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Research

Genetic variation and selfing rate in *Lychnis flos-cuculi* along an industrial pollution gradient

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Summary

- We studied nine populations of a meadow mixed-mating plant *Lychnis flos-cuculi* growing in a gradient of copper smelter emissions. We hypothesize that metal tolerant populations in the polluted areas have experienced a loss of genetic variation and are more selfing than the populations from the unpolluted areas.
- One hundred and thirty-five parental plants and 1059 offspring were genotyped with six microsatellite markers. Selfing rates were assessed manually, with Rmes, Mltr and Colony2. Soil toxicity, population density and pollinators' activity were estimated in the studied areas.
- Populations from the heavily polluted area have experienced a strong founder effect. However, at present, they are characterized by high densities. A recent genetic explosion was registered for the population from the most polluted site, probably due to forest thinning under pollution effects. Selfing rates estimated with different approaches agreed well only for populations with high genetic variation; they comprised 0–0.23 and were similar between polluted and clean areas.
- Self-fertilization in *L. flos-cuculi* hardly represents a mechanism for the fixation of advantageous alleles and a barrier for gene flow from non-tolerant populations. The employment of different methods of selfing rate estimation in populations with low genetic variation appears to be necessary, though not a guarantee of reliable conclusions.

Introduction

Although, heavy metal concentrations in the soil of industrially polluted areas are intolerable for most organisms, a rapid adaptation to heavy metal excess has been documented for plant species from different families. Metal tolerant populations represent striking examples of microevolution (Macnair, 1993) and, more intriguing, they show different scenarios of population genetic processes. In particular, some authors have reported lower genetic variation in polluted areas than in control habitats, implying a founder effect in heavily toxic environments (Bush & Barrett, 1993; Nordal *et al.*, 1999; Mengoni *et al.*, 2001), while others have revealed that metal tolerant populations are highly genetically variable due to successive colonization, a high number of tolerant individuals in the primary population or pollen flow from the neighbouring populations (Wu *et al.*, 1975; Ducousso *et al.*, 1990; Vekemans & Lefebvre, 1997).

Moreover, the directions of the genetic variation's changes depend either on the direct effect of external factors (level of soil toxicity, aridity etc.) or on such species' characteristics as mating system, life history, lifespan and ability to propagate clonally. Specifically, in polluted habitats, species capable of clonal propagation can change the prevailing mode of reproduction (vegetative or sexual) (Antonovics, 1968; Van Rossum *et al.*, 2003), while mixed-mating species exhibit changes in the selfing rate (Antonovics, 1968; McNeilly & Antonovics, 1968; McClure & Whitlock, 2012). An increased selfing rate in metal-tolerant populations of industrially polluted areas has been registered for *Anthoxanthum odoratum*, *Agrostis capillaris* (Antonovics, 1968; McNeilly & Antonovics, 1968), *Armeria maritima* (Lefebvre, 1970) and *Arrhenatherum elatius* (Cuguen *et al.*, 1989). A high self-fertility was, therefore, interpreted as a barrier to gene flow from adjacent populations that were nontolerant to heavy metal excess and/or a mechanism for the fixation of alleles that were favourable under new conditions (Antonovics *et al.*, 1971; Wright *et al.*, 2013), for example at loci associated with metal tolerance. For *Thlaspi caerulescens*, however, a more autogamous breeding system has been found in control populations, which are smaller and lower in density compared with metallicolous populations, implying that a higher selfing rate offers reproductive assurance rather than fixation of metal tolerant alleles or reproductive isolation (Dubois *et al.*, 2003). Such a diversification of population processes during adaptation to industrial pollution indicates that further research in this field is required.

The object of the present research is *Lychnis flos-cuculi* L., a meadow perennial rosette mixed-mating polycarpic plant (Biere, 1991). Genetic processes are well studied for European natural and artificial populations of the species with the main emphasis

on the effects of habitat fragmentation (Galeuchet *et al.*, 2005a; Bowman *et al.*, 2008; Leimu & Fischer, 2010; Aavik *et al.*, 2014). In studies on natural and artificial *L. flos-cuculi* populations from northern and northeastern Switzerland, F_{IS} comprised from 0.30 to 0.59 and from −0.06 to 0.31 (results from Galeuchet *et al.*, 2005a and Aavik *et al.*, 2012; respectively). In the direct assessment of reproductive success under pollinator exclusion in Ulm (Germany), the average fruit set of bagged inflorescences ranged between 0% and 83% with fruits containing between 2 and 147 seeds (T. Witt, pers. comm.). According to observations in an herbivory resistance experiment with the progeny of Swiss *L. flos-cuculi* populations *c.* 90% or more of flowers turned into fruits in a glasshouse mainly due to selfing (Leimu *et al.*, 2008). Based on these estimations we suppose that the selfing rate may vary significantly between and within *L. flos-cuculi* populations. In the present work, we analyse genetic variation in *L. flos-cuculi* populations growing along the gradient of industrial pollution, with a particular focus on the selfing rate.

