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RESEARCH PAPER

Genetic and reproductive characterisation of seasonal flowering morphs of *Gentianella bohemica* revealed strong reproductive isolation and possible single origin

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ABSTRACT

Phenotypic polymorphism represents the most obvious type of intraspecific diversity raising scientific interest in its evolution and maintenance. We studied the regional endemic *Gentianella bohemica*, which exhibits an early- and a late-flowering morph. Genetic variation and structuring were investigated in relation to potential pollination and mating system differences, to verify hypotheses of evolutionary integrity, origin, and reproductive isolation of both flowering morphs. We identified the rarer early-flowering morph as an independent genetic entity, being more selfing, likely stronger pollinator-limited and reproductively isolated. All analysed populations showed strong among population differentiation and low overall genetic diversity due to habitat fragmentation and reduced population sizes. These results indicate likely inbreeding, but we also found evidence for possible outbreeding depression in the late-flowering morph. Both *G. bohemica* morphs are characteristic of traditionally used, nutrient-poor grasslands, but they represent independent conservation units and need temporally adapted management. We, therefore, also briefly discuss our results in the general context of conservation activities in relation to intraspecific polymorphisms and strongly argue for their formal and consequent consideration.

INTRODUCTION

Flowering time is an important trait of higher plants allowing the colonisation of new areas (Lee 2002), the response to global and regional climate change (Parmesan & Yohe 2003; Franks *et al.* 2007) and the formation of new species due to reproductive isolation resulting from temporal differentiation in flowering phenology (Coyne & Orr 2004; Rieseberg & Willis 2007). Generally, reproductive isolation is the result of pre- and post-zygotic mechanisms (Mayr 1963). Differentiation in flowering phenology is a pre-zygotic barrier already preventing successful pollination. However, while reproductive isolation due to geographic isolation can lead to allopatric speciation (Barraclough & Nee 2001; Coyne & Orr 2004), divergence in flowering times (or 'active phases' in animals) on site might open the infrequent pathway to sympatric speciation (Friesen *et al.* 2007; Martin & Willis 2007).

There are numerous examples of closely related taxa, in which differing flowering times are well documented and also used to discriminate species: in the genus *Ophrys*, for instance, specific one to one pollinator–plant relationships often exist, which are not only reflected in different flowering times, but also by different emergence times of pollinating bee species (*e.g.* Paulus 1998, 2006; Ayasse *et al.* 2010). Moreover, there are also cases in which a single species can show differing flowering times within 1 year at the same location. The most striking examples in this respect are annual weeds; some of these species are able to germinate and to subsequently flower at any time throughout the year when (weather) conditions are suitable (*e.g. Poa annua:* Kästner *et al.* 2001; *Capsella bursa-pastoris*: Neuffer *et al.* 2011). However, due to high environmental variability of suitable conditions in time and space, cohorts flowering at different times throughout the year are interconnected by gene flow among cohorts, representing a panmictic continuum, as has been demonstrated for the weedy *Senecio vulgaris* by Haldimann *et al.* (2003).

Finally, differentiation in flowering times within a single species has also been, albeit rarely, reported for non-weedy grassland species. Interestingly, these reports are restricted to certain phylogenetic lineages, like annual species of the genera *Euphrasia*, *Melampyrum*, *Odontites* and *Rhinanthus* (Orobanchaceae; Smith 1963; Zopfi 1993a,b; Koutecký *et al.* 2012) and biennial species of the genus *Gentianella* (Gentianaceae; Wettstein 1895, 1896, 1900; Zopfi 1991). Wettstein (1895) introduced the term 'Saisondimorphismus' (*i.e.* seasonal dimorphism) for taxa showing an aestival, *i.e.* early flowering and an autumnal, *i.e.* late-flowering morph within a year. He postulated reproductive isolation among these flowering morphs and (beginning) speciation. However, subsequent

evidence for the constancy, *i.e.* the genetic determinism, of flowering times and correlated morphological and/or ecological differences among those taxa have remained inconclusive: While some authors found, *e.g.* based on common garden experiments, (almost) no genetic fixation of respective characters (Heinricher 1903; Widder 1957; Smejkal 1962; Campion-Bourget 1982), the work on *Rhinanthus* of Zopfi (1993a,b, 1995) demonstrated consistency of several morphological characters in common garden experiments, and the possibility to differentiate ecological types using multivariate statistics. However, the most recent work of Pleines *et al.* (2013) failed in assessing such ecological *Rhinanthus* types as independent genetic lineages.

In general, intraspecific genetic differentiation might be shallow because such seasonal, morphological and ecological divergences are probably evolutionarily young. Indeed, already Wettstein (1895, 1900) presumed seasonal dimorphism as the result of repeated mowing in summer, forcing populations into flowering either earlier or later in the growing season. Subsequent authors followed this argument of a strong anthropogenic influence on the formation of intraspecific differentiation in the hemiparasitic Orobanchaceae and *Gentianella* (Karlsson 1974; Zopfi 1993a,b; Lennartson 1997).

Regarding *Gentianella bohemica*, taxonomic treatment of the early- and late-flowering morph varies in the literature: at the species level (*e.g.* Skalický 1969), at the subspecies level (*e.g.* Holub 1998; and even below the subspecies level (*e.g.* Greimler *et al.* 2004). However, not only their systematic classification is still debated, but also their evolutionary history and origin. While Wettstein's hypothesis (1895, 1900) of the establishment of early- and late-flowering morphs relates to traditional mowing in summer and implies development during historic times, Krause (1944) hypothesised a prehistoric origin. Moreover, Wettstein's scenario implies the possibility of multiple origins in different regions depending on (similar) land use (Wettstein 1895, 1900; Karlsson 1974), while genetic fixation of relevant and associated characters (Zopfi 1993b) might point to a single origin and subsequent spread across suitable, *i.e.* respectively managed, habitats.

In the present study we analysed *Gentianella bohemica* Skalický, which is endemic to the Bohemian Massif. We included all populations of the early-flowering and most of the late-flowering morphs of *G. bohemica* known from our study region, representing the only region where the early-flowering morph occurs, to answer the following questions:

1 Do early- *versus* late-flowering populations represent independent genetic entities? Finding (well-)defined genetic groups representing the early- and late-flowering morph, might point to a single origin. Note that our sampling includes two locations where both flowering morphs occur in the same meadow; therefore, genetic data should show the extent of reproductive isolation of flowering morphs, at least at these specific sites.
2 Are the two seasonal flowering morphs reproductively isolated? Are there differences regarding phenology, pollinators and/or mating system between early- and late-flowering populations and individuals?

Finally, our findings are also discussed in light of nature conservation practice, by asking whether and how well intraspecific polymorphisms are currently conserved.

MATERIAL AND METHODS

Study species

The European taxa of the genus *Gentianella* have mainly been divided into three morphologically diverse groups: (i) *G. amarella* agg. and (ii) *G. campestris* agg. – these two are both rather coherent – and the more heterogeneous (iii) *G. germanica* agg. (Greimler *et al.* 2004; Jang *et al.* 2005). Our study species *G. bohemica* belongs to the highly diverse last group (Skalický 1969; Wisskirchen & Haeupler 1998) and is described as a geographical and morphological intermediate taxon between *G. germanica* s.str. and *G. austriaca* (Skalický 1969; Greimler *et al.* 2004; Jang *et al.*2005).

