





RESEARCH PAPER

Genotypic and phenotypic distinctness of restored and indigenous populations of *Pimpinella saxifraga* L. 8 or more years after restoration

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Keywords

Common garden experiment; ecological restoration; gene flow; non-local seeds; population genetics.

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INTRODUCTION

Anthropogenic influences seriously impair the biological diversity of the Earth (e.g. Hautier *et al.* 2015). To minimize human impact, ecological restoration aims to assist the recovery of altered or damaged ecosystems and their biodiversity (Nevill *et al.* 2016). This requires large-scale reintroduction of plants, e.g. in Germany in 2015 >21,000 t of perennial seeds, with constantly increasing demand at regional, national and global level (Guerrant & Kaye 2007; Elzenga *et al.* 2019; Mainz & Wieden 2019). In the past, the non-local seed sourcing of naturally occurring herbaceous species was the main strategy for ecological restoration in Germany. The prices for non-local seeds were significantly lower, and large quantities of local seeds were unavailable (Burton & Burton 2002; Kettenring *et al.* 2014). It is only in recent decades that awareness has been raised of the potential consequences of using non-local seed mixtures on the fitness and genetic diversity of indigenous populations (Broadhurst *et al.* 2008; Sgro *et al.* 2011; Breed *et al.* 2013; Aavik *et al.* 2014; Aavik & Helm 2018; Mainz & Wieden 2019). Risks are associated with introgression and hybridization between non-local and indigenous provenances, which may lead to homogenization, coexistence or extinction of regional, potential locally adapted, and/or non-local gene pools (Hughes *et al.* 2008; Jones 2013). On the other hand, locally depleted gene pools

ABSTRACT

- The recovery of altered or damaged ecosystems demands large-scale reintroductions of seeds. In the past, ecological restoration in Germany was carried out with non-local seeds of naturally occurring species. We here analysed whether the genetic pattern of the introduced non-local seeds (R = restored) of *Pimpinella saxifraga* are still detectable several years after application and whether the phenotype differs from that of the regional gene pool (I = indigenous) of the species.
- We collected material from individuals of R and I sites, conducted a common garden experiment and tested for genetic, morphological and phenotypic differences. In a cutting experiment we investigate treatment effects on indigenous and restored populations.
- At all investigated sites we only found *P. saxifraga* individuals with comparatively similar genome sizes. The population genetic analysis revealed two large and quite distinct molecular clusters, separating indigenous and restored individuals along the first axis. None of the vegetative, but two of the reproductive fitness parameters differed between individuals of the R and I sites. Cutting always had a significant influence on all analysed vegetative and reproductive fitness parameters, regardless of the individuals' origin. The effects of mowing always mask origin-specific characteristics, which then disappear.
- Genotypic coexistence reduces the availability of niches for the local genotype and may eventually lead to genotypic competition or introgression. We therefore recommend not to use non-local genotypes of this species in the region. Instead, we recommend using the genetically diverse local genotypes of *P. saxifraga* for restoration purposes.

could benefit from the increase in local diversity through genetic mixing between indigenous and non-local genotypes, as a high level of genetic diversity could be beneficial for possible adaptations to future environmental changes (Rice & Emery 2003; Knop *et al.* 2006; Aviron *et al.* 2009; Verhoeven *et al.* 2010; Sgro *et al.* 2011; Breed *et al.* 2013).

Aavik *et al.* (2012) investigated population genetic admixture between old and restored populations of the grassland plant *Lychnis flos-cuculi* L. (*Caryophyllaceae*) 3–8 years after restoration and discovered only limited gene flow and little genetic admixture between sown and natural populations, which is not indicative for restricted population functional connectivity. In contrast, Reiker *et al.* (2015) were able to show that the use of non-local seed sources of *Daucus carota* L. (*Apiaceae*) after more than 10 years of cultivation with non-local seed sources did not result in genetic differences and differentiation between populations on restored and indigenous sites. Shi *et al.* (2018) investigated the costs and benefits of admixture in non-local and indigenous *Lythrum salicaria* L. genotypes through cross-breeding and reciprocal transplant experiments. They found limited evidence of local adaptation and concluded that in this case admixture can enhance plant performance, which can be particularly beneficial in stressful environments.

In a comparative approach, we here test whether the genetic makeup of the former non-local seed source use of *Pimpinella*

saxifraga L. (*Apiaceae*) in restoration projects at sites in central Germany several years after application (restoration between 1994 and 2004) differs from the species regional gene pool. In a comparative approach, we compare genetic diversity and differentiation using amplified fragment length polymorphisms (AFLP). This molecular method was chosen because of its high polymorphism rate and reproducibility, ease of genotyping and analysis, and low cost (Mattersdorfer *et al.* 2012; Westberg *et al.* 2013). We measured the genome sizes of two randomly selected individuals per site to estimate the ploidy level. Phenological and morphological differences were analysed in a common garden experiment in which we cultivated plants from all sites. In addition, we simulated mowing to investigate whether the treatments had different effects on indigenous and restored populations.

The study was carried out to clarify whether the non-local seed use of *P. saxifraga* in central German populations on restored sites is still detectable several years after restoration and whether it differs from the regional gene pool. We asked: (i) whether and possibly which population genetic differences between indigenous and restored populations are detectable, (ii) whether there are fitness differences between individuals from indigenous and restored sites, and (iii) whether the use of the ten-fold cheaper non-local seeds of *P. saxifraga* can be recommended for ecological restoration.

