

# Genetic variation and plant performance in fragmented populations of globeflowers (*Trollius europaeus*) within agricultural landscapes

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**Abstract** The management of remnant populations in highly fragmented landscapes requires a thorough understanding of the processes shaping population persistence. We investigated relationships between population characteristics (i.e. size, density and pollinator abundance), offspring performance, genetic diversity and differentiation in *Trollius europaeus*, a plant with a nursery pollination system. In 19 populations of different sizes and located in north-east Switzerland, an area which has undergone widespread land use changes over the last decades, we assessed neutral genetic diversity ( $N_{\text{total}} = 383$ ) using AFLPs and plant performance in a greenhouse experiment ( $N_{\text{total}} = 584$ ) using competition and control treatments. Overall genetic differentiation was low ( $F_{\text{ST}} = 0.033$ ) with a marginal significant isolation by distance effect ( $P = 0.06$ ) indicating (historical) genetic connectivity among the populations. Mean expected heterozygosity was  $H_E$  of 0.309 (0.0257–0.393) while inbreeding coefficients ( $F_{\text{IS}}$ ) were significant in only three populations. Genetic diversity was not related to population size, plant density or pollinator abundance. Plant performance was reduced under competition ( $P < 0.001$ ) but the severity of competition was independent of genetic diversity and population size. In summary, remnant populations of *T. europaeus* retain genetic diversity and seem capable of persisting under the present conditions within an agricultural matrix. *T. europaeus* is a perennial herb, thus it may require

several generations for the negative effects of fragmentation and isolation to manifest. Our findings indicate that small populations are as important as large populations for the conservation and management of genetic resources.

**Keywords** Absolute severity of competition · AFLP · Genetic diversity and differentiation · Population size · Plant performance · Nursery pollination

## Introduction

Habitat fragmentation caused by human modification of landscapes is a principal threat to biodiversity and terrestrial ecosystems worldwide (Sala et al. 2000; Fahrig 2003; Reed 2004; Thomas et al. 2004). Today approximately half of the total land surface has been altered by human activities (Ehrlich and Wilson 1991; Saunders et al. 1991; Vitousek et al. 1997), especially in Western Europe where natural habitats have become increasingly isolated within a matrix of intensive agriculture and urbanization (Thompson and Jones 1999).

Habitat fragmentation has three main consequences for plant populations: the direct loss of suitable habitat, reductions in population size and increasing spatial isolation between remnant populations. These changes in the population distribution, structure and size often cause Allee effects (Stephens et al. 1999), limiting reproductive success (Leimu et al. 2006), plant performance and reducing population viability (Fischer and Matthies 1998; Bowman et al. 2008; Schleuning et al. 2009). The resulting small populations are also increasingly vulnerable to environmental and demographic stochasticity (Shaffer 1981; Kery et al. 2000; Hobbs and Yates 2003), rendering them prone to local extinction.

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The vulnerability of fragmented populations might be exacerbated by reductions in genetic variation associated with a reduced population size and increased spatial isolation (Ellstrand and Elam 1993; Lynch et al. 1995; Young et al. 1996; Leimu et al. 2006). In addition, genetic drift increases in small populations as well as inbreeding, reducing genetic variation within populations through a loss of heterozygosity and the fixation of alleles (Ellstrand and Elam 1993). Both processes are enhanced through reduced immigration rates in isolated populations, promoting increased genetic differentiation between populations as well as reduced genetic variation within the single population and limiting the available pool of alleles in a population. Such reduced genetic variation, commonly associated with reduced population size, can lower plant growth and reproduction (Keller and Waller 2002; Leimu et al. 2006). Genetic erosion may also limit a species' ability to adapt to new environments, as its ability to cope with changed conditions depends also on the amount of genetic variation underlying adaptive traits (Young et al. 1996; Booy et al. 2000; Podolsky 2001). Aside from habitat fragmentation, land use changes through an intensification of agriculture cause nutrient enrichment, which leads to increased productivity and hence competition for space and light among component species (Gough et al. 2000; Mittelbach et al. 2001; Schippers and Joenje 2002; Suding et al. 2005). If plants then have a reduced ability to respond to changed environmental conditions due to genetic erosion, their extinction risk might be particularly high (Lynch et al. 1995; Sultan 2000). The genetic effects of habitat fragmentation and its consequences might be mediated by a plants' pollination system. Highly specialized pollination systems might render plants especially prone to fragmentation effects as pollination can easily collapse in the event that fragmentation affects the specialised pollinators or if plant populations drop below a threshold and become unable to support sufficient numbers of pollinators (Bond 1994; Waser et al. 1996; Johnson and Steiner 2000; Ghazoul 2005).