Gentianella bohemica is currently distributed in the northern part of Austria (Mühl- and Waldviertel), the Czech Republic (Šumava Mountains and the Bohemian-Moravian Highlands), in the south of Germany (Bavarian Forest) and in South Poland (Meusel *et al.* 1978; Brabec 2008). With about 65 known populations in the Czech Republic and about 30 populations in Austria (*i.e.* Mühl- and Waldviertel), these regions represent the main distribution area; whereby Germany and Poland cover comparatively few populations (Engleder 2006, 2013; Brabec 2008; Zillig *et al.* 2010).

As common in many taxa of the genus *Gentianella,* our study species forms two seasonal flowering morphs (Wettstein 1896; Janchen 1960; Skalický 1969; Fischer *et al.* 2008). However, extant populations of the early-flowering morph are now only known from Austria (none of the other countries have currently evidence of the existence of early-flowering populations), where they were already reported to be extinct (Fischer *et al.* 2008), but indeed still occur in (at least) four populations in our study area, the Lower Austrian Waldviertel. *G. bohemica* is listed as a priority species on Annexes II and IV of the Habitats Directive (Council of the European Community 2007). Mainly for that reason, we use the epithet *bohemica*, although the valid name is *G. praecox* (A. et J. Kerner) Dostál ex E.Mayer, while *G. bohemica* Skalický in its strict sense only refers to the late-flowering morph [syn. *G. praecox* (A. et J. Kerner) Dostál ex E.Mayer subsp. *bohemica* (Skalický) Holub], whereas the early-flowering morph was described as *G. gabretae* [syn. *G. praecox* (A. et J. Kerner) Dostál ex E.Mayer subsp. *praecox* (Skalický) Holub]. Generally, the two flowering morphs of *G. bohemica* demonstrate different morphological characteristics in their adult stage: the early-flowering morph is sparsely branched, has long internodes and often only a few flowers. In contrast, individuals of the late-flowering morph are usually well branched, have short internodes and many flowers. However, branching in the lower part of the stem can often be observed as a result of mechanical disturbance caused by mowing or grazing (Götz 1991; personal observation). Flowering time of the early-flowering morph is early summer (usually second half of June), whereas the late-flowering morph flowers in autumn (mainly September).

Typical habitats of *G. bohemica* in our study area are traditionally used nutrient-poor grasslands, such as montane and submontane acidophilous *Nardus* grasslands. In the Czech Republic the species has also been recorded from other grassland types, such as *Bromion erecti* and *Arrhenatherion elatioris*, locally also from dry grasslands on acidic soils (*Koelerio-Phleion phleoides*; Brabec 2012).

Gentianella bohemica has a biennial life form, is non-competitive and light demanding (Rösler 2001; Engleder 2006; Dolek *et al.* 2010). Germination rates and survival of seedlings are influenced by the availability of open patches and by drought or too high precipitation during germination (cf. Dolek *et al.* 2010; personal observation). As a consequence, population sizes vary considerably between years (Engleder 2006; Zillig *et al.* 2010; Bucharová *et al.* 2012; Königer *et al.* 2012; personal observation).

Gentianella bohemica is insect-pollinated and partially wind-dispersed. Mechanisms for long-distance dispersal are absent, but grazing cattle or wild animals can aid dispersal of seeds by bending the stem of a mature plant (Dolek *et al.* 2010).

In both flowering morphs the pentamerous flowers are usually 2.0 to 3.5 cm long, reddish-violet and on the inside of petals bearded fringed. This rim of erect, whitish fringes is supposed to prevent small insects from entering the flower (Hegi 1966; Luijten *et al.* 1998). Nectaries are located at the bottom of the funnel-shaped flower (Halbmayr 2006). Nevertheless, a wide range of (not exclusively long-tongued) insects has been documented as pollinators on *G. bohemica* flowers; the most frequent are bumblebees (*Bombus* spp.), honeybees (*Apis mellifera*) and hoverflies (Syrphidae) (Dolek *et al.* 2010; Königer *et al.* 2012).

Plant material and sampling design for genetic analyses

In 2009, leaf samples were collected from four early- ('*e*') and 11 late-flowering ('*l*') *G. bohemica* populations in the Lower Austrian Waldviertel region (see Fig. 1a). This sampling design was somewhat unbalanced because the early-flowering morph, to the best of our knowledge, currently only exists in these four populations. Generally, leaf samples from at least 20 individuals per population were randomly collected; at two sites only leaves from two and four individuals could be collected, given the small population sizes (Table 1).

Leaf material was dried in silica gel and stored at room temperature before DNA extraction. For each population, the number of flowering plants was also recorded as an estimate of the relative population size (*i.e.* census data; see Table 1). As a more appropriate measure of effective population size, we also calculated the harmonic mean of consecutive yearly census data per population (up to sampling year 2009). This estimate of 'effective population size' (cf. Frankham *et al.* 2010) was also used for correlating genetic diversity to population size.

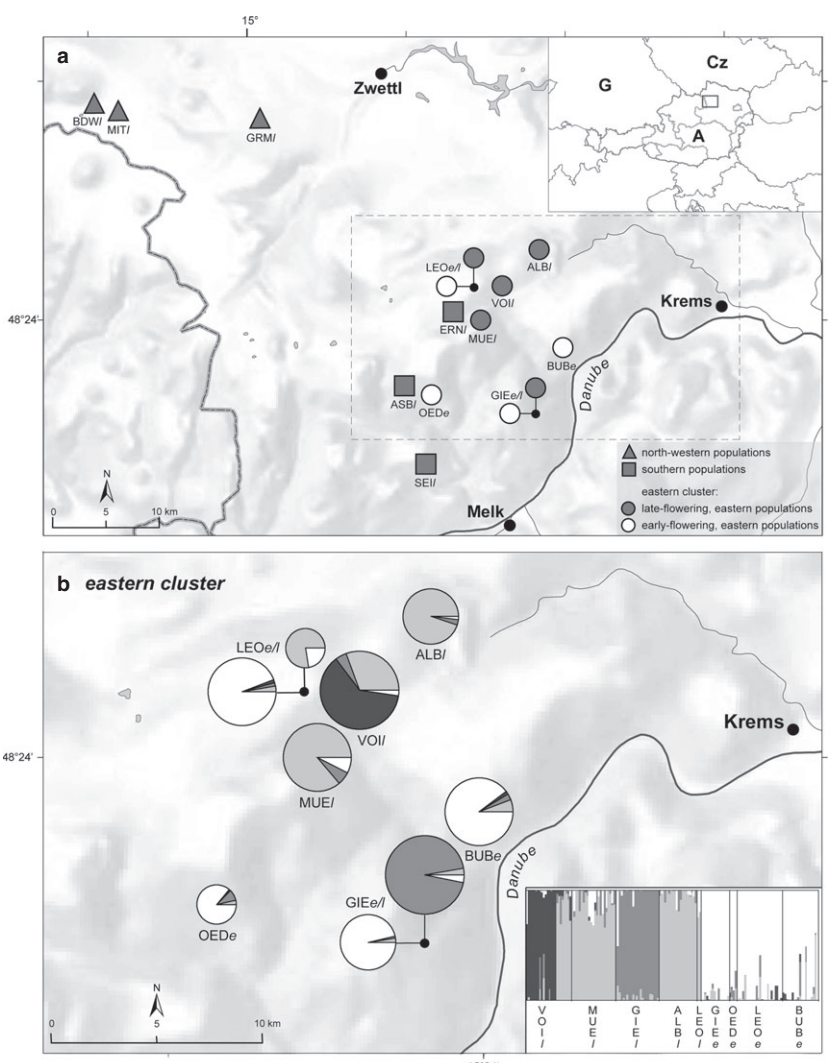

Fig. 1. Location and genetic structuring of *G. bohemica* populations in the Lower Austrian Waldviertel region (both maps based on: www.geodous.com). Details of sampled populations are given in Table 1. a: All early- and late-flowering populations; symbols and colours show the three geographic groups (BAPS, spatial clustering of all individuals) and the two flowering morphs, respectively. b: Populations of the eastern cluster; pie charts represent the proportions of admixture based on $K = 4$ from the Bayesian mixture analysis; the sizes of the circles refer to the population size counts in 2009 (see Table 1).