MATERIAL AND METHODS

Study species

Pimpinella saxifraga is a winter-green perennial herbaceous member of the *Apiaceae*, which forms vegetative rosettes in the first year. From the second year onwards, the flowers develop in a compound umbel. The small hermaphrodite flowers have white petals, and the stamens are protruding (Knuth 1898–1905). Pollinators are beetles, flies, syrphid flies, wasps and bees with medium-length tongues (Willemstein 1987). *Pimpinella saxifraga* is predominantly outcrossing, but according to Knuth (1908), East (1940) and Berger *et al.* (1975), selfing is also possible. *P. saxifraga* mainly grows on sunny, moderately dry, nutrient-poor, calcareous soils in grasslands and on dwarf shrub heathland (Hänsel *et al.* 1994) in lowlands and sub-montane regions across Europe, Central Asia and the Caucasus (Berger *et al.* 1975). The species is morphologically variable. Up to ten subspecies have been defined in its native distribution range, however, none with high confidence levels; thus, the species is sometimes described as *P. saxifraga* agg. Chromosome numbers vary between $2n = 18, 20, 36$ or 40 (<http://ccdb.tau.ac.il/>), however in Germany *P. saxifraga* is only reported as $2n = 18$ (Kumar *et al.* 2012). Morphological and taxonomic investigations in the common garden experiment confirmed only the presence of *P. saxifraga* s. str. in our analysis.

Study region and sampling

The investigation was conducted in central Germany (central and south Hesse, W-Thuringia, NW Bavaria) within an area of $\sim 200 \times 200$ km². Populations were selected, on one hand, on sites which have not been altered during the last 60 years (Prasse *et al.* 2010; indigenous sites, I sites) and, on the other

hand on sites which were formerly restored (restored sites, R sites) with non-local seed sources.

The R sites were created as part of compensatory measures and were restored between 1994 and 2009 (information from road site authorities; Table 1; Fig. 1). Populations are not evenly distributed throughout the investigated area. The original seed mixtures for restoration contained 0.1% *P. saxifraga* seeds (stable mixture since the late 1980s, according to the FLL Bonn; Research Society for Landscape Development and Landscaping, personal communication). The exact source location of seed provenances for restoration is unknown; however, during this period of restoration, seeds were commonly produced in Eastern or Southern Europe, while indigenous seed sources in these large quantities were not available in the late 1980s. The exact origin of the seeds or seedlings of the former restoration projects are no longer traceable.

The I sites were selected on the basis of their regional proximity to the restored sites, but with a minimum distance of 9 km between the indigenous and restored sites. Sites were chosen with information from the Association of German wild plants and seeds producers e.V., and with permission from the local nature conservation authorities (Table 1; Fig. 1). The I sites are mostly maintained under the Habitats Directive of Natura 2000 or are protected nature reserve areas (Table 1), and it is well documented that they have not been modified or re-sown during the last 60 years (Prasse *et al.* 2010).

In autumn 2011 we sampled seven populations per I and R site. Leaf material of 13 individuals per population were sampled for genome size measurements and molecular analyses. The samples were immediately dried and stored in silica gel until further processing (Table 2). The minimum distance between sampled individuals was 2 m so as to best represent site-specific population genetic diversities. For the common garden experiment, we sampled seeds of at least 15 individuals per site.

Genome size estimates

Genome sizes were measured because cytotype differences might be indicative of the different subspecies of *P. saxifraga*. Pre-tests revealed silica-dried leaf material was suitable for 1C value estimates. Genome sizes of two randomly selected individuals per site were analysed. We used the DAPI counterstaining protocol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendation and analysed the samples on a flow cytometer (Cy Flow Sysmex Partec GmbH, Görlitz, Germany). *Latuca sativa* L. (Michaelson *et al.* 1991, <http://data.kew.org/cvalues>) served as reference (Table 2). According to Wang *et al.* (2015), the following formula was used to determine the nuclear DNA content for each sample: Standard 2C value (pg) = (Sample peak mean/Standard peak mean) \times nuclear DNA content of the reference Standard (pg).

Molecular analysis

In the laboratory, ~ 1 cm² of the silica-dried leaf material was homogenized with a mill (Retsch MM 301). Total genomic DNA was extracted (DNeasy Plant mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol. The obtained DNA quality, quantity and purity was checked on 1.5

Table 1. Site-specific information of the investigated *Pimpinella saxifraga* populations.