The study was conducted in the surroundings of a large copper smelter operating since 1940. *L. flos-cuculi* is one of a few species that inhabit either unpolluted territory (UP; 30 km west of the pollution source) or moderately (MP; 4 km) and heavily polluted (HP; 1 km) areas. The extended period of emission and high toxic load in the polluted area caused differentiation in the *L. flos-cuculi* populations of interest in terms of leaf morphology (individuals from the HP area have shorter, roundish, fleshy leaves compared with MP and UP individuals; Dulya & Mikryukov, 2013) and metal tolerance (copper EC_{10}, EC_{50} and EC_{90} are 2–3 times higher for MP and HP populations compared with UP; Dulya *et al.*, 2013).

Three specific hypotheses were tested in this work. First, we hypothesize that the toxic load decreased the *L. flos-cuculi* within-population genetic variation in the gradient of pollution and caused genetic differentiation of HP populations from MP and UP areas. In particular, populations in the HP area should have experienced the founder effect during the colonization of strongly selective metalliferous areas. Second, considering that previous studies of the genetics of plant populations from polluted territories have generally found metal-tolerant ecotypes to be more self-fertile than non-tolerant ecotypes (Antonovics, 1968; McNeilly & Antonovics, 1968; Lefèbvre, 1970; Cuguen *et al.*, 1989), we suppose that metal-tolerant populations from polluted areas are more selfing than their UP relatives. We also use additional information on environmental and population parameters in order to reveal whether self-fertilization in *L. flos-cuculi* may represent a reproductive assurance mechanism in unfavourable conditions for pollen exchange and outcrossing (e.g. small population size, isolation and pollen limitation) or a mechanism for the fixation of advantageous alleles and isolation from nontolerant populations.

Third, we aim to assess indirectly the degree of pollen limitation in the studied populations by analysing selfing rates in flowers at different positions in the inflorescence. This approach is based on the assumption that in protandrous species with basipetal blooming such as *L. flos-cuculi* (when the male reproductive system matures first and flowers open in sequence from upper to lower nodal branches), early opening upper flowers reach the female stage while later lower flowers are in the male stage. This increases the probability of within-inflorescence pollen transfer from later to early flowers, and thus, implies that the selfing rate in early flowers should be higher (Buide & Guitián, 2002). This difference, which is referred to as the flower position effect (FPE), should be especially pronounced in the deficit of outcross pollen upon a reduction in population size or density, or when the number of pollinators is insufficient (Brunet & Charlesworth, 1995). Therefore, it is possible to compare indirectly the levels of pollen limitation in different populations by evaluating the strength of the FPE. In particular, the third working hypothesis implies that FPE should be stronger in populations characterized by an increased selfing rate.

Materials and Methods

Study species

Ragged robin, *Lychnis flos-cuculi* L. (fam. Caryophyllaceae) (ITIS no. 20309, IPNI no. 155082-1; $2n = 24$; synonyms: *Silene flos-cuculi* (L.) Clairv., *Coronaria flos-cuculi* (L.) Braun, *Coccyganthe flos-cuculi* Rchb.), is a cosmopolitan species native to Eurasia that has been introduced and naturalized in North America; it is a rosette perennial, 20–125 cm tall, characteristic of wet meadows (Chaloupecká & Lepš, 2004). Mass flowering is observed in May–June. Flowers are protandrous: five outer stamens mature first, followed by five inner stamens and subsequently the five stigmas mature. Cross-pollination by insects is considered to prevail (Biere, 1991; Van Rossum & Tries, 2010). The inflorescence type is dichasial with up to 50 flowers (Supporting Information Fig. S1). Flowers open basipetally and in sequence of increasing branching order within the same node (Biere *et al.*, 1989). Mature seeds (*c.* 200 per capsule) are dispersed by means of stalk vibration (Bowman *et al.*, 2008). The species is capable of clonal reproduction and produces interconnected vegetative clones that are *c.* 25 cm in diameter (Chaloupecká & Lepš, 2004).

Study sites

The study area was located in the region surrounding the Middle Ural Copper Smelter (Russia, Sverdlovsk region, city of Revda, 50 km from Ekaterinburg). The total polluting emissions from the smelter exceeded 140 000 tons yr^{-1} in 1990–2000, but decreased to 3000–5000 tons yr^{-1} in 2013 (Vorobeichik *et al.*, 2014). These emissions have formed a pronounced 30 km gradient of soil acidity and heavy metal content in the soil (Kaigorodova & Vorobeichik, 1996).

Within each study area (UP, MP and HP), three *L. flos-cuculi* populations were chosen (Figs 1, S2). The studied populations inhabit forest glade meadows (300–5000 m^2) located 130–1520 m from each other within an area (Table S1). In 1989 and 1998, the present habitat of the most close to the smelter population was not inhabited by *L. flos-cuculi*, and the site was forested and covered primarily with *Pohlia nutans* and *Equisetum sylvaticum* (Vorobeichik *et al.*, 1994; E. L. Vorobeichik, pers.