Table 1. Characteristics of all investigated populations of *Gentianella bohemica* including population counts for 2009 (AFLP data) and 2010 (reproduction data), an estimate of effective population size, sample sizes as well as different genetic diversity measures: percentage of polymorphic loci (PL %), Nei's gene diversity (H_E) and Shannon's information index (I); standard deviation (SD).

species/morph	site	code[a]	Altitude (m a.s.l.)	census 2009	census 2010[b]	effective population size[c]	original/final AFLP sample size (2009)	PL	PL %	H_E (SD)	I (SD)
early-flowering morph							**72/62**				
	Buchberg	BUBe	673	130	6[b]	25	24/19	177	32.60	0.0925 (0.1595)	0.1438 (0.2334)
	Gießhübl	**GIEe**	734	26	85[b]	(26)°	20/15	114	20.99	0.0618 (0.1366)	0.0959 (0.2023)
	Leopolds	**LEOe**	798	132	26[b]	43	24/24	151	27.81	0.0756 (0.1455)	0.1187 (0.2154)
	Oed	OEDe	694	4	10[b]	2	4/4	76	14.00	0.0565 (0.1416)	0.0829 (0.2068)
late-flowering morph							**238/228**				
	Albrechtsberg	ALB/	704	20	0[d]	30	20/20	169	31.12	0.0761 (0.1409)	0.1220 (0.2097)
	Aschelberg	ASB/	850	910	143[b]	(910)°	24/21	291	53.59	0.1477 (0.1793)	0.2303 (0.2576)
	Bruderndorfer Wald	BDW/	880	76	7	12	24/24	220	40.52	0.1048 (0.1590)	0.1661 (0.2343)
	Ernst	ERN/	810	35	0[d]	44	24/21	292	53.78	0.1492 (0.1778)	0.2331 (0.2564)
	Gießhübl	**GIE/**	734	201	33[b]	20	24/23	165	30.39	0.0728 (0.1381)	0.1170 (0.2058)
	Groß-Meinharts	GRM/	710	650	90	100	24/24	233	42.91	0.1098 (0.1604)	0.1745 (0.2358)
	Leopolds	**LEO/**	798	2	0	1	2/2	22	4.05	0.0203 (0.0988)	0.0281 (0.1369)
	Mitterschlag	MIT/	860	41	12	12	24/23	305	56.17	0.1500 (0.1749)	0.2360 (0.2525)
	Münichreith	MUE/	825	125	14[b]	65	24/23	188	34.62	0.0898 (0.1522)	0.1423 (0.2248)
	Seiterndorf	SEI/	520	273	33[b]	30	24/23	277	51.01	0.1346 (0.1683)	0.2130 (0.2461)
	Voitsau	VOI/	764	175	33[b]	79	24/24	214	39.41	0.1063 (0.1655)	0.1661 (0.2411)

[a]The two populations where the two flowering morphs (co-)occur on site are marked in bold.
[b]Populations included in reproductive observations and tests of mating system in 2010.
[c]Estimates of effective population sizes were calculated as harmonic mean based on two to eight census years; °only one census year available.
[d]Habitat mown during flowering time.

Amplified fragment length polymorphism (AFLP) analysis

Total genomic DNA was isolated from silica-dried leaf material (ca. 20 mg) using sterilised glass pellets for grinding and the DNeasy Plant Mini Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with minor modifications: an additional centrifugation step to completely remove ethanol was performed, and for the subsequent elution step, 100 µl (instead of 200 µl AE-Buffer) were used to obtain high DNA concentrations. The DNA extracts were then stored at −80 °C at our Institute of Integrative Nature Conservation Research. AFLP profiles were generated for 20–24 individuals per population (for exceptions see Table 1) with additional eight replicated individuals per plate to calculate an error rate (Bonin *et al.* 2004; Meudt & Clarke 2006).

The AFLP analysis followed the original procedure of Vos *et al.* (1995) with some modifications described in Kropf *et al.* (2006) and Kropf (2012). The simultaneous restriction (using the enzymes *Eco*RI and *Mse*I) and ligation (using *Eco*RI- [5′-CTCGTAGACTGCGTACC-3′/5′-AATTGGTACGCAGTC-3′] and *Mse*I-adaptors [5′-GACGATGAGTCCTGAG-3′/5′TACTCAGGACTCAT-3′]) of DNA samples was carried out overnight for 15 h at 23 °C. For the subsequent pre-selective amplification, *Eco*RI and *Mse*I primers with one selective nucleotide (E + 1: 5′-GACTGCGTACCAATTCA-3′, M + 1: 5′-GATGAGTCCTGAGTAAC-3′) were used. Three *Eco*RI/*Mse*I primer combinations (E+ACG/M+CGG [E37/M57], E+AGA/M+CTG [E39/M61], E+ATG/M+CGG [E45/M57]), each with two additional selective nucleotides, were applied for the subsequent selective amplifications; the *Eco*RI primers were labelled with different fluorescent dyes: NED™ (E37), 6-FAM™ (E39) and HEX™ (E45).

A mixture of fluorescence-labelled selective PCR products, together with a diluted internal size standard (ROX™, ET550-R), was run on a MegaBACE DNA Analysis System with 48 capillaries (Amersham Biosciences, Freiburg, Germany). Raw data from the MegaBACE DNA Analysis System were aligned with the internal size standard using the MegaBACE Fragment Profiler 1.2 (Amersham Biosciences) for presence or absence scoring of AFLP fragments in each sample using a peak height threshold of 50 relative fluorescence units (RFUs) within a readable range of 60–550 bp fragment length. The resulting automated presence/absence matrix was then thoroughly checked for misinterpretations (cf. Meudt & Clarke 2006). Further, an error rate was calculated as a relative proportion of mismatches (0 *versus* 1) compared to matches (1 *versus* 1) in AFLP profiles of eight replicated individuals (Bonin *et al.* 2004; Pompanon *et al.* 2005). Reproducibility of AFLP fragments was tested using eight to ten runs with each of these individuals and each primer combination. This method proved that AFLP fragments of *G. bohemica* were reproducible, with an error rate of 0.015% per fragment (cf. Knowles & Richards 2005; Kropf *et al.* 2006).

Analyses of genetic structure and diversity

Genetic structure analysis of the two flowering morphs was initially performed by calculating pair-wise genetic distances among individual AFLP phenotypes. These genetic distances were based on the complementary value of Nei & Li's (1979) similarity coefficient, resulting in an unrooted neighbour-joining phenogram (NJ; Saitou & Nei 1987) constructed with PAUP* (version 4.0; Swofford 2002). Based on the Nei & Li (1979) distance matrix obtained, also a Neighbour-Net phenogram was calculated using standard settings of Splits Tree4 (version 4.11.3; Huson & Bryant 2006) to illustrate patterns of reticulate evolution.