popu- lation code	location	latitude (N)	longitude (E)	protected area no. (status) or date of restoration	management	molecular analysis no. individuals	genome size measurement no. individuals	morphological and phenological analysis	
								no. individuals un-mowed	no. individuals mowed
PI01	Biebertal	50.641687	8.556461	5317305 (FFH)	extensive mowing	13	2	16	15
PI02	Kirchvers	50.690680	8.579207	5317301 (NSG)	extensive mowing	13	2	16	16
PI03	Niederkleen	50.480773	8.616436	5517301 (FFH)	sheep grazing	13	2	16	16
PI04	Eichsfeld	51.220721	10.358348	4728301 (FFH)	sheep grazing	13	2	16	15
PI05	Berka v. d. Hainich	51.039571	10.415881	4828301 (NP)	sheep grazing	13	2	16	16
PI06	Schwarzer Berg	50.292924	9.920461	5526371 (FFH)	extensive mowing	13	2	15	15
PI07	Lauterbach	50.696284	9.359665	5322305 (FFH)	sheep grazing	13	2	16	16
PR01	Ober Erlenbach 1a	50.219039	8.679331	2004	mowing once per year	13	2	15	15
PR02	Ober Erlenbach 1b	50.218477	8.681457	2004	mowing once per year	13	2	17	16
PR03	Ober Erlenbach 2a	50.226271	8.701537	2004	mowing once per year	13	2	16	16
PR04	Ober Erlenbach 2b	50.226831	8.698567	2004	mowing once per year	13	2	17	16
PR05	Herleshausen 1	50.995496	10.153041	2004	mowing once per year	13	2	7	7
PR06	Herleshausen 2	51.002248	10.130403	2003	mowing once per year	13	2	No data	No data
PR07	Fernwald	50.561315	8.757745	2003	mowing once per year	13	2	13	12
					total I	91	14	111	109
					total R	91	14	85	82
					total I + R	182	28	196	191

Location in accordance to nearest village or town; normal = number of un-mowed individuals for the elevation of reproductive Fitness, cut = number of mowed individuals; Management = Type of maintenance.

% TAE-agarose gel and measured for each individual using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). A total of 10 µl genomic DNA (30 ng·µl⁻¹) was double-digested in a final volume of 25 µl (0.25 µl *EcoRI* (10 U·µl⁻¹; Thermo Fisher Scientific), 0.15 µl *MseI* (10 U·µl⁻¹; Thermo Fisher Scientific), 2.5 µl 10x buffer (Thermo Fisher Scientific) and 12.1 µl purified H₂O at 37 °C for 3 h. Reaction was terminated by exposure to 65 °C for 10 min. Ligation was carried out with 0.5 µl T4 ligase (10 U·µl⁻¹; Thermo Fisher Scientific), double-stranded adapters 0.5 µl *EcoRI* (5 pmol·µl⁻¹; Metabion), 0.5 µl *MseI* (50 pmol·µl⁻¹; Metabion Planegg, Germany), 1.2 µl ATP (10 mM; Thermo Fisher Scientific), 0.5 µl 10x ligation buffer (Thermo Fisher Scientific) at 20 °C for 16 h. Two consecutive PCR amplifications were performed using primers with one (+1) and three (+3) selective nucleotides at their 3' ends, the *EcoRI* + 3 forward primer of the second PCR being fluorescence-labelled. For the first pre-selective PCR, 1.5 µl of restriction ligation product was used as template in a total volume of 7 µl containing 0.2 µl primers (50 ng·µl⁻¹; Metabion), 0.7 µl 10x buffer (Qiagen), 0.14 µl dNTPs (10 mM; Thermo Fisher Scientific), 0.04 µl Taq polymerase (1000 U·µl⁻¹; Qiagen) and 4.2 µl ddH₂O. PCR conditions for the pre-selective amplification comprised an initial step of 94 °C for 2 min followed by 30 cycles at 94 °C for 20 s, at 56 °C for 30 s, at 72 °C for 2 min, and a final extension step at 72 °C for 30 min. Samples were sent to LGC (Cologne, Germany) for fragment visualization. An initial primer screening with six different primer combinations and two individuals per different I and R sites was conducted, and for the final analyses, the following primer combinations were used: E35/M48, E35/M51 and E35/M62 (Table S1). These primers proved to be variable and informative, provided clear and reproducible

bands and were sufficiently polymorphic to show variation within and among populations.

A data matrix was established using GENOGRAPHER 2.1.4 (Benham *et al.* 1999). Each AFLP fragment was scored using the 'thumbnail' option, which allows for the comparison of signals per locus over all samples. If possible, peaks of low intensity were additionally scored by eye and included into the analysis. Standard lanes, carrying identical samples on each plate, were added as quality check. For ten samples, the AFLP analysis for each primer combination was repeated and the error rate was calculated as number of differences related to the total number of comparisons and subsequently averaged over the three combinations (Pompanon *et al.* 2005). Although we carefully analysed the data twice from two independent researchers and the AFLP gel images were very good and thorough, the final overall error rate was relatively high (12%). This is due to the low number of repetitions because of plate restrictions.

Genetic diversity estimates were analysed by using AFLPSurv 1.0 (Vekemans 2002) by applying the default options (Table 3). Genetic variation within and among populations was evaluated using Analysis of Molecular Variance (AMOVA) in ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010), with 10,000 permutations (Table 4). Patterns of genetic population structure were visualized with a Principal Coordinates Analysis (PCoA) using the R package ADEGENET version 1.4-2 (Jombart 2008). Pairwise F_{ST} was calculated with Gen ALEx 6.5 (Peakall & Smouse 2012; Table 5). To further explore the genetic affiliation of individuals to genetic clusters, we applied the Bayesian clustering approach implemented in STRUCTURE version 2.3.3 (Pritchard *et al.* 2000; Fig. 3). Genetic clusters were detected by applying default settings, the admixture model, with 100,000