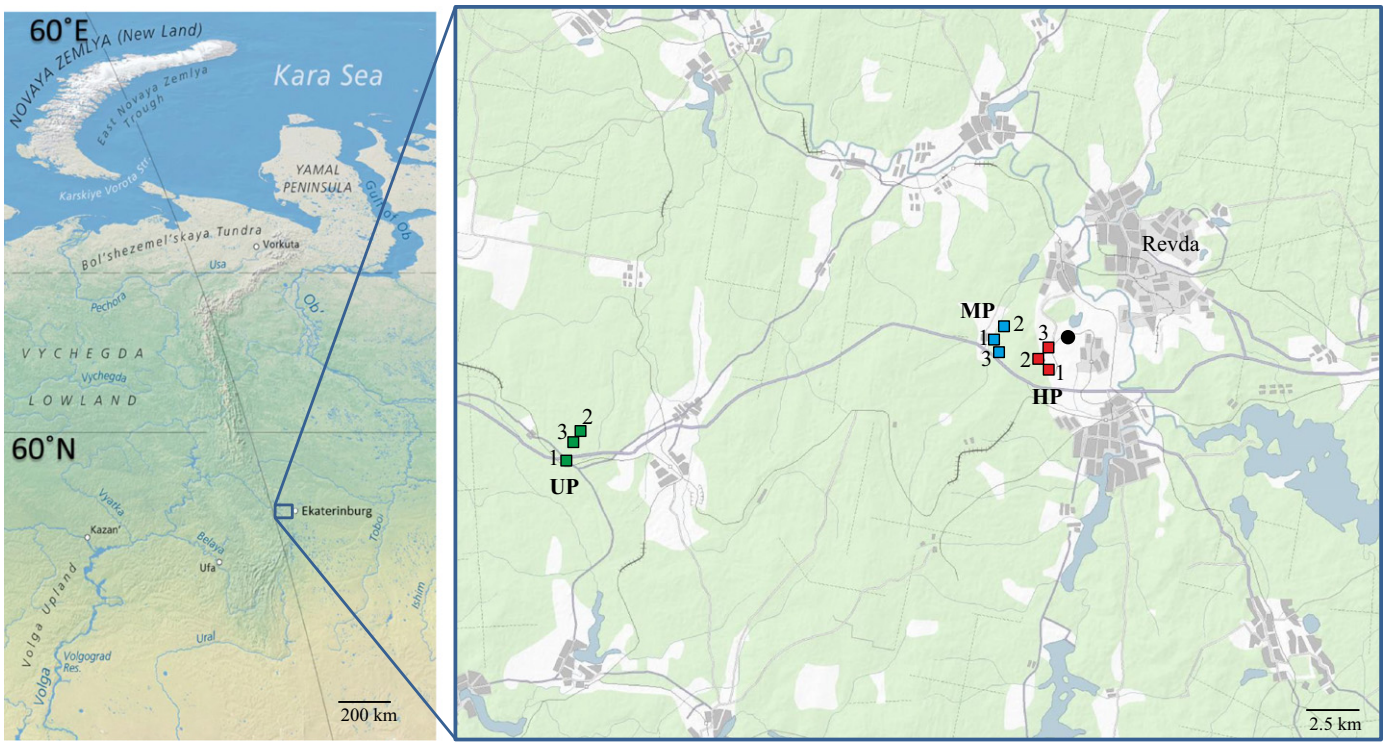

Fig. 1 Location of study populations of *Lychnis flos-cuculi* in the Urals. Empty squares and corresponding numbers denotes populations within each area (UP, unpolluted; MP, moderately polluted; HP, heavily polluted). Solid circle denotes location of the pollution source (Middle Ural Copper Smelter). Map is provided by the The Federal Service for State Registration, Cadastre and Cartography (Rosreestr; http://maps.rosreestr.ru/PortalOnline/).

comm.). Today it is characterized by the highest *L. flos-cuculi* density among the studied populations (Dulya *et al.*, 2013) (Table 1).

Herb coverage, height and species composition were assessed in each meadow. To determine heavy metal concentrations, the root soil layer (0–10 cm) was taken, air dried and sieved through 2 mm mesh. Two gram aliquots of the samples were extracted with 20 ml of 5% HNO_3. Measurements of the metal concentrations were made in a Vario 6 atomic absorption spectrometer (Analytik Jena, Germany).

The pollinators' activity assessment was performed on 21 and 22 June 2013 (i.e. in the period of mass flowering in the studied populations). Pollinators visiting the flowers located within sight of a motionless observer were recorded within 10 min of observation in each area synchronously from 11:00 h to 17:00 h. Before each observation the observer stayed motionless for 10 min counting visible flowers. In total 110 observations (8–14 per population) were made with a mean number of flowers per observation 23.4, and ranging from 4 to 115 depending on the population density. Each observation was performed on different plants. The weather on those days was similar in all areas: fair, with some clouds and gusty wind, average air temperature of 29.5°C (24–34°C).

Genetic analysis

In July to August 2012, in the studied populations, seeds and leaves were collected from mature *L. flos-cuculi* plants (Table 1). In each population, the distances between the collected plants were measured using an eTrex 20 GPS receiver (Garmin, Olathe, KS, USA), a compass and a measuring tape. These distances ranged from 10 cm to > 100 m (Table S2). Plants collected < 1 m apart were confirmed to be separate, that is, different individuals. Leaf samples were air-dried and stored at −20°C for subsequent genetic analysis.

