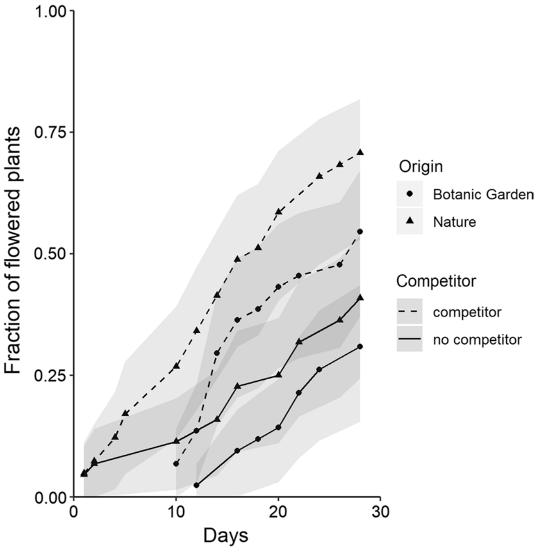

Fig. 3 Proportion of *B. grossus* plants that started flowering over time. Plants originated from two different origins and grew with and without a competition treatment. Cox proportional hazards model showed significant effects of the origin ($Chi_1^2 = 8.9$, $p = 0.003$) and competition ($Chi_1^2 = 15.8$, $p < 0.0001$) but these factors did not interact

Discussion

Germination experiment

Trifolium spadiceum seeds from the botanic garden had a lower fraction of dormant seeds compared to seeds re-collected from nature. Loss of dormancy is a typical result observed in various studies comparing seeds from commercial nurseries and from wild plants (Schröder and Prasse 2013; Herget et al. 2015; Gallagher et al. 2016), but there are only few studies investigating changes in dormancy during cultivation in ex situ collections (Ensslin et al. 2011, 2017). Loss of dormancy may be explained by unintentional or intentional selection of non-dormant seeds during cultivation (Humphrey and Schupp 2002). Because dormancy is generally interpreted as a bet-hedging strategy to maintain long-term survival of a population in unpredictable environments (Evans and Dennehy 2005; Satterthwaite 2010; Simons 2011) we argue that seed material from the botanic garden may have reduced fitness when transferred to the wild as the concerted germination may cause complete loss of the population after a stressful environmental episode.

Seed origin also had a strong effect on seed dormancy in *Sisymbrium austriacum*, albeit in the opposite direction, with a generally higher fraction of seeds from the botanic garden being dormant compared to seeds from nature. However, there was also a strong temperature treatment effect, such that warmer temperatures generally increased germination. Indeed, irrespective of dormancy, seeds and their germination behaviour are often strongly adapted to local temperature conditions (Okagami 1986; Huang et al. 2003). Therefore, we argue that we are not dealing with year-to-year dormancy here but rather with altered temperature requirements for germination. A change in temperature requirement for germination could have evolved since seeds in the ex situ collection of this species are usually being germinated in the greenhouse in a comparatively warm climate before seedlings are transplanted outside. Thus, we conclude that, through cultivation in the botanic garden, *S. austriacum* seeds may have shifted their germination response to temperature, such that it demands higher temperatures to reach the same germination rate as seeds from nature. This could have negative consequences for restoration of natural populations when using seeds from cultivation collections, as these seeds may germinate later in spring and therefore would experience a decreased growing season length.

In the outdoor germination experiment with *B. grossus*, no differences in germination behaviour between the seeds from the botanic garden and from nature were observed. Interestingly, the cultivation environment does not mimic the natural environment very well, since *B. grossus* has been sown each year in spring whereas under natural conditions germination happens in autumn. We therefore had expected to see differences in germination traits, in particular a more rapid germination in seeds from the botanic garden since their growing season is markedly reduced through spring sowing. Also, given that germination took place over a period of 3–5 weeks for both seed origins, there may be substantial genetically based variation in germination traits on which selection could have acted. If the seeds had germinated already in autumn in our experiment, we could have tested whether there is a difference in the survival of seedlings from nature versus from the botanic garden, expecting the former to survive better.