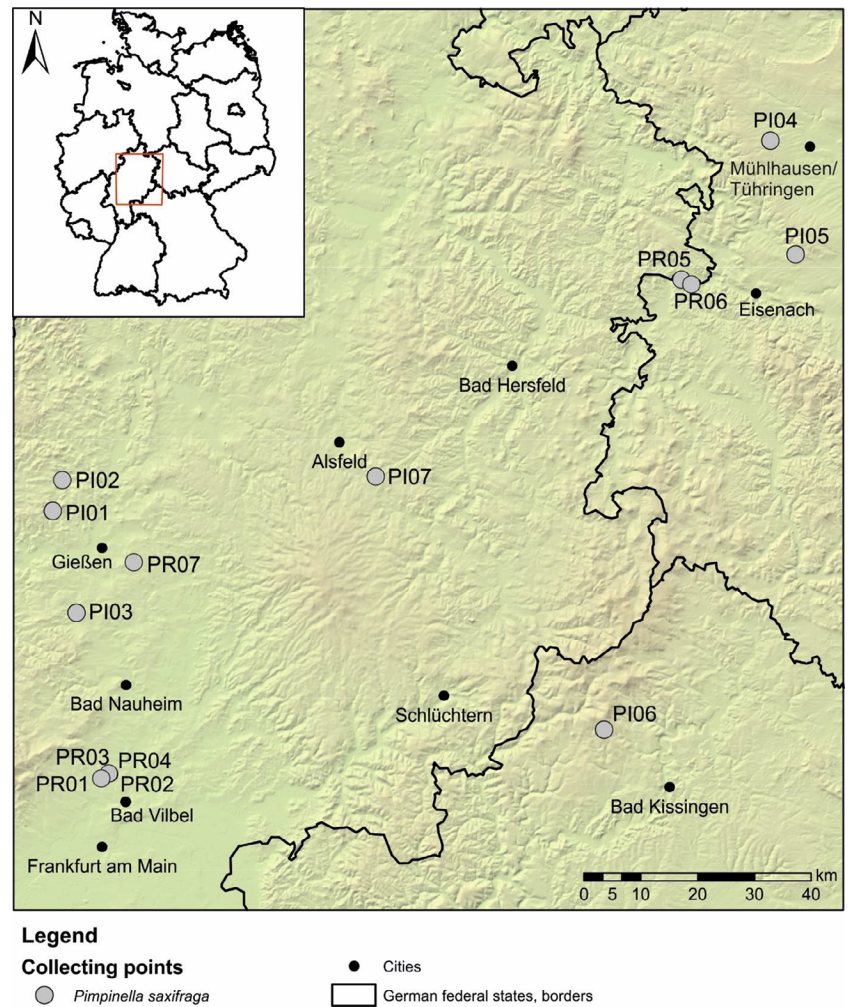


Fig. 1. Map depicting sampling sites in the study region in central Germany (Hesse, Thuringia and Bavaria). PI01–PI07 are the sampled indigenous sites, PR01–PR07 are the sampled restored sites (see also Table 1). Map was prepared with ArcGIS Desktop (ArcGIS Desktop 10.2.2., Esri).

Table 2. Genome size estimates of *Pimpinella saxifraga* ($n = 2$) from indigenous (I) and restored (R) sites.

individuals from I sites				individuals from R sites			
population code	(genome size in pg)			population code	(genome size in pg)		
	individual 1	individual 2	mean		individual 1	individual 2	mean
PI01	3.38	3.45	3.42	PR01	3.01	3.2	3.11
PI02	3.20	3.39	3.30	PR02	3.10	3.82	3.46
PI03	3.50	3.62	3.56	PR03	3.75	3.90	3.83
PI04	3.31	3.82	3.57	PR04	3.60	3.80	3.70
PI05	3.55	3.60	3.58	PR05	3.30	3.56	3.43
PI06	3.54	3.64	3.59	PR06	3.20	3.60	3.40
PI07	3.44	3.85	3.65	PR07	3.30	3.48	3.39
mean I			3.52	mean R			3.47

Markov Chain Monte Carlo (MCMC) replicates, with a burn-in period of 50,000 and 20 repeats per run for each chosen cluster number (*i.e.* $K = 1-14$), Ploidy = 2, and Recessive alleles = 1. To identify the most likely K modal distribution, DK (Evanno *et al.* 2005) was determined using STRUCTURE HARVESTER (Earl & von Holdt 2012). To verify the most probable cluster membership coefficient among the 20 runs of STRUCTURE and STRUCTURE HARVESTER, we used CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007).

Corresponding graphs were constructed with DISTRUCT (see Fig. 1; Rosenberg 2004).

Morphological analyses

In spring 2012, 60 seeds were sown and cultivated in a common garden experiment at the university nursery in Giessen (Germany). The germinated seedlings from 13 of the 14 examined populations were planted in single pots with uniform

Table 3. Population specific genetic diversities of *Pimpinella saxifraga* from indigenous and restored sites with calculated percentage polymorphic loci (PLP) and genetic diversities (H_e) after Lynch & Milligan (1994), calculated with a Bayesian method with non-uniform prior distribution of allele frequencies (Zhitovskiy 1999; Vekemans 2002). Paired *t*-tests of the indigenous and restored sites of both species were not significant.