The seeds of each plant were collected separately from two lateral flowers on the upper branch and two lateral flowers on the lower branch of the inflorescence (Fig. S1). In February 2013, the seeds of *c.* 10 plants from each population (5 seeds per flower, 20 seeds per plant) were planted in a glasshouse, and the leaves of 32-d-old seedlings were collected, dried and frozen for analysis. Overall 1194 plants were included in analysis. The data structure is presented in Table 1.

Air-dried leaf samples were frozen in liquid nitrogen and homogenized in an MM 400 mixer mill (Retsch, Haan, Germany) at 30 Hz for 30 s. The total DNA was extracted from 16 to 25 mg of the homogenate using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Union City, CA, USA). Genotyping with six microsatellite markers (Galeuchet *et al.*, 2002) was performed using two PCR multiplexes (Tables S3, S4) developed on the basis of information provided in Aavik *et al.* (2012). The reactions were performed with a Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) in a GeneAmp 2720 thermocycler (Applied Biosystems, Foster City, CA, USA); the volume of the reaction mixture was 10 µl. Capillary electrophoresis with fluorescence detection was performed using a 3130 Genetic

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Table 1 Environmental parameters of the study sites, characteristics of *Lychnis flos-cuculi* populations and summary of the sampling scheme

	UP populations			MP populations			HP populations		
Parameter	1	2	3	1	2	3	1	2	3
Coordinates	59.4244°N, 56.7935°E	59.4293°N, 56.8026°E	59.4272°N, 56.8013°E	59.8276°N, 56.8521°E	59.8315°N, 56.8616°E	59.8290°N, 56.8476°E	59.8748°N, 56.8402°E	59.8715°N, 56.8439°E	59.8731°N, 56.8485°E
Herb vegetation parameters[a]									
Height, cm	37.1 ± 5.9	41.9 ± 3	33.2 ± 4.3	24.1 ± 6.1	24.6 ± 7	26.1 ± 5.3	17.5 ± 5.3	32.7 ± 6.5	20.9 ± 4.3
Projective cover, %	95.5 ± 2.4	92.2 ± 3.7	86.1 ± 5.3	75.8 ± 7.9	67.2 ± 10.3	82 ± 6.9	29.8 ± 11.5	41.4 ± 14.4	28.9 ± 10.9
α-diversity	10.8 ± 0.14	17.1 ± 0.64	17.5 ± 0.17	15 ± 0.81	9.3 ± 0.75	13.7 ± 0.87	2.9 ± 0.17	1.9 ± 0.12	2.4 ± 0.17
Metal concentrations ($\mu g\ g^{-1}$)[b]									
Copper (Cu)	22.9 ± 0.8	22.3 ± 1.9	22.2 ± 1.9	186.4 ± 38.2	307.9 ± 51.5	394.9 ± 55.8	357 ± 73.7	871.9 ± 46.9	363.9 ± 85.8
Cadmium (Cd)	0.6 ± 0.003	0.7 ± 0.1	0.6 ± 0.1	1.7 ± 0.2	3.0 ± 0.8	4.3 ± 0.8	3.5 ± 0.6	3.5 ± 0.3	1.8 ± 0.2
Lead (Pb)	18.7 ± 1.9	22.4 ± 1.9	19.1 ± 2.2	50 ± 10.5	96.3 ± 25.6	151.4 ± 28.7	88.3 ± 25	346.5 ± 30.1	92 ± 12.3
Zinc (Zn)	36.3 ± 1.7	44.6 ± 7.5	35 ± 4.4	89.3 ± 10	147.5 ± 22.1	195.7 ± 37.9	171.4 ± 23.9	150.6 ± 5.9	88.4 ± 8.9
Density of *L. flos-cuculi* populations[c]									
N of vegetative rosettes per m^2	1.39 ± 0.59	2.19 ± 0.79	3.49 ± 0.97	0.20 ± 0.10	1.80 ± 1.00	–	3.92 ± 3.92	16.92 ± 10.13	18.69 ± 9.41
N of generative stalks per m^2	0.04 ± 0.02	0.11 ± 0.07	0.43 ± 0.41	0.14 ± 0.04	1.21 ± 0.45	–	1.40 ± 1.40	6.45 ± 5.60	0.27 ± 0.18
Pollinators visits, visits per flower per hour	1.43 ± 0.88	0.40 ± 0.18	1.27 ± 0.47	1.46 ± 0.28	1.65 ± 0.38	3.59 ± 0.74	1.85 ± 0.40	3.69 ± 0.57	1.14 ± 0.25
Number of flowers per stalk	24.7 ± 4.4	15.9 ± 1.6	18.4 ± 1.8	20.4 ± 2.6	11.5 ± 1.2	18.5 ± 1.3	18.5 ± 1.8	16.1 ± 1.3	14.6 ± 1.5
Sample sizes for genetic analysis									
N of parental plants[d]	15 (10)	15 (10)	15 (9)	15 (10)	15 (11)	15 (10)	15 (12)	15 (10)	15 (11)
N of progeny from maternal plants	115	143	124	53	75	61	183	158	147
N of progeny from upper/lower flowers	51/64	71/72	54/70	24/29	33/42	30/31	94/89	78/80	85/62

UP, unpolluted; MP, moderately polluted; HP, heavily polluted.
[a]$n = 3$ plots 25 × 25 m with 10 microplots 0.5 × 0.5 m inside each, $n = 2$ for UP populations 1 and 2; α-diversity, species number per 0.25 m^2.
[b]$n = 5$ soil samples for each population, $n = 8$ for MP population 1.
[c]$n = 3$ plots 10 × 10 m each with 100 microplots 0.5 × 0.5 m inside, $n = 6$ for MP population 1.
[d]Number of maternal plants whose progeny was genotyped is shown in parentheses.