Moreover, we hypothesised genetic structure for the whole AFLP dataset; using BAPS (Bayesian Analysis of Population Structure, version 4.14; Corander *et al.* 2003, 2004) and determined the most probable number of clusters (K) within the dataset by means of stochastic optimisation. The analysis was carried out using $K = 2$–16 clusters (*i.e.* one more than the 15 populations studied), with seven replicates for each K. Calculations were performed with and without geographic coordinates of the populations as an informative prior. Moreover, Bayesian admixture analyses (Corander & Marttinen 2006) were performed with 100 iterations to estimate the admixture coefficients for each individual, and 20 iterations for the estimation of admixture coefficients for each of 100 reference individuals (Corander *et al.* 2004).

To demonstrate genetic differentiation of individuals from the early- and late-flowering morph growing at the same location, we also performed a principal coordinates analysis (PCoA) based on squared Euclidean distances, as implemented in NTSYS-pc (Rohlf 2000). This analysis was conducted with all AFLP phenotypes of the population GIE, the population with the highest number of individuals of both flowering morphs available (Table 1). A minimum length spanning tree (MST) computed from the distance matrix was superimposed onto the two-dimensional PCoA plot to elucidate similarities among individuals when all dimensions were taken into account.

Hierarchical analyses of molecular variance (AMOVA; Excoffier *et al.* 1992) were performed to estimate genetic differentiation among and within the 15 populations using ARLEQUIN (version 2.000; Schneider *et al.* 2000). Different subsets (*i.e.* representing the results of Bayesian clustering or the two flowering morphs of *G. bohemica*) were calculated with significance tests on the basis of 10,100 permutations. In addition, based on the AMOVA-derived matrix of pair-wise F_{ST}-values, a population-based NJ analysis was performed using PHYLIP (version 3.65; Felsenstein 2005). We also used those pair-wise F_{ST}-values to test for isolation by distance (Wright 1943). The significance was evaluated by comparing the observed normalised Mantel statistic Z (Mantel 1967) with its random distribution obtained after 9999 permutations using NTSYS-pc (Rohlf 2000).

Analyses of genetic diversity estimators were performed for each population separately. The following diversity values were calculated: (i) Nei's (1973) gene diversity (H_E); (ii) Shannon's information index (I, Shannon & Weaver 1949) and (iii) number and percentage of polymorphic loci (PL) using POPGENE (version 1.32, Yeh *et al.* 1997).

Phenological analyses and pollinator observation

In the growing season 2010 the duration of *G. bohemica* flowering was documented in the four known early- plus five selected late-flowering populations (see Table 1). Dates of the first day of flowering and the last day of intact inflorescences of these nine populations were recorded based on controls on a daily basis. Moreover, 15 flowers of the early- (in LEO*e* and GIE*e*) and 52 flowers of the late-flowering (in GIE*l*, SEI*l*, VOI*l* and MUE*l*) morph were marked to survey flowering times. Whenever possible, the third flower to bloom was chosen. After marking individual flowers, the floral phase was controlled every day. Sampling size varied between populations because of damage caused by herbivores (*e.g.* roe deer, slugs and/or insects), therefore, results were merged for each morph. Phenological analyses partly include the observation of other phenomena within single flowers: i.e. dichogamy and herkogamy. Especially in the early-flowering population GIE, a conspicuous proportion of non-herkogamous flowers (i.e. showing the stigma not clearly located above the anthers) was observed and therefore quantified.

Pollinator observations took place preferably on sunny and warm days during anthesis. Referring to Pontin *et al.* (2006), a patch of about 1 m², hosting as many *G. bohemica* individuals as possible, was observed for 15 min every full hour during the day to coincide with diurnal variation of insect activity. Each insect that entered the funnel-shaped flower was categorised as a potential pollinator. Pollinator and visitor observations for the early-flowering morph took place on 24 June between 06:00 and 20:15 h in LEO*e* and on 25 June between 12:00 and 17:15 h in BUB*e*. On 18, 21 and 23 September pollinator visits were studied for the late-flowering morph in ASB*l* between 08:30 and 18:00 h; two different non-adjacent patches in ASB*l* were observed half an hour later. The duration of observation was defined by daily flower opening and closing time, as there was no insect activity detected before or after.

Mating systems

The mating system of both morphs was tested for agamospermy (*Agam*), spontaneous (*SpSlf*) and manual self-pollination (*MSlf*), cross-pollination within the same (*Cr*) and among populations (*CrPop;* in case of an individual population, *Pop* is replaced by the respective population code, *e.g. CrSEI*), in comparison to open-pollination (OP) as control group (cf. Kearns & Inouye 1993; for manipulation details see Table 2). Only intact buds were randomly chosen for treatment. Fine forceps, which were sterilized between each flower treatment in a flame, were used for emasculation. Pollination was performed with clipped off anthers using medium-sized to high pollen loads (cf. Ornelas & Lara 2009). A finely woven small bag was put around the manipulated flowers to prevent any insect activity.

Within the treatment *MSlf,* pollen of the very same flower was used; for *Cr*, pollen from individuals ca. 3 m distant was used. Within the early-flowering morph, pollen for *CrPop* was randomly chosen, whereas for the late-flowering morph, plants at ASB*l* (receptor) were cross-pollinated with two donor populations, *i.e.* SEI*l* and MUE*l*, which are differently related (see AFLP results below). All tests of the mating system were performed at the four early-flowering populations and at the

Table 2. Manipulations for tests of the mating system within the two flowering morphs.

treatment	code	emasculation	flower manipulation	bagging
agamospermy	*Agam*	Yes	No	Yes
spontaneous self-pollination	*SpSlf*	No	No	Yes
manual self-pollination	*MSlf*	Yes	Self-pollinated	Yes
manual cross-pollination, within population	*Cr*	Yes	Cross-pollinated	Yes
manual cross-pollination, between populations	*CrPop*	Yes	Cross-pollinated	Yes
open-pollination	*OP*	No	No	No

late-flowering population ASB/due to the limited number of flowers, with up to three manipulations per individual.

In June/July capsules matured about 1–2 weeks after manipulation, while maturation of capsules lasted up to 1 month in the late-flowering morph, due to moister and colder weather conditions (early-flowering morph n = 85, late-flowering morph n = 149). For the final tests of differential success of differing manipulations, 60 capsules of the early-flowering morph and 95 capsules of the late-flowering morph were collected and dried at room temperature (*i.e.* 10–15 capsules per specific treatment). The capsules were weighed and seeds per capsule counted. As all treatments had capsules (see Results below), seeds were categorised depending on their vitality to obtain quantitative data on relative mating success of different treatments. Therefore, all seeds within a given capsule were counted and categorised as normally developed, wrinkled or aborted; assuming that the proportion of normal well-developed (*i.e.* showing no reduction in volume, uniform shape) seeds will perform best. In this sense, we finally calculated an autofertility (AF) index based on the proportion of normally developed seeds by dividing the value obtained from spontaneous selfing by the value following manual selfing. The AF index will range between 0 and 1; with index values close to 0 indicating no capacity for autonomous selfing and values reaching 1 meaning strong selfing capacity. Data obtained from the mating system experiments were tested for normality, and variance analysis and Kruskal-Wallis tests were performed for comparison of means using the statistic programme SPSS (version 19; SPSS, Chicago, IL, USA).