Globeflower *Trollius europaeus* (Ranunculaceae), a plant with a specialised pollination system that has been subject to widespread habitat fragmentation, represents an interesting case study for the effects of habitat fragmentation on population genetics and performance. While still common in many European alpine regions, the species is becoming increasingly threatened in lowland regions due to its requirement for moist meadows (Muncaciu et al. 2010; Lemke 2011). In Switzerland moist habitats have undergone drastic changes during the last century, with up to 90 % of the former areas being lost due to drainage, deterioration and fertilization (Broggi and Schlegel 1989; Bowman et al. 2008). In our main study region, *T. europaeus* populations have undergone substantial reduction in

the last decades, dropping from frequent to rare occurrences (Artendatenbank Canton Zurich, <http://www.aln.zh.ch>), as many habitats are undergoing considerable changes due to urban sprawl and agricultural intensification. The remaining populations in the Canton Zurich are now mainly found on nature protection sites. In addition, *T. europaeus* has a highly specialized nursery pollination system in which *Chiastocheta* flies act almost as exclusive pollinators. *Chiastocheta* depends on *T. europaeus* as their larvae develop within the flower and consume a portion of the developing seeds (Pellmyr 1989).

Since plant population size, density and pollinator abundance are all potentially influenced by habitat fragmentation, influencing plant population persistence, we tested how these variables affect neutral genetic variation in plant populations, plant performance in a greenhouse experiment and their interaction. We chose to explore such interactions on *T. europaeus* not only because it is a species of conservation concern in many regions of Europe (including Switzerland), but also because its pollination system might render this species particularly vulnerable to land use changes. As *Chiastocheta* is expected to have short flight ranges (Després 2003; Johannesen and Loeschcke 1996), this pollination system might have resulted in highly differentiated plant populations. Such differentiation might either add negatively due to increased population isolation or alleviate ongoing fragmentation processes due to earlier purging effects (Byers and Waller 1999; Ouborg and Vantreuren 1994). Besides genetic differentiation we tested whether small populations of *T. europaeus* exhibit reduced genetic variation and plant performance and whether plant performance is positively correlated with genetic diversity. As it has been suggested that small populations might show a reduced competition ability (Fenster and Dudash 1994; Young et al. 1996), we assessed plant performance in a competition treatment as well as a control treatment to determine the severity of competition.

## Methods

### Study species

*Trollius europaeus* L. (Ranunculaceae) is a long-lived perennial, self-incompatible plant, occurring in moist habitats throughout northern and mid-Europe (Lauber and Wagner 2001). It usually has one (sometimes two or more) yellow globose and tightly closed flowers per stalk (Dorszewska 1974), and reaches first bloom after approximately 2–3 years. The flower shape excludes insects other than a few species of *Chiastocheta* flies, rendering pollination dependent on up to six *Chiastocheta* sp. (Pellmyr 1989; Jaeger and Després 1998). While adult *Chiastocheta*

flies use the flowers to forage, mate, shelter and oviposit; the larval stage feeds and develops on ovules within the ripening seed heads, forming a nursery pollination system (Pellmyr 1989, 1992; Jaeger and Després 1998; Pompanon et al. 2006; Dufay and Anstett 2003).

### Study sites

Plant material was collected from 19 *T. europaeus* populations in northeast Switzerland, on elevations ranging from 537 to 1,250 m a.s.l (Table 1). Populations were situated north-east and south-west of Lake Zurich (Fig. 1). Pollinator abundance, plant population size and density (Table 1) were determined in a parallel study during 2006 to 2008, where we investigated amongst others the effect of pollinator abundance on seed set (Klank et al. 2010). Flower number as a surrogate for population size was found to range from 140 to 820,700 flowers (median 3,930), with densities from 0.11 to 14.44 flowers m<sup>-2</sup> (median 2.11). Pollinator abundance was determined by counting *Chiastocheta* flies in either all flowers in the small populations or at least 200 flowers in large populations over 3 years at one of two time points each (2006–2008, see Klank et al. (2010) for a detailed description). On average 0.069 to 0.393 *Chiastocheta* flies/flower (median 0.13) were found.

### Genetic survey

Genetic diversity and differentiation were assessed with AFLPs (amplified fragment length polymorphisms). Leaves from 22 randomly sampled individuals were collected from each population in 2006 and 2007 with a minimum inter-individual distance of ~4 m to avoid sampling from the same individual. Leaf material was dried in silica gel immediately after collection. DNA extractions were done with the DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol using 10 mg of dried plant material. DNA concentrations were measured with a Nanodrop 1000 (Thermo Scientific) and averaged at ~24 ng/μl. Selective AFLP primer pairs were *Eco*-ATC–*Mse*-CAG, *Eco*-ATC–*Mse*-CAT and *Eco*-AGA–*Mse*-CTC as used by Després et al. (2002). For the digestion-ligation step, first 10 μl extracted DNA (~240 ng) was digested for 1 h at 37 °C in a 27 μl restriction mix consisting of 2 U EcoR1, 2 U Mse1, 0.4 μl bovine serum albumin (10 mg/ml), and 4 μl NEB2 buffer (10×, New England Biolabs). Second, PCR adaptors were ligated to the DNA fragments for 3 h at room temperature by adding 14 μl ligation mix consisting of 15 pmol EcoR1 adaptor, 136.35 pmol Mse1 adaptor, 0.1 μl bovine serum albumin, 5 μl ATP (10 mM), 1 μl NEB2 (10×, New England Biolabs), and 20 U T4 ligase (New