For *T. spadiceum* and *S. austriacum* a delayed mean germination time for seeds originating from the botanic garden compared to the wild origins was observed. These results are contrasting with the results of Ensslin et al. (2017) who compared 72 species from botanic garden collections and their wild origins with regard to mean germination time and found no differences. A potential explanation for our results may be unintentional selection during sampling or cultivation. Depending on the time of seed sampling in nature or in cultivation collections, plants with ripe fruits may represent an early or a late cohort due to early or late germination in spring, respectively, and this variation may have a genetic basis. However, this explanation is more likely for plants that flower in the first year than for perennial species that do not yet flower in the first year (as our focal species). Alternatively, rapid germination at the beginning of the growing season is a successful strategy in competitive environments to gain a head start over later seedlings (Metz et al. 2018). Therefore, *T. spadiceum*, originally growing in a competitive habitat, may have experienced relaxed selection (Lahti et al. 2009) under the non-competitive conditions in the botanic garden showing a delayed mean germination time. Furthermore, under cold germination conditions, seeds from *S. austriacum* from the botanic garden show a much stronger delay in germination than seeds from nature. Therefore, seeds from the botanic garden seem to have lowered their ability to germinate under abiotically less benign conditions, which may be an effect of adaptation to warmer germination conditions.

Seed viability was influenced by the origin of *T. spadiceum*, with seeds from nature being more viable than seeds from the botanic garden. The main cause for this is likely mortality of viable seeds through fungal infection (*Alternaria* spec.) which we observed on the seeds in some Petri dishes. Seeds are likely to bear fungal spores (Neergaard 1977), but seeds from nature appear to have a higher resistance towards pathogens than seeds from the botanic garden. These results correspond with results by Maass (2006) who showed that seeds from wild accessions of *Lablab purpureus* are less infected by fungi than cultivated accessions. Seed resistance towards pathogens is related to seed size or thickness of seed cover and to phenolics content (Plitmann and Kislev 1989), which could well be influenced by inbreeding in small populations, reducing resistance towards infection (Menges 1991; Heschel and Paige 1995). Since germination rate is also increased in seeds with thin seed coats (Baskin and Baskin 2014), this may explain why we found that seeds from the botanic garden had not only a lower seed viability but also a higher germination rate.

Greenhouse experiment

Plants from cultivation collections and from natural origins showed clear trait differentiation in all three species in the greenhouse experiment. *Trifolium spadiceum* plants from nature had higher shoot length and number of branches than plants from the botanic garden but did not differ in aboveground biomass. In environments rich in nutrients or lacking competition, plants tend to increase branch density and decrease branch length (Bonser and Aarssen 2001, 2003). Observations on *T. spadiceum* in our experiment did not conform to this expectation, but it could be that *T. spadiceum* has been under strong influence of inbreeding and genetic drift (Armbruster and Reed 2005), explaining the smaller size of plants from the botanic garden compared to plants from nature. Relaxed selection (Lahti et al. 2009) due to sufficient supply with nutrients, water and through reduced competition under cultivation may also remove or weaken selection for specific trait values and thereby broaden trait variability. However, in this small population in the botanic garden we expect relaxed selection to increase the effect of random genetic drift.

The origin of *S. austriacum* had a significant effect on the rosette diameter and on the number of leaves. Plants from the botanic garden had less but longer leaves than plants from nature, whereas the aboveground biomass did not differ, which indicates that the changes in leaf size and number reflect an altered resource investment (Wang et al. 2014). The natural *S. austriacum* population grows on loose substrate along a steep slope, which may be subject to strong dynamics, e.g. after rains or when wild animals pass by. Thus, plants at such locations may easily get buried under the substrate, and an increased rate of leaf development may help plants to cope with such dynamics. In dune vegetation, which is subject to similar substrate dynamics, Zhang and others have shown that plants develop more branches and leaves to decrease the risk that plants get buried with sand (Zhang and Maun 1992; Shi et al. 2004). However, substrate dynamics are likely much weaker in the habitat of *S. austriacum*. In case the observed differentiation is indeed an adaptation to substrate dynamics, it might have been lost through relaxed selection, genetic drift, inbreeding, or adaptation to the stable soils in the botanic garden. Nevertheless, we suspect that selection for a decreased leaf production rate, whatever its cause, is negligible over the few generations in the botanic garden, so relaxed selection, genetic drift or inbreeding are more likely explanation than genetic adaptation.