Analyzer (Applied Biosystems). The peaks were sized using the GeneMapper v.3.7 software (Applied Biosystems) and internal size standards S450 (Sintol, Moscow, Russia) or LIZ-500 (Applied Biosystems). Among 1100 genotyped individuals, none was identified as a blank individual at any locus, indicating that the proportion of null alleles in the sample is negligible. However, using the Brookfield algorithm in Micro-Checker v.2.2.3 (Van Oosterhout *et al.*, 2004), we found the evidence for higher than zero frequency of null alleles in locus *Cuculi 12* (0.14–0.24) in UP populations and in loci *Cuculi 12* and *17* (0.18 and 0.12) in HP population 3 (Table S5). It is worth noting that various estimators proposed to detect null alleles are based on testing for Hardy–Weinberg proportions, which assumes panmixia, and thus are not applicable to mixed-mating species.

Data analysis

Estimation of population differentiation with analysis of molecular variance (AMOVA) in the parental generation was performed with the GenAlEx v.6.501 (Peakall & Smouse, 2012).

Progeny and parental samples significantly differed in number and structure which did not allow us to compare directly genetic variation between the generations. For this purpose, subsamples of nine plants (equal to the minimum number of families per population) were randomly selected with replacement from each population within each generation with 1000 iterations using the R software v.3.2.1 (R Core Team, 2015). Each subsample of progeny consisted of individuals from different families. The measures of within-population diversity were estimated in each subsample and averaged by iterations for each locus and then averaged by loci. A between-generation difference of pairwise F_{ST} (ΔF_{ST}) was considered significant if its CI did not overlap zero.

Recent changes in the effective population size were tested using Bottleneck v.1.2.02 (Piry *et al.*, 1999). Expected equilibrium heterozygosity (i.e. Nei's gene diversity) was assessed with the infinite allele model (IAM), stepwise mutation model (SMM) and two-phase mutation model (TPM, with 95% of stepwise mutations, and a variance among multiple steps of 12).

The selfing rate (S) in each family was estimated by means of four approaches: manually, and with Mltr, Colony2 and Rmes. In manual inference, in each progeny array (consisting of the maternal genotype and its offspring), an offspring with alleles not found in its maternal plant was considered outcrossed. To obtain the estimate of S based on the distribution of multilocus heterozygosity for each progeny array, we used the Rmes program (David *et al.*, 2007). This method should be insensitive to null alleles (David *et al.*, 2007). Using the Mltr v.3.4 program (Ritland, 2002), for each offspring we estimated the selfing probability based on the multilocus outcrossing rate ($1-t_m$). This analysis was conducted for each area with a Newton–Raphson optimization algorithm; pollen and ovule allele frequencies assumed to be equal. Standard errors were estimated with the bootstrap (1000 iterations with resampling at the family level). In Colony v.2.0.5.9 (Wang *et al.*, 2012) we performed pedigree reconstruction for each offspring. The input data included progeny arrays and other parental plants (Table S6). Candidate paternal plants are inferred by the program from parental generation and 'expected' plants whose genotypes were calculated on the basis of allele frequencies. Pedigree reconstruction was performed either for each population, assuming that cross-pollination takes place mainly within populations, since the average distance of *L. flos-cuculi* pollen transfer in open habitats is *c.* 135 m (Van Rossum & Tries, 2010), or for an area, considering that the maximum distance of pollen transfer reaches 524 m and, hence, pollen exchange between populations in the same area may also be relatively active. Two approaches for pedigree reconstruction were used: pairwise-likelihood score (PLS) and PLS combined with full likelihood method (FPLS), for other parameters see Table S7. The accuracy of S estimates produced by Colony was evaluated in simulation module (Wang, 2013) and comprised 76–85% depending on the population (for details and results see Table S8, Figs S3, S4).

Based on the results of manual inference, Mltr and Colony, S was calculated as the proportions of offspring produced by selfing. The relationship of S with flower position in the inflorescence and pollution level was evaluated by generalized linear mixed-effects models (GLMM with a binomial distribution and a logit link function) in package lme4 v.1.1-8 (Bates *et al.*, 2015). 'Flower position' and 'area' or 'population' were considered fixed effects, and 'maternal plant', a random effect (see Table S9). The dependent variable was the number of offspring that resulted from self-fertilization and outcrossing in flowers from upper and lower positions. R^2 for the models was estimated according to Johnson (2014).