England Biolabs). This step was finished by inactivating the ligase for 10 min at 65 °C. For preselective amplification 1.5 μl restriction–ligation mix were added to 19 μl PCR reaction mix containing 157.5 ng of each preselective primer *Eco*-A and *Mse*-C, 0.3 μl dNTP mix (25 mM), 1.5 μl MgCl<sub>2</sub> (25 mM), 4 μl Promega GoTaq<sup>®</sup> reaction buffer (5×, no MgCl<sub>2</sub>) and 0.375 U GoTaq polymerase (Promega). PCR reactions cycling conditions were as follows: 1 cycle (94 °C for 3 min), 20 cycles (94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s) and 1 cycle (72 °C for 1 min). This preselective PCR product was then 1:20 times diluted with 1× TE buffer. Selective amplifications were performed by adding 3 μl of the diluted preselective PCR product to 11 μl selective PCR mix containing 1.5 μl MgCl<sub>2</sub> (25 mM), 0.5 μl dNTPs (25 mM), 3 μl GoTaq reaction buffer (5×, no MgCl<sub>2</sub>) and 0.375 U GoTaq polymerase. PCR cycling conditions were 1 cycle (94 °C for 3 min), 10 cycles (94 °C for 30 s, 65 °C for 30 s with a reduction of the annealing temperature of –1 °C from 65 to 56 °C), then 25 cycles (94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min) and 1 cycle (72 °C for 60 s). All PCRs were carried out using a Bio-Rad dyad cycler. Fragments were separated on an ABI3720xl (GeneScan<sup>™</sup> 500 LIZ<sup>®</sup> sizing standard) and Genemapper 3.5 software (Applied Biosystems) used for fragment reading. We used the following procedure to generate the fragment absence/presence matrix: Preliminary peak positions were selected manually for each primer pair in Genemapper 3.5 (ABI) and peak height data were recorded. Using AFLPScore 1.4 (Whitlock et al. 2008) we retained those loci that had an error rate of <5 % using 28 duplicated samples, resulting in a total of 101 loci. As recommended by Lynch and Milligan (1994), we retained only loci with a frequency smaller than 1 – (3/n) with n being the number of total samples, leading to a set of 76 polymorphic loci for analysis.

### Greenhouse experiment

To test plant performance in relation to population origin we determined the absolute severity of competition in a greenhouse setting. We selected the fast growing yellow oat-grass *Trisetum flavescens* (Poaceae) as the competitor species as it occurs naturally with *T. europaeus* (Ellenberg 1996). In 2007, one mature seed head per individual from 16 plants (i.e. seed families)/population were collected. The seeds were placed on standard germination soil in petri dishes and cold stratified for 4 months in the dark at 4 °C and then placed in growth cabinets (MLR-351H, Sanyo Electric Co. Ltd.) for germination. The cabinets were run with an alternating 12 h light cycle at 15 °C, 50 % relative humidity and 14.4 k lx. The petri dishes were left in the growth cabinets for 10 weeks and seedlings were then transplanted into trays in the greenhouse. The greenhouse

**Table 1** Population size, flower density ( $m^{-2}$ ), pollinator abundance (mean number of *Chiasocheta* flies/flower), location of the studied *Trollius europaeus* populations, inbreeding coefficient  $F_{IS}$  for each population, 95 % confidence intervals (Bonferroni corrected), percentage of polymorphic loci (PLP) and expected heterozygosity ( $H_E$ ) at overall  $F_{IS} = 0.117$  (95 % CI 0.064–0.170)