Bromus grossus plants from the botanic garden had shorter shoots but more branches than plants from nature. Similar to *S. austriacum*, the aboveground biomass did not differ between the two origins, indicating that the changes in shoot length and branch number is due to an altered resource allocation (Wang et al. 2014). The observed trait differences fit general expectations of adaptation to the cultivation conditions, since plants cultivated in a less competitive environment are likely selected to invest more resources into number of shoots than in the length of shoots (Bonser and Aarssen 2001, 2003). In this context it is important to mention that seed sampling during cultivation also promotes selection for well-adapted phenotypes since gardeners generally collected all seeds leading to seed representation according to plant reproductive success.

During the greenhouse experiment only *B. grossus* started flowering and plants from the botanic garden flowered significantly later than plants from nature. Under natural conditions, seeds germinate in autumn and undergo vernalisation before flowering in spring and ripening in summer. In the botanic garden under ex situ cultivation, seeds were sown in spring rather than in autumn, so plants did not experience vernalisation in the first year. Only some plants managed to flower in late summer of the first year whereas other plants waited until spring next year after vernalisation (S. Bauer, pers. comm.). During the experiment, however, all seeds were vernalised, which may explain why plants from nature flowered earlier than plants from the botanic garden. For grassland species it is typical that flowering starts when the plant reaches a specific size (Mooney et al. 1986; Vile et al. 2006; Sun and Frelich 2011). Later flowering of plants from the botanic garden is therefore in line with their observed smaller shoot length, as these plants may reach their flowering size threshold later, some of them only in the second year of growth under cultivation conditions. Thus, plants grown in the botanic garden have more time for development as they have a longer period until vernalisation and therefore may have relaxed their growth rate.

An alternative explanation for the delayed flowering in plants from the botanic garden may be the lack of competition during cultivation, which may have led to changes in resource allocation. Plants growing without competition tend to invest more resources into branches than into shoot length (Bonser and Aarssen 2001, 2003). Therefore, these plants reach their threshold size for flowering later but at this stage there are more branches, each of which may bear inflorescences, leading to increased fitness. This relationship between flowering phenology and competition is also supported by the fact that the plants grown with competition, flowered on average 3 days earlier than plants grown without competitors (see below).

The competition and nutrient treatments had significant effects on plant growth in the three investigated species. *Trifolium spadiceum* grew smaller under competition and had less branches and lower biomass, which is in line with many other studies (e.g. Kiaer et al. 2013; Wang et al. 2014). For *B. grossus*, competition had a negative effect on biomass and number of shoots but shoot length was positively affected. The response of plants under competition to invest more resources into growth than into branches is also shown by other studies (Bonser and Aarssen 2001, 2003). Furthermore, there was an interaction between origin and competition in number of branches for *B. grossus*. Only in the competition-free environment did plants from the botanic garden invest more resources into branches than plants from nature. It is possible that plants from the botanic garden evolved such plastic responses as an adaptation, even over only few generations. Alternatively, the observed plasticity may be passive responses to the treatments (Van Kleunen and Fischer 2005), e.g. reduced growth under competition and nutrient limitation.

Sisymbrium austriacum plants growing with fertiliser grew larger compared to plants without fertiliser. However, the additional phosphorus treatment did not cause the expected toxic effect. Here, the total amount or the applied concentration may not have been high enough or, alternatively, phosphate may have been bound to soil components and, therefore, may not have affected the plants.