RESULTS

Amplified fragment length polymorphisms

For AFLP analyses we originally investigated 310 individuals from 15 populations representing two seasonal morphs of *G. bohemica*. Finally, analyses were based on 290 individuals (*i.e.* 62 early and 228 late flowering) since we failed to generate reliable AFLP patterns for 20 individuals. Using three primer combinations (M61/E39; M57/E37; M57/E45), 200, 178 and 177 AFLP fragments (mean: 185 ± 13 fragments, ±SD) were observed, respectively, resulting in a total of 555 AFLP fragments. Twelve monomorphic fragments (*i.e.* 2.2%) were excluded from further statistical analyses, which were therefore based on 543 polymorphic AFLP fragments.

Population relationship and population structure

Generally, the individual-based neighbour-net analysis (Fig. S1) and the population-based NJ analysis (Fig. 2) showed that the *G. bohemica* populations were mostly well-defined groups reflecting flowering time and, within flowering morphs, geographic origin. In both analyses the four early-flowering populations formed one independent group, whereas populations of the late-flowering morph basically showed clustering into three geographic groups. Subsequently, we therefore refer to these groups as: early-flowering eastern populations, late-flowering eastern populations, southern populations and northwestern populations (Fig. 1a). Nevertheless, in the population-based phenogram (Fig. 2) the late-flowering northwestern and southern populations were not clearly separated, but form a grade. Furthermore, the late-flowering population VOI*l* showed some heterogeneity in the individual-based analysis, as indicated by variable allocation to the northwestern and eastern populations (cf. Fig. S1). Performing a Mantel test on the matrices of pairwise F_{ST}-values and respective geographic distances (km) between populations revealed no isolation-by-distance pattern (Mantel $r = 0.074$; $P = 0.329$).

The spatial clustering of individuals (BAPS), which includes geographic coordinates of populations as an informative prior, revealed an optimal number of K = 3 groups (Figs 1a and 2): (i) GRM*l*, BDW*l* and MIT*l* (*i.e.* northwestern populations); (ii) ASB*l*, SEI*l* and ERN*l* (*i.e.* southern populations); and (iii) all other populations (*i.e.* the 'eastern cluster' including early- and late-flowering populations). Without this additional geographic information, a higher optimal number of $K = 5$ groups was found within the whole dataset (*i.e.* clustering of 290 individuals), where ERN*l* and all early-flowering populations in the eastern cluster each formed an additional separated group (not shown).

Furthermore, a detailed investigation of the eastern cluster (comprising 154 individuals and 378 polymorphic AFLP fragments) revealed K = 4 as optimal number of groups (*i.e.* clustering of 154 individuals): (i) all early-flowering populations; (ii) MUE*l*, LEO*l* and ALB*l*; (iii) GIE*l*; and (iv) VOI*l*. Based on these results, an admixture analysis clarified the proportions of admixture (Fig. 1b). Unsurprisingly, VOI*l* showed notable admixture (30.6%) with the three spatially neighbouring populations MUE*l*, LEO*l* and ALB*l*, but also to a lesser extent with the late-flowering GIE*l* population (4.5%). Within the late-flowering LEO*l* population we found a notable proportion of admixture (22.5%) with the early-flowering group. All other populations of the eastern cluster showed relatively low proportions of admixture compared to those in populations VOI*l* and LEO*l*. This pattern is much the same when testing admixture in the whole dataset (not shown). The PCoA of early- and late-flowering individuals at location GIE (Table 1) clearly indicated two groups separated along the first axis representing, without exception, the two morphs. Furthermore, a superimposed MST tree connected the two flowering groups only through a single branch (Fig. 3).

The AMOVA resulted in an appreciable differentiation of 31% among all populations. Within population variation reached

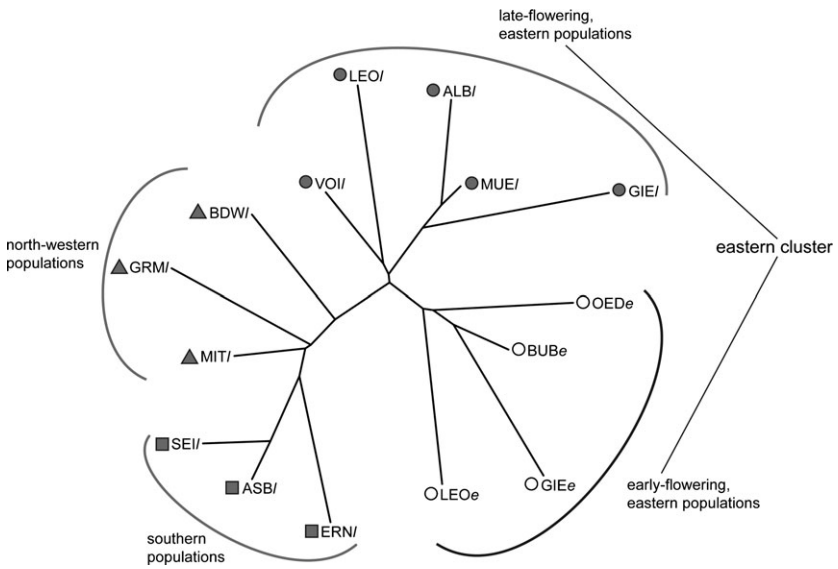

Fig. 2. Unrooted NJ tree based on pair-wise genetic differentiation (*i.e.* F_{ST}-values) between populations (for abbreviations see Table 1).

more than two-thirds (Table 3). A hierarchical AMOVA based on Bayesian clustering of individuals (K = 5, n = 290, see above) led to differentiation of 17.4% among groups. Analysing the data with respect to differentiation among early- and late-flowering populations resulted in an obvious lower differentiation of 6.2%. AMOVA calculations for the eastern cluster also revealed significant differentiation (26.8%) among populations. Beyond that, hierarchical AMOVA based on Bayesian clustering of individuals (K = 4, n = 154, see above) of the eastern cluster showed relatively strong differentiation between the early- and the late-flowering populations (9.6%) compared to the differentiation found between the four AFLP-defined geographic groups (12.2%).

In addition, differentiation between populations was calculated using pair-wise population F_{ST}-values (Table S1). Within the early-flowering morph relative low F_{ST}-values (0.15–0.19) were found between population BUB*e* and all remaining populations. However, differentiation between the early- and late-flowering populations was comparatively high (0.22–0.43). Altogether the highest differentiation was found between the late-flowering populations GIE*l* and SEI*l* (0.45), while the lowest pair-wise F_{ST}-value was observed between the late-flow-

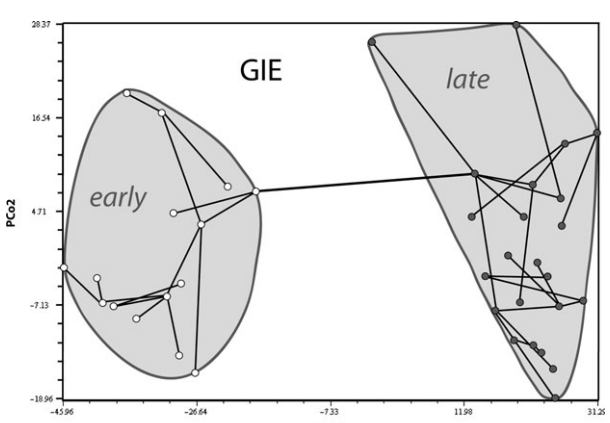

Fig. 3. Principal coordinates analysis (PCoA) of *G. bohemica* individuals of the GIE population covering the early- (n = 15) and late-flowering (n = 23) morphs at the same location.