<i>Pimpinella saxifraga</i> from indigenous sites				<i>Pimpinella saxifraga</i> from restored sites			
population code	N	PLP	H_e	population code	N	PLP	H_e
PI01	13	96.9	0.363	PR01	13	89.5	0.342
PI02	13	88.9	0.358	PR02	13	93.2	0.364
PI03	13	91.4	0.352	PR03	13	93.8	0.377
PI04	13	90.7	0.347	PR04	13	91.4	0.379
PI05	13	91.4	0.361	PR05	13	96.3	0.395
PI06	13	92.6	0.359	PR06	13	95.1	0.372
PI07	13	91.4	0.355	PR07	13	95.1	0.379
Ø H_e I	13	91.9	0.356	Ø H_e R	13	93.5	0.373

nutrient-poor soil. Mice destroyed our experiment for PR06 and reduced the number of samples in PR05. The randomly selected plants of each population were arranged without grouping and cultivated with regular irrigation and weed control. One-third per study site grew up without treatment and another third was cut to 15 cm on 31.07.2013 to simulate grazing or mowing as natural conditions of grassland habitats. All main shoots were cut down, no matter whether they were in flower or not. The last third was harvested on 20.06.2013 to count and measure the vegetative morphological variation. This allowed for testing of two models of influence on morphological and phenological variation: (i) the effect of origin (I, R) and (ii) the combination of treatment (mowed, unmowed) and origin (I, R).

Investigated parameters of vegetative morphological variation were: (1) number of leaves, (2) size of the longest leaves, by measuring length and width, (3) length and (4) diameter of the root, and leaf weight of (5) fresh and (6) dry matter, and (7) fresh and (8) dry root (7 and 8). The data collections of reproductive morphological variation were measured twice per week in 2013 throughout the season. They were: (9) day of first flowering, (10) day of maximum number of flowering umbels, (11) day of maximum number of fruiting umbels (12) number of flowering

umbels, (13) number of fruiting umbels, (14) number of umbels with ripe fruits only, (15) weight of ripe umbels, (16) day of death (after seed set, plants turn brown and die off). Additionally, the following derivatives of the former reproductive parameters were calculated: (17) duration from first flowering to highest amount of fruiting umbels, (18) number of simultaneously flowering and fruiting umbels, (19) the date of this, (20) duration from first flowering to the first ripe umbel and (21) duration from first flowering to the end of the growing season (all significant traits provided in Table 6; Fig. 4).

Data analysis

STATISTICA (version 10.0; Statsoft, Tulsa, OK, USA) was used to calculate effects of the single factors 'origin' ($k = 2$, indigenous and restored) and 'treatment' ($k = 2$, mowed versus unmowed) based on raw data. The factor combinations were assessed with a multi-factorial ANOVA. Subsequently, the significance of the differences between the treatments was assessed using a Tukey test (Table 6).

For all other statistical analyses, we used R 3.5.1 (R Core Team 2013) and the R packages 'nlme' (Pinheiro *et al.* 2020) and 'lme4' (Bates *et al.* 2015) based on the raw data (Fig. 4). Figures were generated with R 'ggplot2' (Wickham 2016).

RESULTS

Genome sizes

All 28 analysed *P. saxifraga* individuals had, on average, comparable 1C values of 3.50 ± 0.18 pg on indigenous sites and of 3.47 ± 0.36 pg on restored sites (Table 2).

Genetic diversity and differentiation

The AFLP dataset with three different primer combinations and 182 analysed individuals resulted in 162 unambiguously scorable loci, ranging from 50 to 450 bp. The mean genetic diversity of the indigenous *P. saxifraga* populations was $H_e = 0.356$, with genetic diversities ranging from $H_e = 0.347$ (PI04) to $H_e = 0.363$ (PI01; Table 3). The mean genetic diversity of the restored *P. saxifraga* populations was $H_e = 0.373$, with genetic diversities ranging from $H_e = 0.342$ (PR01) to $H_e = 0.395$ (PR05; Table 3).

Table 4. AMOVA for populations of restored and indigenous *Pimpinella saxifraga*. Statistics include degrees of freedom (df), AMOVA sums of squares (SS), variance components (VC), percentage variation, differentiation values (F_{CT} , F_{SC} , F_{ST}) and significance level ($P < 0.001$).

	source of variation	df	SS	VC	% variation	<i>F</i> -statistics ($P < 0.001$)	
all sites	among groups (I, R)	1	459.735	4.945	14.253	F_{CT}	0.143
	among populations	12	536.654	1.374	3.961	F_{SC}	0.046
	within populations	168	4356.770	28.375	81.786	F_{ST}	0.182
	total	181	5353.159	34.695	100		
indigenous sites only	among populations	6	335.747	1.511	3.996		
	within populations	84	3050.077	36.310	96.004	$F_{ST(I)}$	0.040
	total	90	3385.824	37.822	100		
restored sites only	among populations	6	306.495	1.336	3.811		
	within populations	84	2832.077	33.715	96.189	$F_{ST(R)}$	0.038
	total	90	3138.571	35.051	100		

Table 5. Pairwise F_{ST} values for the different provenances of *Pimpinella saxifraga*.

species	source of variation	range of pairwise F_{ST} values
<i>Pimpinella saxifraga</i>	indigenous (I)	0.011–0.086
	restored (R)	0.003–0.095
	all (I + R)	0.003–0.219

The PCoA depicted two large and quite distinct clusters that separated indigenous and restored individuals along the first axis (Fig. 2). Notably, very few individuals reflect intermediate genotypic patterns. Overall, the first three principal coordinates accounted for 10.17%, 3.84% and 3.08% of genetic variation.