Population	Elevation (m s.s.l)	Co-ordinates <sup>a</sup>	Population size	Flower density ( $m^{-2}$ )	Pollinator abundance	<i>N</i> (AFLP)
ARV	1,250	732,336/223,768	820,700	14.44	0.119	21
BII	678	683,267/235,908	230	0.32	0.201	20
B15	656	712,323/236,612	840	0.11	0.393	20
B3	537	702,884/244,312	57,780	1.79	0.267	21
F1	639	680,308/236,502	3,930	5.24	0.219	20
FII	612	705,796/241,768	800	10	0.101	22
F13	764	705,729/245,752	1,140	1.2	0.128	19
F15	712	705,852/257,047	310	1.24	0.253	15
F2	745	682,861/235,022	230	0.18	0.13	22
F23	703	707,460/244,360	24,350	2.11	0.071	20
F24	732	708,000/250,470	1,920	1.4	0.129	20
F25_27	612	707,931/254,335	1,120	1.72	0.078	22
F29	800	708,330/242,391	27,120	3.98	0.292	22
F30	771	709,023/253,436	4,260	2.34	0.069	21
F34	597	709,868/257,254	43,360	2.77	0.236	15
F41	752	711,868/241,711	86,460	4.93	0.2	21
F7	852	694,443/225,287	12,110	2.73	0.091	21
Kafer	740	708,454/252,875	140	0.15	0.239	19
Kriens	1050	662,502/206,591	238,040	6.9	0.113	22
# loci	PLP	$F_{IS}$	$F_{IS}$ lower CI	$F_{IS}$ upper CI	$H_E$	SE ( $H_E$ )
76	98.7	0.184	-0.050	0.419	0.33	0.017
76	100	0.242	-0.265	0.749	0.36	0.014
76	100	0.099	-0.040	0.238	0.31	0.018
76	100	-0.052	-0.184	0.080	0.26	0.019
76	100	0.361	-0.031	0.752	0.39	0.013
76	100	<b>0.151</b>	0.006	0.296	0.32	0.017
76	100	<b>0.263</b>	0.035	0.492	0.37	0.015
76	98.7	0.121	-0.063	0.305	0.3	0.018
76	100	<b>0.125</b>	0.016	0.234	0.28	0.018
76	100	0.030	-0.109	0.169	0.28	0.017
76	97.4	-0.003	-0.125	0.118	0.28	0.019
76	100	0.012	-0.187	0.210	0.28	0.017
76	100	0.119	-0.069	0.307	0.3	0.019
76	100	0.000	-0.184	0.184	0.29	0.018
76	100	0.005	-0.158	0.168	0.3	0.017
76	100	0.100	-0.058	0.258	0.32	0.017
76	98.7	0.224	-0.087	0.536	0.31	0.018
76	100	0.120	-0.081	0.322	0.32	0.017
76	100	0.077	-0.040	0.194	0.29	0.018

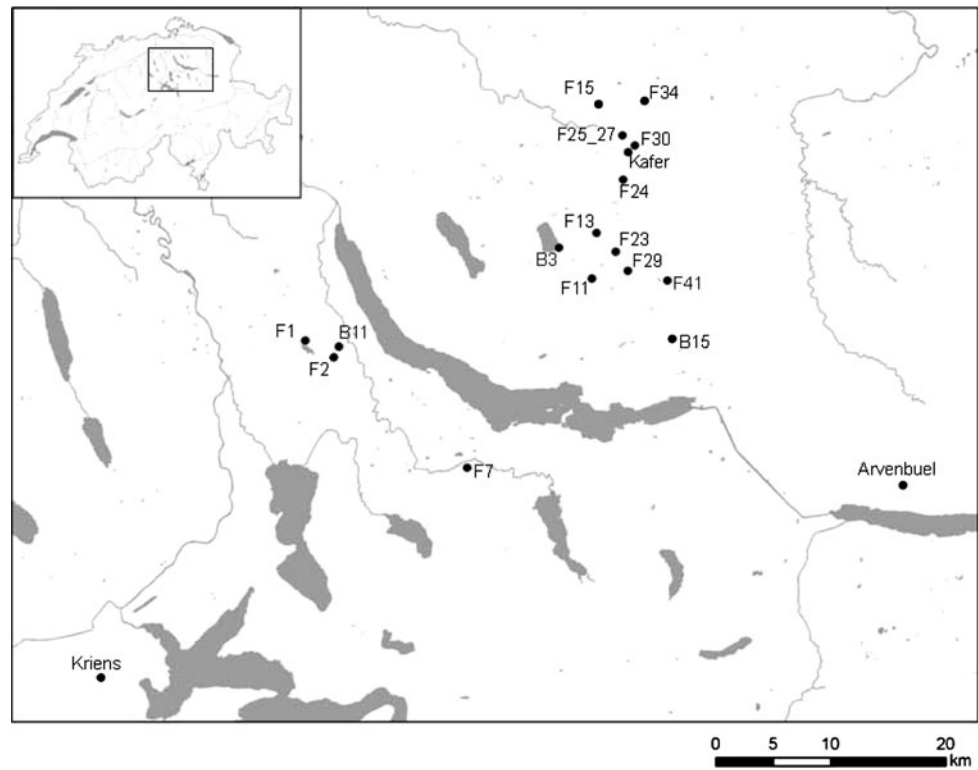
Bold  $F_{IS}$  are significant

<sup>a</sup> Coordinates in meters according to the Swiss topographical maps (Bundesamt für Landestopographie, Wabern, Switzerland)

compartments were run with 20 k lx from 08:00 to 18:00 and temperatures of 20 °C during daytime and 16 °C at night. Plants were watered automatically every day using watering mats. After 2 weeks two similar sized seedlings/

seed family were planted in 1 L pots (13.5 cm height, Ø 11 cm) filled with standard potting soil (Klasmann-Deilmann). *Trisetum flavescens* seeds were sown into half of the pots 2 days after transplanting the seedlings. Thus from