Table 3. Analyses of molecular variance (AMOVA) for the whole dataset and separately calculated for the eastern cluster. Hierarchical AMOVAS were calculated for the groups found in the Bayesian clustering analysis and between the early- and late-flowering morphs.

source of variation	df	sum of squares	variance components	% total variance
analyses of the whole dataset				
among all populations	14	4014.629	13.40626	30.97***
within populations	275	8219.026	29.88737	69.03
five groups (*i.e.* one early- and four late-flowering groups)				
among groups	4	2437.237	7.7613	17.38***
among populations within groups	10	1577.392	6.99804	15.67***
within populations	275	8219.026	29.88737	66.94***
two groups (early-flowering *versus* late-flowering)				
among groups	1	548.089	2.80638	6.23*
among populations within groups	13	3466.540	12.38633	27.48***
within populations	275	8219.026	29.88737	66.30***
analyses of the eastern cluster				
among populations	8	1320.460	8.51943	26.78***
within populations	145	3378.138	23.2975	73.22
four subgroups (*i.e.* one early- and three late-flowering groups) within the eastern cluster				
among groups	3	848.193	3.95544	12.15***
among populations within groups	5	472.267	5.28909	16.25***
within populations	145	3378.138	23.2975	71.59***
two subgroups (early-flowering *versus* late-flowering) within the eastern cluster				
among groups	1	396.152	3.18756	9.59**
among populations within groups	7	924.308	6.74559	20.30***
within populations	145	3378.138	23.2975	70.11***

Significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ering geographically close populations SEI*l* and ASB*l* (0.09; Fig. 2; see also cross-pollination experiments below).

Genetic diversity within populations

Basically, calculations of three different diversity parameters revealed similar results (Table 1). Compared to other studies on short-lived perennial plants with restricted distribution (*i.e.* regional endemics), we found comparatively low absolute diversity values (cf. Nybom 2004) with moderate differences among populations from the various regions. If we compare populations with similar sample size (n = 19–24; *i.e.* disregarding the individual-poor populations LEO*l*, GIE*e* and OED*e*), the late-flowering population GIE*l* had the lowest Nei's gene diversity ($H_E = 0.073$) and Shannon's information index ($I = 0.117$; Table 1). The highest diversity was in the northwestern population MIT*l* ($H_E = 0.15$), although its number of individuals was rather low (*i.e.* 41 in 2009). The values of GRM*l* and BDW*l*, which form together with MIT*l* the northwestern group, were, in general, slightly lower (Table 1).

In the early-flowering morph genetic diversity within populations was slightly lower ($H_E = 0.06$–0.09; Table 1). To examine whether there is a relationship between population size and genetic diversity, a correlation analysis was carried out. We used census data from the sampling year 2009 as well as an estimate of the effective population size (Table 1) for this analysis to deal with fluctuating population sizes of *G. bohemica*. Both analyses showed no significant correlation between the three diversity parameters (*i.e.* PLP, H_E and I) and population size.

Phenology and mating system

Flowering time and duration

In the individual-rich, early-flowering population GIE*e* (85 individuals) flowering time was longest at ca. 3 weeks (*i.e.* starting around 18 June and finishing on 7 July). However, in all other early-flowering populations anthesis started around 20 June and was completed by the first week in July (Fig. 4). The late-flowering population ASB*l* (143 individuals) flowered for about 7 weeks (starting around 3 September and finishing on 22 October), while the population with the shortest flowering period was VOI*l* (27 individuals), flowering for only about 3 weeks. Generally, anthesis of the late-flowering morph started early in September and was completed in the second half of October. Considering a flowering gap of about 8 weeks (*i.e.* first week of July to first week of September; Fig. 4), the two morphs of *G. bohemica* are reproductively isolated by flowering time.

On average, a single flower of the early-flowering morph was open for about 2.8 ± 0.8 days (n = 10). The mean duration of flowering of a single flower in the late-flowering morph was about 5.1 ± 2.2 days (n = 44). However, Mann–Whitney *U*-tests showed no significant difference, probably due to small sample size of the early-flowering morph.

Most daily assessed flowers were herkogamous as expected (*i.e.* stigma located conspicuously above anthers); however, we infrequently also observed reduced herkogamy in both morphs, especially at GIE*e* where 2.5% of all flowers in the population showed no herkogamy. Moreover, in both morphs we observed some rare cases of dichogamy, where the stigma protruded from the still closed, twisted petals, while the anthers were not dehisced.

Potential pollinators

In the early-flowering morph no potential pollinators were observed. During observations of the late-flowering morph, 115 single approaches of insects entering the funnel-shaped flowers were noted: 51% were by bumblebees, 35% the honeybee *Apis mellifera*, 10% the hoverfly *Myathropa florea* and 4% the day-flying moth *Autographa gamma*. We observed at least four different potentially pollinating bumblebee species: *Bombus pascuorum* (27% of all approaches), *Bombus terrestris/lucorum* (12%; species not differentiated in the field), *Bombus hortorum* (9%) and *Bombus lapidarius* (3%).

Mating system

Apart from capsule-loss in the field (*e.g.* through herbivores), all manipulations led to the development of mature capsules. On average, the early-flowering morph's capsules weighed 9.6 mg, maximum of 22.5 mg and minimum of 1.2 mg (n = 60). The highest mean weight was achieved through open pollination (*OP*); high capsule weights were also observed for all other treatments except for agamospermy (*Agam*: mean: 3.71 mg; Fig. S2a). The capsule weight of the late-flowering morph showed a similar mean of 9.55 mg but with higher variation: maximum 27.5 mg and minimum of 0.8 mg (n = 95; Fig S2b). Like the early-flowering morph, the highest mean capsule weight was reached through *OP* at 14.53 mg, and all mean capsule weights of the other treatments differed significantly from the *Agam* treatment (3.67 mg). Interestingly, comparison of the mean weights between *CrMUE* and *CrSEI* was close to significantly different, a statistical result obviously weakened by the smaller sample size of only ten replicates each (Fig. S2b).

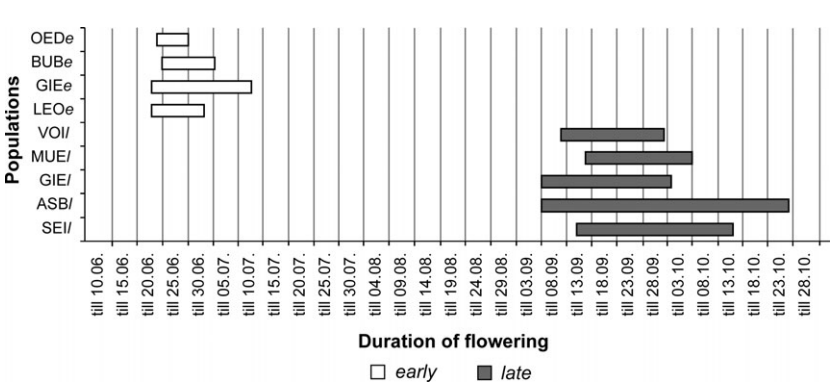

Fig. 4. Flowering time of the four early- and five late-flowering populations observed in 2010 (for abbreviations see Table 1).