Besides the above-mentioned explanations for trait changes in the cultivation collections, the wild populations may also have experienced drift, inbreeding and adaptation. However, populations of *T. spadiceum* and *S. austriacum* are larger than the cultivation collections in the botanic garden such that drift processes in the wild populations are likely negligible. Moreover, the cultivation conditions pose a much more drastic change of environment than potential mean environmental changes in nature, so we expect novel and strong selection pressures in the cultivation collections. The wild population of *B. grossus*, however, is very small and may be subject to drift. However, in this small population drift would be balanced by strong selection against maladapted individuals in the natural environment, whereas selection by novel cultivation conditions is expected to lead to trait changes in the cultivation collection.

Conclusions

We observed genetic differentiation in phenotypic traits in three species between plants from natural populations and from their derived cultivation collections. These changes have occurred in only two to seven generations. Through this, the cultivation collections may have reduced or even lost their ability to grow successfully in their natural environments, but this remains to be tested. Potential problems for reintroduction into nature are changes in seed dormancy, changes in the germination requirements, decreased seed resistance to fungi, and maladaptive changes in morphological traits. It is not possible to pinpoint the mechanism for these changes based on our study, but causes may be relaxed selection, genetic drift, inbreeding, adaptation or combinations of these.

We assume that most of the phenotypic changes occurred during cultivation in the botanic garden. Although we cannot exclude that phenotypic change may also have occurred meanwhile in the natural populations, either through genetic drift in these small to medium sized populations or through adaptation to changing natural conditions, we expect that changes in the cultivation collections are much stronger. Genetic drift during cultivation may have occurred through strong bottlenecks year after year, since only 10–20 individuals were grown each generation. Whereas relaxed selection in the competition-free environment with plenty of water and nutrients may have allowed for drift processes, novel selection pressures may have led to adaptation, for instance selection for rapidly germinating seeds and adaptive changes in plant height and number of branches. Maternal effects and heritable epigenetic changes may also have contributed to trait changes, and their effects may likewise pose a risk for reintroduction efforts, at least during the first few generations when such effects can still be significant (Groot et al. 2017).

We could not include replicates of different populations for each species as in similar studies (Ensslin et al. 2011; Lauterbach et al. 2012), but the fact that differentiation in phenotypic traits occurred in all three functionally different species, suggests that phenotypic differentiation is common in cultivation collections. Our study cannot be used to infer local adaptation, for which reciprocal transplantations are necessary (Kawecki and Ebert 2004). Only in this way can loss of adaptation to natural conditions be proven. Nevertheless, the applied treatments were chosen to reflect major differences between nature and the botanic garden, which proved useful to explore possible adaptive strategies.

The phenotypic differentiation and presumed adaptations to cultivation conditions can negatively affect the success of reintroduction of plants into nature. Therefore, the crucial work of botanic gardens to conserve species richness can be further improved. However, in order to provide effective recommendations, we need broader studies across more species, populations and botanic gardens. Aside from common garden studies to investigate genetic differentiation in functional traits, we should also conduct reciprocal transplant experiments between original and ex situ populations—e.g. alongside reintroduction projects—to test for local adaptation. Moreover, molecular studies can give insight into the speed of neutral processes such as genetic drift and inbreeding, and can be used in Q_{ST}–F_{ST} comparisons (Leinonen et al. 2008) to infer past selection.

Acknowledgements We thank the *Regierungspräsidium Tübingen* for issuing the permits for seed sampling of the threatened species, Martin Engelhardt (Tübingen) and Bernd Junginger (Botanic Garden of the University of Tübingen) for locating the original natural populations, Simon Bauer (Botanic Garden of the University of Tübingen) and Michael Koltzenburg (University of Tübingen) for sharing knowledge on the species and cultivation techniques, and Andreas Ensslin (University of Bern) for his critical review of a previous version of our manuscript. This study benefited from a mobility fund from the European Union programme *Knowledge Education Development* to L.S.

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