The STRUCTURE (Pritchard *et al.* 2000) analysis resulted in a distinct modal maximum of ΔK at $K = 2$ (Evanno *et al.* 2005) and a saturation value of mean log-likelihood at $K = 2$. The STRUCTURE analysis clearly confirmed the PCoA results (Fig. 2) and depicted two large and distinct clusters that separated I and R site individuals (Fig. 1). Only faint signatures of introgression from I to R could be detected (*e.g.* in PR01, PR04; Fig. 3), while R to I introgressions seem to be more common (*e.g.* in PI02, PI03, PI06, PI07).

The AMOVA resulted in a global F_{ST} of 0.182 (Table 4), indicative of high genetic differentiation between all sites. Separately,

the variation within the respective indigenous and restored sites is negligible ($F_{ST(I)}$ of 0.040 and $F_{ST(R)}$ of 0.038, both $P < 0.001$). The highest percentage of genetic variation was consistently largest within populations. The hierarchical AMOVA (Table 5) showed that the range of pairwise F_{ST} values between all sites are very high and varied between 0.003–0.229, whereas it was low within I and R sites ($I = 0.011$ –0.086, $R = 0.003$ –0.095).

Morphology

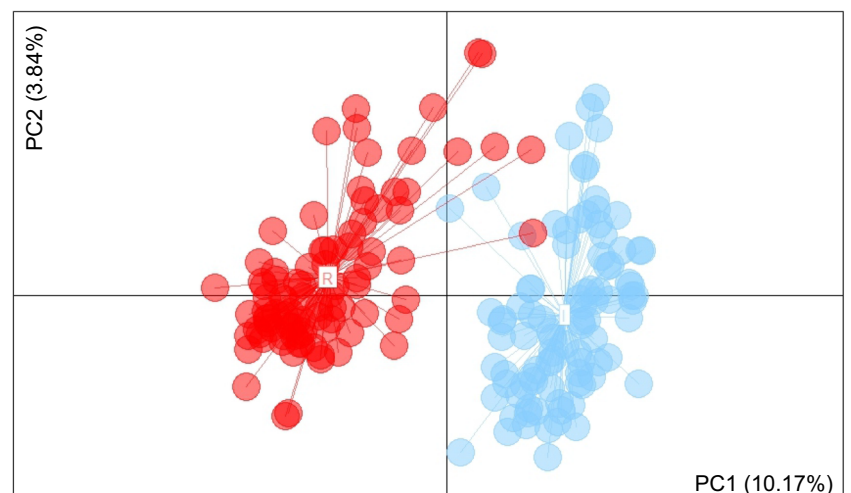
In total, 387 individuals were examined with the ANOVA. A total of 196 individuals were un-mowed, to test the effect of origin, and 191 were cut, to simulate mowing, which is the typical management treatment on the restored sites (Table 1). A total of 111 individuals from I and 85 individuals from R sites were un-mowed; 109 individuals from I and 82 individuals from R sites were mowed individuals (Table 1). For the un-mowed individuals, the analyses of vegetative characters (traits 1–8) did not reveal any significant differences between populations of the R and I sites. However, of the 13 reproductive fitness parameters analysed, we found two significant site-specific differences. Comparing the duration from the first flowering to the maximum number of flowering umbels (traits 9 and 10; Fig. 4a), the individuals of the R sites reached the maximum number of umbels earlier in summer, on average after

Table 6. Effects of treatment (T: mowed, un-mowed) and origin (O: from indigenous (I) or restored (R) sites) or a combination of treatment and origin (O \times T).

<i>Pimpinella saxifraga</i>	duration from flowering to maximum umbels				number of flowering and fruiting umbels				number of fruiting umbels with ripe fruits			
	df	MQ	<i>F</i>	<i>P</i>	df	MQ	<i>F</i>	<i>P</i>	df	MQ	<i>F</i>	<i>P</i>
all	1	81039.4	790.8	<0.001	1	18640.4	410.2	<0.001	1	11799.6	376.2	<0.001
treatment (T)	1	642.0	6.3	0.013	1	3039.2	66.9	<0.001	1	2664.2	84.9	<0.001
origin (O)	1	449.6	4.4	0.037	1	421.2	9.3	0.003	1	157.0	5.0	0.026
T \times O	1	15.3	0.1	0.700	1.000	68.5	1.5	0.221	1	33.8	1.1	0.300
error	299	102.5			315	45.4			305	31.4		

Significant *P*-values < 0.05 are in bold.

df = degrees of freedom, *F* = variance ratio, *P* = error probability.

**Fig. 2.** PCoA summarizing the AFLP marker results for *Pimpinella saxifraga* individuals. Each circle represents one individual. Individuals from restored sites (R) are depicted in red, and from indigenous sites (I) are depicted in blue.

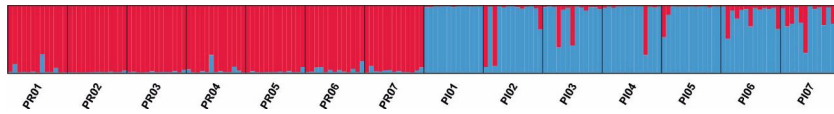


Fig. 3. AFLP results evaluated in a STRUCTURE analysis for the surveyed populations of *Pimpinella saxifraga*.