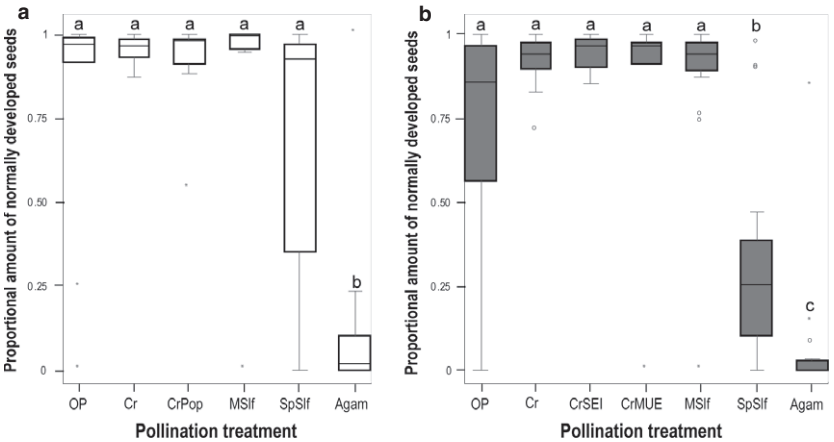

Fig. 5. Proportions of normally developed seeds obtained after different pollination treatments. The six tests are: open-pollination (*OP*), cross-pollination within the same population (*Cr*), cross-pollination among populations (*CrPop*, *CrSEI*, *CrMUE*), manual self-pollination (*MSlf*), spontaneous self-pollination (*SpSlf*) and agamospermy (*Agam*). Differing small letters indicate significant differences among treatments. a: Early-flowering morph: statistical comparison of means indicated a highly significant difference of the *Agam* test to all other treatments ($P < 0.0001$); b: late-flowering morph: statistical comparison of means showed a significant difference of each for both treatments (*SpSlf* and *Agam*) to all other treatments ($P \leq 0.021$ and $P < 0.001$).

Both morphs showed strong variation in the number of seeds per capsule. The 60 capsules collected from the early-flowering morph had a mean of 50 ± 18 seeds per capsule, whereas all 95 capsules collected of the late-flowering morph had a mean of 73 ± 26 seeds per capsule. The highest number of seeds in all three categories counted was 183, whereas the lowest number was only 11. However, to further assess the success of each mating strategy, the percentage of normally developed seeds was estimated and statistically compared across treatments (Fig. 5). In both morphs, cross-pollination gave the highest percentage of normally developed seeds per capsule (early-flowering: average 95% in *Cr* and 92% in *CrPop;* late-flowering: 94% in *Cr* and 92% in *CrSEI*). Furthermore, in both morphs the lowest proportions of normally developed seeds were at the *SpSlf* (early: 67%; late: 33%) and the *Agam* treatment (14% and 8%; Fig. 5, S3). However, we cannot completely exclude a possible leak in the pollinator exclusion system that might have enabled minor seed development, as in the *Agam* treatment.

While we did not find significant differences in mean capsule weights nor for most of the treatments in the proportion of normally developed seeds between the two flowering morphs, the percentages of normally developed seeds were significantly different between the two morphs in the two selfing treatments (*MSlf*, *SpSlf*; both $P = 0.036$; Fig. S3). In particular, spontaneous selfing (*SpSlf*) worked better in the early-flowering morph (67% normally developed seeds), whereas it reached only 33% in the late-flowering morph; this is also reflected in an AF index of AF = 0.76 for the early-flowering morph compared to AF = 0.39 for the late-flowering morph.

DISCUSSION

Genetic and phenological coherence of early-flowering *G. bohemica*

All AFLP analyses resulted in the genetic independency of the early-flowering morph. We found clear differentiation between the two morphs within the eastern cluster, the only regional group in which the two flowering morphs co-occur. Studying the entire dataset, however, this differentiation is obscured by a distinct geographic pattern. For the early-flowering morph we therefore assume that the principle of reciprocal monophyly applies (Avise 2000; see also Kropf *et al.* 2006, 2008). Highlighting the individual-rich GIE location, genetic differentiation between the two flowering morphs did not indicate gene flow among flowering morphs on site. Moreover, phenological observations verified a time lag of about 8 weeks between the flowering times, suggesting an effective pre-zygotic barrier between the two morphs. Furthermore, gene flow *via* time-delayed germination from the soil seed bank can be excluded, since we have evidence for the late-flowering morph being late-flowering again under standard conditions in common garden experiments (K. Plenk, F. Göd, M. Kriechbaum & M. Kropf, unpublished data), as observed by Lennartson (1997) in *Gentianella amarella*. These results confirm reproductive isolation between the two flowering morphs (*i.e.* the seasonal dimorphism) of *G. bohemica,* as originally described by Wettstein (1895).

Despite considerable geographic distances, all currently known early-flowering populations clustered together in one genetic group within the eastern cluster. This homogeneous genetic constitution, observed within the early-flowering morph, strongly differs from the geographic structure discovered within the late-flowering morph. Regarding the two locations where the two morphs co-occur, the late-flowering GIE*l* and LEO*l* were placed in different genetic groups, whereas the early-flowering GIE*e* and LEO*e* were both within the single early-flowering group. In these two specific cases, flowering times were also observed as clearly not overlapping. The genetic coherence of the four early-flowering populations might be seen as an indicator of successful gene flow among those populations. However, given basically similar pollen and seed dispersal mechanisms, the strong geographic structure found within the late-flowering morph in the same area contradicts such an assumption. In addition, the pollination system (entomophily) as well as the low potential for seed dispersal and the disjunct distribution of *G. bohemica* generally does not indicate frequent long-distance-dispersal of pollen and/or seeds (cf. Königer *et al.* 2012). The proceeding spatial isolation, through abandonment of traditional grassland management or intensification of land use, further aggravates inter-population pollination, since plant–pollinator mutualisms might be disturbed and pollinators often are not able to overcome large geographic distances and/or specific barriers, such as forests (cf. Rathcke & Jules 1993; Fischer & Matthies 1998; Zurbuchen & Müller 2012). Nevertheless, (single origin and) recent spread might represent an alternative explanation for the genetic similarity

of early-flowering populations. Such a spread could have occurred along historical transport routes, *e.g.* between Bohemia (today Czech Republic) and Austria, where several trails for salt trade existed (Hajná 2011; see also Königer *et al.* 2012). For our study region, at least the population at ASB is adjacent to a historically important post station (personal communication with tenant). Königer *et al.* (2012) also discussed the harvest and transport of hay together with cattle drives as possible dispersal vectors in former times. They found low genetic differentiation between Austrian and Czech populations, which they mainly explain by earlier periods of connectivity between these trading regions. Considering that Neilreich (1859), Beck von Managetta (1893) and Wettstein (1896) noticed *Gentianella* as frequently occurring around GIE, BUB and OED in historical times, this region might be the origin of such spread. However, the highly patchy distribution of the current early-flowering populations might lead to increasing differentiation among populations due to genetic drift (or local adaption) in the future (cf. Lienert 2004; see also Zopfi 1993b; Lennartson 1997), as already evident for the late-flowering populations. Beside random genetic drift, the probably most important selection pressure will be traditional grassland management, *i.e.* notably the timing and frequency of mowing and/or grazing. In this context, the genetically heterogeneous population VOI*l* is conspicuous due to its comparatively high amount of admixture and varying allocation to two different groups (Fig. 1b, S1). As this admixture is mainly with the neighbouring populations MUE, LEO and ALB, there might be possible gene flow *via* seeds mediated by grassland management, *e.g.* haying or grazing using the same machines or livestock. However, intentional dispersal of seeds from other populations cannot be totally excluded.