**Fig. 1** Location of the 19 *Trollius europaeus* populations in Switzerland. The grey areas are lakes; the largest lake in the centre is Lake Zurich. Reproduced with the permission of swisstopo (JA100120)



each seed family one seedling was grown with *T. flavescens* and another was grown alone (i.e. control treatment). Per treatment, plants were randomly assigned to two blocks which were kept in two different greenhouse compartments. The blocks of each treatment were formed to avoid interference of above ground competition by *T. flavescens* between the two treatments. Blocks were exchanged biweekly between compartments and plants within blocks repositioned randomly.

We recorded leaf width and number of leaves/seedling at the start of the experiment. After 10 weeks, above and below ground biomass were harvested. We measured the total leaf area/plant using a LI-3000C leaf scanner with a LI-3050C Transparent Belt Conveyer (Li-Cor Biosciences). We further measured stalk lengths and recorded the dry weight of above and below ground biomass. We also assessed the influence of the competition treatment: According to Snaydon (1991), the proportional reduction in plant performance due to the competition treatment for each parameter (leaf area, average stalk length within an individual, above and below ground biomass) is expressed as the absolute severity of competition (ASC) for each seed family, calculated as  $\log_{10}(\text{trait measure without competition}) - \log_{10}(\text{trait measure with competition})$ . Positive ASC values indicate a better performance in the control treatment, while negative values indicate a higher performance for plants grown in competition.

#### Statistical data analysis

#### Genetic data

Given that the Hardy–Weinberg equilibrium conditions cannot be readily assumed in remnant populations that have been subjected to fragmentation and increasing isolation, we applied the approach of Dasmahapatra et al. (2008) by estimating the inbreeding coefficient ( $F_{IS}$ ) of each individual and calculating the overall mean and mean/population for later analysis. Mean  $F_{IS}$  were tested for significance by calculating 95 % Bonferroni corrected confidence intervals (CI) with the null hypothesis of no difference. The derived overall  $F_{IS}$  value was implemented in AFLPSURV (Vekemans et al. 2002) for further analysis. We calculated population differentiation  $F_{ST}$  (mean overall  $F_{ST}$  and population pairwise  $F_{ST}$ ) as well as expected heterozygosity ( $H_E$ ). Calculations were done using the Bayesian method with non-uniform prior distribution of allele frequencies. Permutations ( $N = 4999$ ) of individuals among populations were done to test the hypothesis of no genetic differentiation among populations. Significances for the observed pairwise  $F_{ST}$  were evaluated using 95 % Bonferroni-corrected CI of the bootstrapped data. In case of mainly significant pairwise  $F_{ST}$ , a Mantel test to test for isolation by distance based on pairwise population genetic distances ( $F_{ST}/(1 - F_{ST})$ ); calculated in AFLPSURV) and

log transformed geographic distances (Rousset 1997) with 4,999 permutations available in IBD (Bohonak 2002) was carried out. We further evaluated the relationship between  $H_E$  and population size (log-transformed), population density and pollinator abundance by applying a linear regression in R (R Development Core Team 2009).

### Greenhouse data

To test whether plant performance was reduced in the competition treatment and if populations differed in their response as well as whether greenhouse compartments had an effect we first analysed the harvest data of each parameter. Eleven seedlings from eight populations independent of population size died during the experiment, which were removed from the analysis with their half-sib counterparts. We applied a type III Ancova with populations included as a random factor and treatment, compartment, as well as their interaction with population as fixed factor. Initial leaf width was also fitted as a fixed factor to account for variations in seedling size at the beginning of the experiment. Treatment was tested on the interaction term of treatment: population, and compartment was tested on the interaction term of compartment: population, whereas all other terms were tested on the residual.

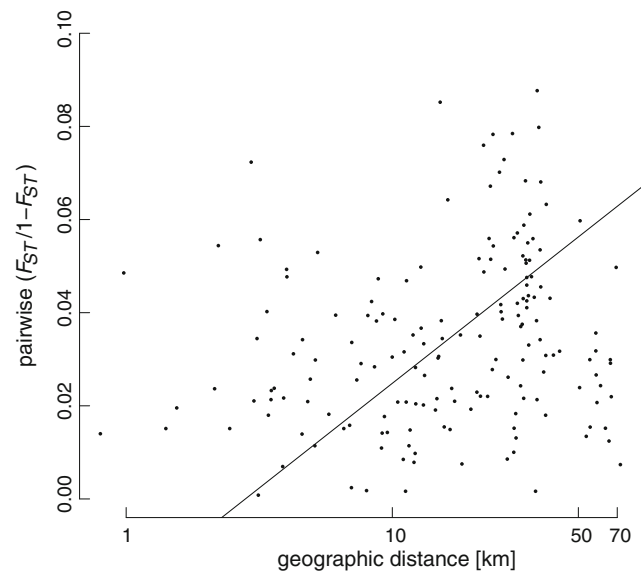
After calculating the absolute severity of competition (ASC) we used a linear mixed-effects model for analysis. As individual data points are not independent and nested within populations, we fitted the factorized population origin as a random factor. We used the ASC of the initial leaf width as a covariable, and population size (log-transformed), population density and expected heterozygosity as fixed effects. Both analyses were carried out using R (R Development Core Team 2009) and fixed effects showing a pairwise correlation coefficient above 0.8 were centred (Zuur et al. 2009). Varying degrees of freedom result from the exclusion of missing data points.