Lychnis flos-cuculi seeds disperse over a short distance and the species also forms compact clones (Chaloupecká & Lepš, 2004). Pollen exchange between siblings or clones may bring bias in S in populations with different spatial genetic structure. Therefore, in each population, matrices of pairwise geographic and genetic distances between parental plants were generated for the analysis. Autocorrelation was estimated between these matrices for each distance class using the Mantel coefficient of correlation (*rM*; Borcard & Legendre, 2012) in the vegan v.2.3-0 package (Oksanen *et al.*, 2014). Genetic distances are presented by Queller and Goodnight's (1989) pairwise relatedness coefficients calculated with the Coancestry v.1.0.1.2 (Wang, 2011).

Between-population and between-area differences in pollinators' visits (square-root transformed) were estimated with ANOVA in R. Pairwise comparisons of areas and populations in pollinator spectra were performed with permutational multivariate analysis of variance (Anderson, 2001) using Bray–Curtis dissimilarity matrices.

Results

Parameters of the studied populations and sites

Polluted areas differed most drastically from the unpolluted area in the concentration in the soil of the main component of emissions, Cu; that is, in the HP area the Cu concentration exceeded the UP level by a factor of 16–39 and by a factor of 8–18 in the MP area (Table 1). Vegetation of *L. flos-cuculi* habitats in the UP

and MP areas is presented with 61 and 56 herb species, respectively, while only 14 species were found in the HP area. The density and height of the herb vegetation in the studied meadows also decreased along the pollution gradient, that is, the total herb coverage in the MP and HP areas was 1.2–3 times lower than that in the UP area. The studied *L. flos-cuculi* populations differed in density. In particular, in the HP area the density of vegetative rosettes was 1.2–93 times higher than those in the MP and UP areas.

The main diurnal pollinators of *L. flos-cuculi* in the study area are insects of the orders Lepidoptera (*Aporia crataegi*, *Coenonympha glycerion*, *Ochlodes sylvanus*; families Hesperiidae, Satyridae), Diptera (mainly Syrphidae), Hymenoptera (superfamily Apoidea, genus *Bombus*; family Chrysididae) and Coleoptera (family Mordellidae; *Trichodes ircutensis*, *Malachius bipustulatus*). Pollinators spectra differed significantly between the areas: $F_{UP/MP}(1, 54) = 4.1$, $P = 0.007$; $F_{UP/HP}(1, 54) = 3.5$, $P = 0.004$ and $F_{MP/HP}(1, 74) = 20.8$, $P = 0.001$. The most diverse spectrum of pollinators with the highest proportion of Apoidea (*c.* 37%) was found in the HP area (Figs S5, S6). In the UP area Lepidoptera and Apoidea comprise up to 50% and 25% of all pollinators. In the MP area *c.* 90% of all pollinators are presented by Lepidoptera. The spectrum of pollinators in HP population 3 differed from the other HP populations (Tables S10, S11) with higher Coleoptera frequency and lower Lepidoptera and Apoidea frequencies in it. Within the MP area, a significant difference in pollinators' spectra was found between populations 1 and 3, mainly due to the higher frequency of Lepidoptera in the latter population. In populations of the UP area, the pollinators' composition was similar.

The frequency of flower visits in populations within the same area were similar (Table 1; $F(2, 105) = 0.38$, $P = 0.681$). The frequency of flower visits in the HP and MP areas was almost twice as high as that in the UP area ($F(2, 107) = 8.14$, $P < 0.001$). For the UP–MP and UP–HP comparisons with Tukey's test, $P < 0.001$, while for the HP–MP comparison, $P = 0.994$.

Within-population genetic variation

The *Cuculi 13* locus showing polymorphism in European *L. flos-cuculi* populations (Galeuchet *et al.*, 2002) was monomorphic and, hence, was excluded from further analysis. The five other markers had, on average, 9.3 alleles each and were highly sensitive for the identification of genotypes (Table S12; Fig. S7).

In the MP and UP areas, no clones were found, from 15 parental individuals per population. Five pairs of genetically identical individuals were registered in HP population 2, whereas three and six identical individuals were found in HP population 3. Two of the most frequent genotypes are shared between populations in the HP area. The number of alleles and proportions of heterozygotes are significantly higher in the UP and MP than in the HP areas (Fig. 2a–c). Coefficients of inbreeding (F_{IS}) did not differ significantly from zero in both generations. However, significantly higher F_{IS} were revealed in