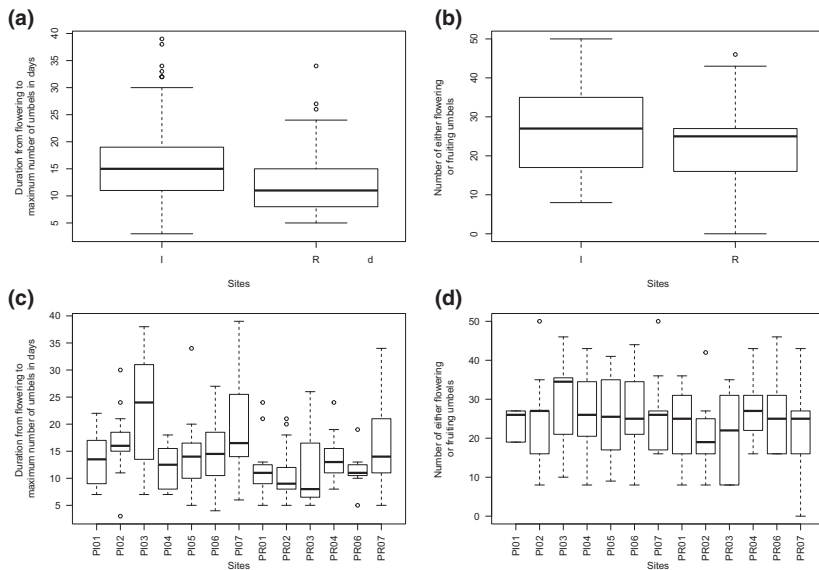


Fig. 4. Summarized results of two significant reproductive fitness parameters of *Pimpinella saxifraga* on restored (R) and indigenous (I) sites. (a) Mean values of trait 9 (day of first flowering) and trait 10 (day of maximum number of flowering umbels) combined; (b) mean values of trait 12 (number of flowering umbels) and trait 13 (number of fruiting umbels) combined; (c) site specific values of trait 9 and 10 combined; (d) site specific values of trait 12 and 13 combined. Bars indicate mean fitness parameter \pm SE. *P*-values for comparisons between R and I sites are from ANOVA using the residuals of linear mixed-effect models for factor origin.

22.9 days, while the individuals of the I sites reached this maximum after an average of 26.5 days. At the same time, the plants of the R sites had fewer flowering and fruit-bearing umbels (characteristics 12 and 13; Fig. 4b), on average 12.5 umbels per individual, than those of the I sites, with an average of 16.6 umbels per individual.

The ANOVA showed that cutting always had a significant influence on all analysed vegetative and reproductive fitness parameters, regardless of the origin of the samples. Thus, effects of mowing always mask origin-specific characteristics, which then disappear (Table 6).

DISCUSSION

Even more than 8 years after the compensation measures, we can still detect population genetic patterns indicating the previous use of non-local seed sources of *P. saxifraga*. At all investigated sites in central Germany we found only *P. saxifraga* individuals with comparatively similar genome sizes (Table 3), which on average are slightly lower than the earlier cited 1C value of 3.82 pg (Temsch *et al.* 2010). The deviation from previous results, however, are within potential reading inaccuracies of the available CyFlow device. Our analysis is limited to estimation of the genome size of only two individuals per population, so it is possible that additional cytotypes occur in the respective populations.

Morphological identification of the plants in the common garden experiment supported the assignment of all individuals to *P. saxifraga* subsp. *saxifraga* L. None of the vegetative fitness parameters differed between individuals of the R and I sites. Our results support earlier findings of Reiker *et al.* (2020) in *Daucus carota* and Bucharova *et al.* (2017) in a transplant experiment with seven different herbaceous plant species, who

also discovered mostly non-significant vegetative fitness differences in populations from R and I sites.

In *P. saxifraga*, only two of the analysed reproductive fitness parameters differed significantly between individuals of the different origins. One of the site-specific significant traits relates to phenology (number of days from first flowering to maximum of flowering; Fig. 4). The plants from the R sites reached their maximum flowering umbels, on average after 22.9 days, which was significantly earlier than the plants from the I sites (on average after 26.5 days). The earlier maximum flowering of plants that originally came from non-local seed sources still reflected in our common garden experiment an adaptation of these plants to their original and different environments, climate, latitudes and seasonality (Durka *et al.* 2016). Flowering time is known to differ strongly along geographical gradients (*e.g.* Montague *et al.* 2008; Kawakami *et al.* 2011) and may be indicative of the R site individuals' non-local origin. Nevertheless, the exact origin of the seeds is no longer traceable today.

Plants from I sites had a 1.3-fold higher number of either flowering or fruit-bearing umbels at the maximum of flowering or fruiting, with an average of 16.6 umbels, while plants from R sites had an average of only 12.5 umbels (Fig. 4). Our results are in line with the results of the transplant experiment of Bucharova *et al.* (2017), who found that *Centaurea cyanus* L. and *Lychnis flos-cuculi* L. plants of indigenous origin generally had an average reproductive performance 1.3-fold and 1.4-fold higher than the non-native plants, respectively. The values are not significant if the number of flowering and fruit-bearing umbels at the time of maximum flowering or fruiting, respectively, are analysed independently. Overall, we found no difference in the weight of mature umbels at the end of the growing season; both provenances had a surprisingly similar number of

mature umbels, indicating an overall similar reproductive success.