## Results

### Genetic survey

Calculating the overall inbreeding coefficient over all 383 individuals used in this study gave a significant mean  $F_{IS}$  of 0.117 (SE = 0.018, 95 % CI [0.064–0.170]). Mean  $F_{IS}$  for each population were non-significant expect for three populations (Table 1).

Using the overall mean  $F_{IS}$  value (0.117), a mean expected heterozygosity ( $H_E$ ) of 0.309 (SE 0.008) was calculated, with  $H_E$  ranging from 0.260 to 0.393 (Table 1). The percentage of polymorphic loci (PLP) varied between 97.4 and 100 % (Table 1).



**Fig. 2** Isolation by distance for 19 *Trollius europaeus* populations in north-eastern Switzerland. Pairwise ( $F_{ST}/(1 - F_{ST})$ ) and log-transformed geographic distances, based on IBD analysis with a correlation coefficient of 0.209 ( $P = 0.0598$ )

We further found our study populations to have a relatively low overall  $F_{ST}$  of 0.033 and the 95 % CI of the permuted data set of randomly assigned individuals being [−0.006–0.002] showing that our populations were significantly differentiated. As all population pairwise  $F_{ST}$  were significant (Table A1, electronic supplementary material) we carried out a Mantel test for isolation by distance and found a marginally significant correlation coefficient of 0.209 ( $P = 0.0598$ , Fig. 2).

The multiple linear regression for expected heterozygosity showed no significant relationship with either population size ( $P = 0.130$ ), population density ( $P = 0.079$ ) or pollinator abundance ( $P = 0.437$ ).

### Greenhouse experiment

All measured traits were influenced by the treatment and the population origin ( $P > 0.001$ , Table 2). The compartment assignment was not significant ( $P > 0.18$ ) except for average stalk length ( $P = 0.004$ , Table 2). Interactions between treatment: population and compartment: population were non-significant, except for below ground biomass which showed a compartment: population interaction ( $P = 0.005$ ; Table 2). The total leaf area of *T. europaeus* generally decreased when grown with *T. flavescens* (ranging between −12.47 and −44.42 %). The same was found for above and below ground biomass (between −9.87 and −41.37 %, between −19.65 and −46.00 %, respectively). Average stalk length showed an increase of 15.50–69.12 % when grown in combination with *T. flavescens*. An

**Table 2** Analysis of raw harvest data of the greenhouse experiment of 19 *Trollius europaeus* populations grown with and without competition by *Trisetum flavescens* and using an Ancova type III

	DF	Sum Sq	F value	P
<b>Leaf area</b>				
Intercept	1	117,062	46.6722	<0.0001
Initial leaf width	1	525,108	209.3591	<0.0001
Treatment	1	417,489	133.7272	<0.0001
Compartment	1	1132	0.5268	0.4773
Population	18	200,304	4.4367	<0.0001
Treatment:Population	18	56,195	1.2547	0.2126
Compartment:population	1	2,149	0.8638	0.3510
Residuals	536	1,333,690		
<b>Average stalk length</b>				
Intercept	1	36,403	94.9148	<0.0001
Initial leaf width	1	22,005	57.3733	<0.0001
Treatment	1	58,420	11.7581	<0.0001
Compartment	1	18	9.0000	0.0040
Population	18	40,678	5.8923	<0.0001
Treatment:Population	18	9,937	1.4580	0.0997
Compartment:Population	1	2	0.0044	0.9473
Residuals	542	205,226		
<b>Above ground biomass</b>				
Intercept	1	2.882	47.7448	<0.0001
Initial leaf width	1	11.656	193.1322	<0.0001
Treatment	1	8.081	135.0585	<0.0001
Compartment	1	0.026	2.0000	0.1781
Population	18	3.84	3.5352	<0.0001
Treatment:Population	18	1.077	0.9896	0.4700
Compartment:Population	1	0.013	0.2190	0.6400
Residuals	543	32.828		
<b>Below ground biomass</b>				
Intercept	1	9.358	81.9745	<0.0001
Initial leaf width	1	37.746	330.6584	<0.0001
Treatment	1	34.895	429.9179	<0.0001
Compartment	1	0.327	0.3438	0.5575
Population	18	8.577	4.1743	<0.0001
Treatment:Population	18	1.461	0.7136	0.7985
Compartment:Population	1	0.951	8.0447	0.0047
Residuals	541	61.55		

overview of all population means and differences between treatments can be found in Table A2 in the electronic supplementary material.