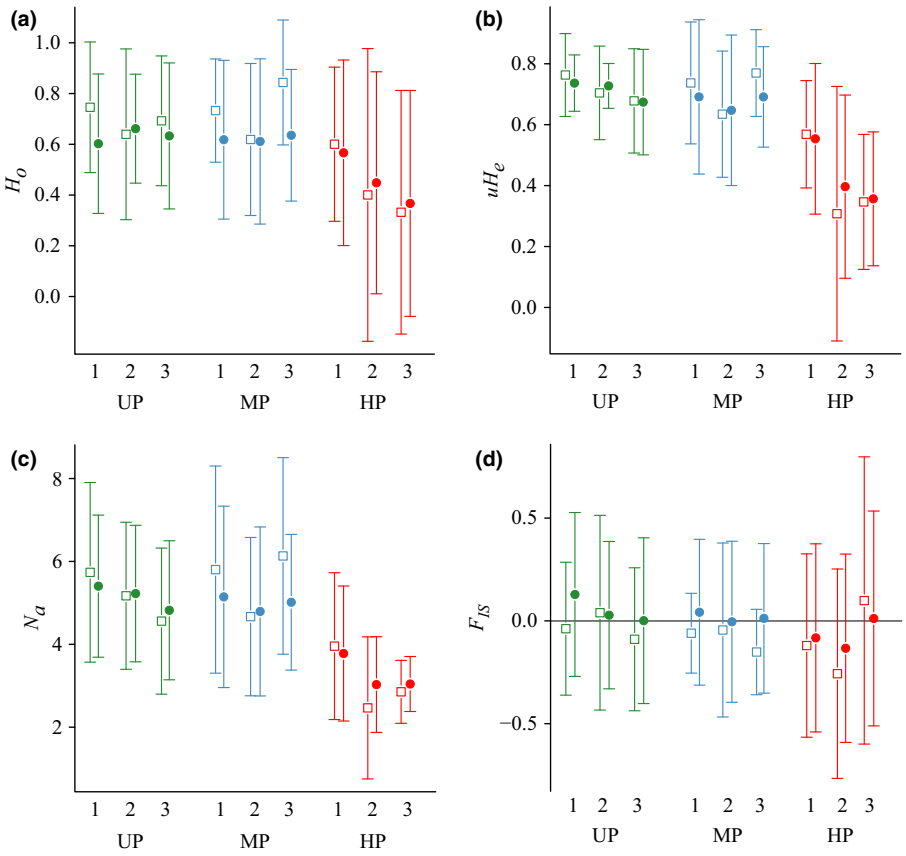

Fig. 2 (a) Observed (H_o) and (b) expected proportion of heterozygotes (uH_e), (c) allelic variation (N_a), and (d) Wright's inbreeding coefficient (F_{IS}) in the parental generation (empty squares) and progeny (solid circles) in *Lychnis flos-cuculi* populations of different areas. Mean and 95% confidence interval are shown, with a locus used as a sampling unit. Numbers along the horizontal axis (1–3) denote population identification numbers; UP, unpolluted; MP, moderately polluted; HP, heavily polluted area. The pairwise comparisons are presented in Supporting Information Table S19.

UP population 1 and MP population 2 compared with the other populations (Fig. 2d).

No significant difference in genetic variation between the two generations was found (Fig. 2; Table S13), though the difference between F_{IS} values in the parental generation and progeny was negative in all the populations, except for HP population 3 (mean ΔF_{IS} for nine populations was −0.062 with CI ranging from −0.115 to −0.009). This provides some evidence for more outcross adults and thus, possibly, lower fitness of inbred individuals. A positive (though insignificant) difference of F_{IS} between parents and progeny, revealed in HP population 3 possibly illustrates the increased frequency of outcrossing in the last generation, which, we suppose, resulted from population explosion or an income of new alleles (see next section).

Changes in effective population size

For population 1 in the MP area, heterozygosity excess or deficiency was detected depending on the mutation model used (Table 2). This is not surprising since the true model of mutation for most loci is unknown, and different mutation models are prone to different biases in the estimation of heterozygosity disequilibrium (i.e. IAM, to heterozygosity excess detection, and SMM, to deficiency; Piry *et al.*, 1999). No changes in the effective population size were found for populations 1 and 2 in the HP area.

For population 1 and 3 in the UP area a significant heterozygosity excess was shown under IAM, which may indicate a recent genetic bottleneck in these populations. For population 2 in the UP area and population 2 in the MP area a significant heterozygosity deficiency was indicated under the TPM and SMM. This finding may suggest that these populations could have experienced a recent expansion in size or a recent influx of rare alleles from immigrants. Only for population 3 in the HP area was the heterozygosity deficiency registered irrespective of the used model, strictly indicating its recent explosion in size or influx of new alleles from genetically distinct populations.

Table 2 Heterozygosity excess analyses of *Lychnis flos-cuculi* populations

		IAM		TPM		SMM	
Area	Population	P_{exc}	P_{def}	P_{exc}	P_{def}	P_{exc}	P_{def}
UP	1	**0.02****	1	0.50	0.59	0.59	0.50
	2	0.11	0.92	0.97	**0.05****	0.98	**0.03****
	3	**0.02****	1	0.89	0.31	0.95	**0.08***
MP	1	**0.02****	1	0.98	**0.03****	1	**0.02****
	2	0.50	0.59	0.98	**0.03****	0.98	**0.03****
	3	0.11	0.92	0.95	**0.08***	0.95	**0.08***
HP	1	0.59	0.50	0.95	**0.08***	0.95	**0.08***
	2	0.84	0.44	0.91	0.16	0.91	0.16
	3	0.98	**0.03****	1	**0.02****	1	**0.02****

IAM, the infinite allele mutation model; TPM, the two-phase mutation model; SMM, the stepwise mutation model; UP, unpolluted; MP, moderately polluted; HP, heavily polluted; P_{exc} and P_{def}, *P*-values for heterozygosity excess (i.e. genetic bottleneck) and deficiency (i.e. explosion) in one-tailed Wilcoxon sign rank test; significant deviations from heterozygosity equilibrium are depicted in bold (**, $P < 0.05$; *, $P < 0.1$).