Reproductive traits and genetic diversity of *G. bohemica* flowering morphs

Both morphs display a mixed mating system with pollination by insects such as bumblebees, honeybees, hoverflies and moths, but are also spontaneously capable of self-pollination. Although there are no obvious morphological and mating differences between the two flowering morphs, there is indirect evidence for a difference in their reproductive performance. As we could not observe any pollinator of the early-flowering morph, we assume scarce pollinator visitations and/or higher annual fluctuations in pollinator abundances; thus, these populations face strong pollinator limitation (Bierzychudek 1981). Low visitation rates are often a result of population decline, small population sizes and/or fragmentation and isolation of populations due to disturbed mutualism between pollinators and plants (*e.g.* Luijten *et al.* 1999; Mustarjärvi *et al.* 2001; Becker *et al.* 2011). Given only four early-flowering populations today, such a scenario seems very likely.

Low genetic diversities as a consequence of these reduced (effective) population sizes can also lead to morphological differences, *e.g.* during formation of flowers. Reduced herkogamy was observed in some flowers of both morphs, making spontaneous self-pollination very likely. Even though dichogamy is mostly seen as prevention of self-pollination (*i.e.* favouring outcrossing), the indication of protogyny, as observed here, does not seem to be an effective barrier to self-pollination (cf. Luijten *et al.* 1999). It could actually facilitate self-pollination by favouring geitonogamy with respect to the plants' usually multi-flowered appearance (cf. Gargano *et al.* 2009). The duration of a single flower of the different morphs varied slightly. Even though it was not proved statistically, the flower duration of the late-flowering morph was approximately 2.3 days longer compared to the early-flowering morph. The benefit of long flower duration lies in a higher likelihood of pollinator visitation and therefore successful reproduction through outcrossing, but also requires a higher amount of resources, which may have negative effects on seed production (*e.g.* Ashman & Schoen 1997; Giblin 2005; Duan *et al.* 2007). Faced with very few pollinator visits, the short flower duration might also indicate decreased overall fitness of the early-flowering morph, currently known from only four individual-poor locations, which are already prone to strong genetic drift (cf. Kalisz & Vogler 2003).

In this overall context of pollinator limitation, the mixed mating system of the two *G. bohemica* morphs can basically be interpreted as a strategy for reproductive assurance. The early- and the late-flowering morph both show high levels of seed set and a high proportion of normally developed seeds per capsule within cross-pollination treatments, but spontaneous selfing works better in the early-flowering morph (Fig. S3; $AF_{early} = 0.76$ compared to $AF_{late} = 0.39$). This may lead to genetic erosion and inbreeding depression (Aguilar *et al.* 2008; Levin 2012), especially with respect to small population sizes where mating with close relatives is very likely (Barrett & Kohn 1991; Ellstrand & Elam 1993; Dudash & Fenster 2000). Moreover, higher selfing capacity of the early-flowering morph further supports the hypothesis of effective reproductive isolation among morphs (see above; cf. Martin & Willis 2007; Brys *et al.* 2014).

The high capsule weights and proportions of normally developed seeds for any of the two cross-pollination results further indicate that both morphs are predominately outcrossing and that habitat fragmentation and isolation may have led to a shift in their mating system due to strong pollinator limitation (cf. Fischer & Matthies 1997; Luijten *et al.* 1999; Kalisz & Vogler 2003). Hence, this process is having an even stronger effect on the early-flowering morph of *G. bohemica*, possibly escalating its threatened status.

Apart from inbreeding depression, an additional possible genetic threat for formerly common and now fragmented species is the phenomenon of outbreeding depression, for which we found the first, but weak, evidence in our study system. If populations become locally adapted or have adapted to a specific environment, gene transfer among strongly isolated populations may result in reduced fitness of offspring (Fischer & Matthies 1997; Dudash & Fenster 2000; Frankham *et al.* 2010). In our investigation we cross-pollinated ASB/with the genetically differently related populations SEI/and MUE/, as demonstrated in our AFLP analysis (Fig. 2), which resulted in differences in capsule weights. The cross-pollination between ASB/and SEI/, the genetically more similar populations, yielded slightly higher capsule weights as compared to the more distantly related cross. Nevertheless, this first sign of possible outbreeding depression should be considered in future research and conservation activities. Outbreeding depression, generally, seems to be a surprisingly common phenomenon in rare grassland species (*e.g.* Fischer & Matthies 1997; *Gentianella germanica*; Becker *et al.* 2011: *Astragalus exscapus*).

Conservation aspects

In contrast to other studies on species characterised by seasonal dimorphism (*e.g.* Tali *et al.* 2006; *Neotinea* (*Orchis*); Pleines *et al.* 2013: *Rhinanthus*), we found two independent genetic entities representing the early- and the late-flowering morph of *G. bohemica*. Furthermore, these entities are reproductively isolated. Strong genetic differentiation among populations, lack of an isolation-by-distance pattern, low overall levels of genetic diversity and pronounced geographic structuring within the late-flowering morph in our AFLP dataset are signs of increasing isolation and fragmentation (*i.e.* reduced gene flow), which is a serious threat to our study species and may cause (local) extinction.

As *G. bohemica* is a rare, regional endemic conservation target species and listed as priority species in the EU Habitats Directive, there is a need for conservation measures. Therefore, fact sheets have been prepared, monitoring programmes established and management recommendations developed (Zillig *et al.* 2010; Brabec 2012; Engleder 2013). However, these basically do not consider the intraspecific polymorphism regarding its seasonal differentiation. This applies to listings in the respective Annexes at the EU level (administrative nature conservation), as well as to regional level of conservation management activities. For example, management from mid-October to the end of June in the following year is considered to be ideal (Brabec 2012), which exclusively applies to the late-flowering morph, but would be counterproductive for the survival of the early-flowering morph. From a conservation point of view, it is therefore necessary to acknowledge the seasonal morphs both as important conservation units, regardless of their systematic/taxonomic treatment. A simple solution in the case of *G. bohemica* would be a listing of the two morphs in the respective nature conservation guidelines and lists (*e.g.* Annexes of the EU Habitats Directive). In general, consistent treatment of intraspecific polymorphisms is fundamental for protecting multiple gene pools (as the two flowering morphs in *G. bohemica*) representing an important intraspecific genetic level of biodiversity, as is explicitly intended in the CBD (Convention on Biological Diversity 1992).

OUTLOOK

Future investigations will focus in more detail on the origin (spatial) and probable age of the early- and late-flowering morphs, especially in relation to (historic) grassland management and trading routes throughout the species' distribution range. Such a historic formation of seasonal dimorphism, as postulated by Wettstein (1895, 1900), would imply a rather young age of differentiation as well as a highly dynamic spread over the study region Waldviertel and a very recent dramatic decline of *G. bohemica* populations. As we found a considerable proportion of the early-flowering morph's gene pool within the late-flowering LEO*l* population, a single origin of the early-flowering morph (at this site) might be a possible scenario; especially because LEO is one of the two rare sites where both flowering morphs still occur today. Even though Wettstein's (1896) hypothesis cannot yet be finally verified, it is likely that in former times there were more locations with both flowering morphs of *G. bohemica* on site (cf. Neilreich 1859; Beck von Managetta 1893; Wettstein 1896; Janchen 1977).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Individual-based Neighbour-Net of the investigated *G. bohemica* populations, based on pair-wise genetic distances complementary to the similarity coefficient of Nei & Li (1979). For abbreviations see Table 1.

Fig. S2. Capsule weights obtained by different pollination treatments: (A) the early-flowering morph; (B) the late-flowering morph.

Fig. S3. Comparison between the two flowering morphs of the mean proportion of normally developed seeds obtained after different pollination treatments.

Table S1. Genetic distances between the 15 *Gentianella bohemica* populations based on pair-wise FST-values.

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