The absolute severity of competition (ASC) showed no significant correlations with population size, population density or expected heterozygosity (Table 3). The only significant effect came from the initial leaf width at the start of the experiment ( $P > 0.0001$ ), and population density was marginally significant for below ground ASC (Table 3).

**Discussion**

Genetic diversity and differentiation of *Trollius europaeus* populations

For *T. europaeus*, we found a relatively low  $F_{ST}$  of 0.033 and populations contained similar levels of genetic diversity independent of their sizes. Given the apparent isolation of populations across the landscape and the specialized pollination system we expected a higher degree of genetic

**Table 3** Linear mixed-effects model for analysis of the absolute severity of competition (ASC) of *Trollius europaeus*

ASC leaf area					
Random effects	Intercept	Residual			
	0.0000	0.2572			
Fixed effects	Estimate	SE	DF	<i>t</i> value	<i>P</i>
Intercept	0.2399	0.0625	265	3.8406	0.0002
ASC (initial leaf width)	0.9878	0.1491	265	6.6265	<0.0001
Population size <sup>a</sup>	−0.0072	0.0085	15	−0.8419	0.4141
Population density	0.0028	0.0060	15	0.4753	0.6414
Expected heterozygosity <sup>b</sup>	−0.9682	0.5179	15	−1.8695	0.0812
ASC average stalk length					
Random effects	Intercept	Residual			
	0.0321	0.1288			
Fixed effects	Estimate	SE	DF	<i>t</i> value	<i>P</i>
Intercept	−0.0914	0.0443	271	−2.1242	0.0346
ASC (initial leaf width)	0.3438	0.0756	271	4.5476	<0.0001
Population size <sup>a</sup>	−0.0007	0.0058	15	−0.1254	0.9019
Population density	−0.0056	0.0041	15	−1.3851	0.1863
Expected heterozygosity <sup>b</sup>	−0.0839	0.3571	15	−0.2351	0.8173
ASC above ground biomass					
Random effects	intercept	Residual			
	0.0296	0.2732			
Fixed effects	Estimate	SE	DF	<i>t</i> value	<i>P</i>
Intercept	0.1982	0.0709	272	2.7924	0.0056
ASC (initial leaf width)	1.0408	0.1584	272	6.5694	<0.0001
Population size <sup>a</sup>	−0.0003	0.0096	15	−0.0361	0.9717
Population density	−0.0006	0.0067	15	−0.0938	0.9265
Expected heterozygosity <sup>b</sup>	−0.4608	0.5903	15	−0.7807	0.4471
ASC below ground biomass					
Random effects	Intercept	Residual			
	0.0320	0.2339			
Fixed effects	Estimate	SE	DF	<i>t</i> value	<i>P</i>
Intercept	0.3000	0.0642	270	4.6706	<0.0001
ASC (initial leaf width)	1.1143	0.1375	270	8.1025	<0.0001
Population size <sup>a</sup>	−0.0097	0.0087	15	−1.1082	0.2852
Population density	0.0126	0.0061	15	2.0690	0.0562
Expected heterozygosity <sup>b</sup>	−0.4098	0.5297	15	−0.7737	0.4512

<sup>a</sup> Log-transformed<sup>b</sup> Centred

differentiation, which was not confirmed by our results. Nevertheless, the retention of genetic variation within populations seems to be a common pattern in outcrossing perennial plants (Nybom and Bartish 2000). The degree of genetic differentiation often increases with the area under study (Nybom and Bartish 2000). Indeed, we found much smaller differentiation than Després et al. (2002), whose study area covered the Pyrenees, Alps and Fennoscandia ( $F_{ST}$  of 0.033 vs. 0.152; same marker systems used).

Isolation by distance was marginally significant for our study populations ( $P = 0.06$ ), suggesting that gene flow can extend over several kilometres and/or accumulates over time. In contrast, an allozyme study of *Chiastocheta* in discrete populations of *T. europaeus* (minimum distance to the next *T. europaeus* patch of 1 km) in Denmark found local differentiation and low dispersal between the sampled insect populations (Johannesen and Loeschke 1996). This indicates that pollination by *Chiastocheta* occurs primarily



on a local scale, as also suggested by Després (2003), most probably resulting in the isolation of plant populations. Seed dispersal as a source for gene flow is probably also limited. Globeflower seeds do not have any morphological characteristics that aid dispersal, and usually drop to the ground in the close vicinity of the mother plant. Therefore, historical gene flow in an only moderately fragmented landscape seems to be responsible for the observed genetic pattern in *T. europaeus*. We further cannot rule out the possibility that the assumed isolation of populations is counteracted by small undetected populations of *T. europaeus* distributed throughout the landscape, acting as stepping stones for *Chiastocheta* and facilitating pollen movement, even though it seems unlikely for our study region. In addition, larger Diptera and other insects very occasionally visited *T. europaeus* flowers (personal observation CK). Although such visits were rare, they might contribute disproportionately to long distance gene flow due to a greater foraging range.