Our mowing experiment was conducted to simulate management on the restored sites, which are all compensatory sites that are mowed once a year. However, it was not possible to simulate the management strategies on the indigenous sites, which had different intervals of sheep grazing as well as intensive or extensive mowing. The mowing experiment was conducted because management practices can have a major impact on grassland species, e.g. Reisch & Poschlod (2009, 2011) discovered a shift towards earlier flowering of *Scabiosa columbaria* L. on mown sites compared to grazed sites. However, this could not be confirmed in our common garden experiment for *P. saxifraga*.

Despite the use of non-local seed sources for restoration purposes and up to 10 years after the first initial seed sowing, it was still possible to detect specific genetic patterns for the *P. saxifraga* individuals on R and I sites without significant mixing (Fig. 2). Both genetic patterns are comparatively diverse ($\bar{O} H_e = 0.365$), with higher genetic diversities than typical for perennials ($H_e = 0.16 \pm 0.10$), common plant species ($H_e = 0.20-0.10$, or dicotyledons ($H_e = 0.16 \pm 0.08$; Reisch & Bernhardt-Römermann 2014). The populations of non-local origin (R sites) featured, on average, higher population genetic diversities ($\bar{O} H_e R = 0.373$) than the indigenous (I) plants ($\bar{O} H_e I = 0.356$; Table 3), and thus are not genetically depleted. This may be because, for seed production, seeds from different populations are often mixed to increase genetic variation (Bucharova *et al.* 2019).

Sympatric cryptic lineages and genotypic coexistence in the region is an indicator of strong gene flow barriers, as a result of either reproductive isolation and/or limited dispersal capacity of the species. *Pimpinella saxifraga* is self-compatible (East 1940), which, according to theory, fosters low outcrossing rates (Nybohm & Bartish 2000; Duminil *et al.* 2009). The percentage variation among groups (I, R) was decisive for the high differentiation ($F_{ST} = 0.182$, $P < 0.001$) of *P. saxifraga* in the region, while both genetic signatures separately feature patterns of negligible population genetic differentiation ($F_{ST(I)} = 0.040$, $P < 0.001$; $F_{ST(R)} = 0.038$, $P < 0.001$). The large to very large pairwise F_{ST} values between *P. saxifraga* populations of different origins indicate that the effects of random genetic drift are not outweighed by gene flow and result in unpredictable and strong genetic isolation among provenances.

The STRUCTURE analysis (Fig. 3) revealed very few indications of potential recombination between genotypes from I and R sites, with slightly lower crossing barriers in the indigenous populations, where very few individuals (PI02, PI03, PI04, PI06, PI07) reflect some genetic pattern typical for the R sites but not *vice versa*. Only two individuals in PI02 feature genotypes that are potentially indicative of introgression *via* seed dispersal or potentially originated from the initial seed bank prior to restoration. The smooth seed surfaces in *P. saxifraga* lack specialized dispersal structures, indicating primarily barochorous dispersal in the near vicinity of the mother plant (Knuth, 1908), while Tóth (2014) claimed that *P. saxifraga* only sporadically produces viable seed banks. Thus, the origin of the genetic R site pattern in the individuals of the I site remains unknown. Pollinators potentially responsible for recombination events in populations PI03, PI04, PI06 and

PI07 are beetles, flies, syrphid flies, wasps and bees with a medium length proboscis (Willemstein 1987; Rader *et al.* 2011), and all with a foraging distance rarely above 1 km (Kwak *et al.* 1998; Peterson *et al.* 2008). We did not sample all populations in the regions, thus, potential stepping-stone populations might have provided bridging functions between genotypes of similar provenances. In a previous study in *Daucus carota* L. (Reiker *et al.* 2015), we analysed the population genetic diversity of the same R and I sites and detected negligible population differentiation in our study region. However, the opposite patterns between these two species from the same sites can be explained by the different breeding systems of the respective species, as *D. carota* is an obligate outcrossing species.

GENERAL RECOMMENDATIONS

The fact that the non-local genotypes of *P. saxifraga* flourish on the restored sites for at least 10 years after population establishment is indicative for the genotype persistence capacity even for those of non-local origin. However, the genetic distinctiveness reduces the availability of niches for the local genotype and may eventually lead to genotypic competition or introgression. A subsequent loss of genetic incompatibility between the two genotypes may affect the gene flow–drift equilibrium over time, which can result in adverse intrinsic genetic diversity, resulting in phenomena such as heterosis, as shown for F_1 populations of *Mimulus guttatus* DC (van Kleunen *et al.* 2015), *Silene vulgaris* (Moench) Garcke (Keller & Taylor 2010) and *Alliaria petiolata* (M.Bieb.) Cavara & Grande (Mullarkey *et al.* 2013). The onset of an introgression of non-local genotypic diversity into the local gene pool is already evident. Potential genetic benefits associated with population admixture are increased genetic variation and the formation of novel trait combinations due to segregation and recombination, which could result in increased individual fitness and population growth rates (e.g. Hufford & Mazer 2003; Verhoeven *et al.* 2004; Bucharova *et al.* 2019). However, considering restoration objectives and goals, as well as the efficient use of resources concerning cost and seed availability (Ehrenfeld 2000; Kaye *et al.* 2001; Wilkinson 2001; Doede 2005; Miller *et al.* 2011), for *P. saxifraga* we recommend refraining from introducing non-local genotypes of species in the region in order to avoid genotypic competition. Instead, we recommend using the genetically diverse local individuals for restoration purposes.

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