Species distribution range is likely influencing not only genetic differentiation but also diversity. Després et al. (2002) found significantly higher within-population genetic diversity of *T. europaeus* in the Alps than in the Pyrenees or Fennoscandia. This higher diversity was similar to the diversity levels found in our study ( $H_E = 0.229$  compared to  $H_E = 0.309$  in our study). Després et al. (2002) interpreted this to be caused by the proximity to suggested glacial refugia and suggested that the alpine populations represent moderately fragmented relics of a large southern ancestral distribution area. As our study populations are located within the same geographical range, it is plausible that the vicinity to potential refugia shaped within-population genetic diversity.

Despite an overall  $F_{IS}$  of 0.117, only three within population inbreeding measures were significant. Even though, those populations contained all less than 5,000 flowers, another eight populations of this size range had no indication for inbreeding, indicating that the loss of genetic diversity due to matings among relatives is of minor importance in small populations of *T. europaeus*.

#### Effects of population size, plant density and genetic diversity on plant performance

*Trollius europaeus* plants in the greenhouse experiment did respond to competition with *T. flavescens* with decreased leaf area and biomass, confirming a reduced performance under competition. The severities of competition in the different traits under study, however, were not affected by the population size, the population density or the expected heterozygosity. Likewise, the individual plant traits were not affected by population size and expected heterozygosity (details not shown). Leaf stalks reacted to

competition by elongation, a likely response to competition for light, but this was independent of population size, population density or expected heterozygosity (details not shown). The similar levels of genetic diversity across populations might explain the lack of differences in plant performance.

While many studies have found positive effects between population size, neutral genetic diversity and plant fitness (Gaudeul et al. 2000; Lienert et al. 2002; Galeuchet et al. 2005; Leimu et al. 2006; de Vere et al. 2009; Jacquemyn et al. 2009; Krauss et al. 2010), the absence of such a relationship, as in this study, has also been found for other species across a wide range of habitats (Schmidt and Jensen 2000; Podolsky 2001; Kuss et al. 2008; Brownlie et al. 2009; Qiu et al. 2009; Spigler et al. 2010). Furthermore, while reduced population size and decreased genetic variability often occur jointly (Oostermeijer et al. 1994; Fischer and Matthies 1998; Lienert et al. 2002; Vergeer et al. 2003a; Vergeer et al. 2003b), other studies did not find such relationships (Ouborg and Vantreuren 1995; Lammi et al. 1999; Fischer et al. 2000; Luijten et al. 2000; Pluess and Stöcklin 2004; Dostalek et al. 2010), indicating more complex mechanisms regulating plant fitness and performance.

#### Conclusions and implications for conservation

Given our results, it seems most likely that the retention of historical genetic patterns or sufficient gene flow between populations are counteracting negative effects of changes in population sizes and habitat alteration. In Switzerland, major fragmentation and land conversion events have taken place in the last 60 years (Schulz and Dosch 2005), the time period in which a decline of *T. europaeus* has been observed in our study region (Artendatenbank Canton Zurich, <http://www.aln.zh.ch>). Even though *T. europaeus* reaches reproductive maturity after  $\sim 2$  years, its lifespan is thought to be considerably longer, thus probably only a few population turnovers (i.e. exchange of all individuals) took place. As populations often lose variability very slowly (Amos and Balmford 2001) and perennial plants are assumed to have intermediate rates of population declines (Eriksson and Ehrlen 2001), it could also be that we will see more pronounced effects in the future. Due to the approach used here, we cannot infer current gene flow among our study populations. The observed results might thus be considerably influenced by historic gene flow patterns preserved in the present day populations. As *T. europaeus* is becoming increasingly rare across Europe due to the loss of suitable, moist habitats, especially in areas of intense agriculture and dense settlement structures, a main aim of our study was to understand possible threats to this species triggered by habitat fragmentation, especially given

the higher expected vulnerability due to its nursery pollination system. Here as well as in a parallel study of in situ reproductive success (Klank et al. 2010) we found no negative effects of small population size on plant performance.

*Trollius europaeus* ability to persist in relatively small remnant habitats in combination with its easily recognizable characteristic globose flowers might make it an eligible candidate as a flagship species for the conservation of moist meadows (as also suggested by Lemke 2011). Furthermore, the fact that for *T. europaeus* population size had no effect on population genetic diversity or performance suggests that management for its conservation should acknowledge that small populations retain considerable viability and therefore conservation value.

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