Spatial genetic structure

From one to five pairs of plants growing < 1 m apart were collected in each population. From 26 such pairs, only two plants growing 46 cm apart in HP population 2 were found to be genetically identical (possibly resulting from clonal propagation). Other genetically identical plants found in the HP populations (see the section on Within-population genetic variation) grew 3–61 m apart (mean 35 m). *rM* between Queller–Goodnight relatedness and geographic distances in each population were insignificant (Table S14).

In correlogram analysis the relatedness of plants from a certain distance class did not differ from the relatedness expected by chance (Fig. 3), except for a significantly low relatedness in the last distance class of 20–105 m in two populations from the MP

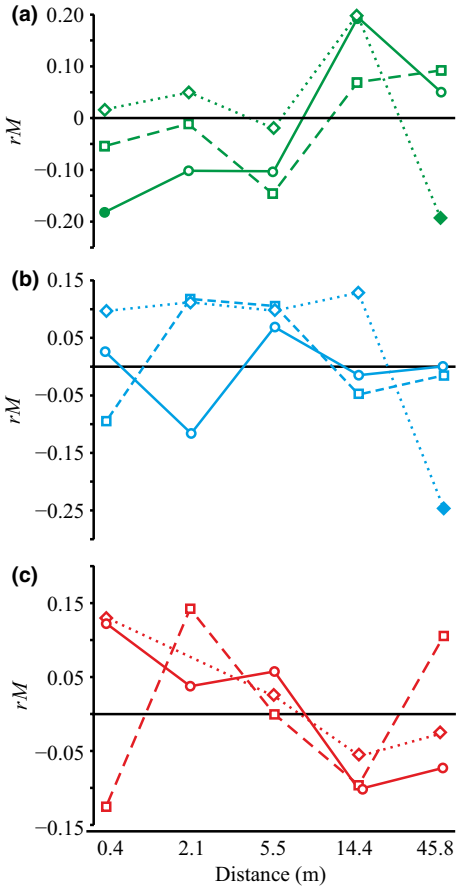

Fig. 3 Spatial genetic structure of *Lychnis flos-cuculi* populations in (a) unpolluted (UP), (b) moderately polluted (MP), and (c) heavily polluted (HP) areas. Circles, squares and diamonds refer to population identification numbers 1, 2 and 3, respectively, within the areas. When interpreting the correlograms, one should not look for high values of Mantel *rM* statistics, but for the shape drawn by its significant values. A positive and statistically significant *rM* (filled markers) indicate that for the given distance class the genetic relatedness among plants in the population is higher (for positive *rM*) or lower (for negative *rM*) than expected by chance (i.e. the mean within-class genetic relatedness is different from the mean among-class similarity).

and UP areas. Furthermore, in the UP population 1 significantly low relatedness was found between neighbouring plants. These results indicate the absence of a pronounced genetic autocorrelation in the studied populations.

Between-population genetic variation

According to fixation indices a significant genetic differentiation in the parental sample was observed between populations, areas and between populations within areas (Table 3). Individual variation accounted for 90% of the total variance; variation between areas, for *c.* 9%; and variation between populations, for *c.* 2%.

In the parental generation, the highest pairwise F_{ST} values (up to 0.23) were recorded between populations from the HP area and populations from the other areas (Fig. 4; Table S15). The populations in the UP and MP areas were differentiated from each other to a lesser degree (F_{ST} 0–0.04). Significant differences were also revealed between all populations within the HP area (F_{ST} 0.04–0.10).

Considering that populations in the MP and UP areas are located 25 km apart from each other, while the distance between the HP and MP areas is only 3 km, the observed level of between-area genetic differentiation is unlikely to be a consequence of isolation by distance, but rather of a decrease in the within-population genetic variation in the HP area.

The mean between-generation ΔF_{ST} was negative for the UP and MP populations showing their higher differentiation in offspring than in the parental generation (Fig. 4; Table S16). However, most of the ΔF_{ST} for HP populations was positive, possibly indicating a lower isolation of HP populations from each other and genetically distinct neighbours.

Selfing rate

The *S* values estimated for each family with most of the methods correlated with $r > 0.6$ within the UP and MP areas and population 1 from the HP area (Table S17), though within UP population 1 and MP population 1 the correlation was found to be less ($-0.10 < r < 0.45$). Moreover, *r* between the results of Colony (PLS) and the other methods within HP populations 2 and 3 were negative.

Generally, the highest number of similar *S* (i.e. differing by < 5%) was registered between Colony (FPLS), manual inference and Mltr (43–57% of equal *S* when all populations were taken into account and 59–63% for MP and UP populations only). However, the methods corresponded predominantly at zero values of *S*. Rmes showed higher *S* in all the populations compared with the other methods by *c.* 25% (Fig. 5). Colony (FPLS) assigned all progeny in the HP area to outcrossing and showed more (by 20–70%) zero values for *S* than the other methods in the MP and UP areas. Colony (PLS) also showed lower *S* in the HP area compared with the manually inferred and Mltr results by 15% and 25%, respectively, but demonstrated higher *S* (by *c.* 15%) in the UP and MP areas and HP population 1. It is worth noting that the results of pedigree reconstruction in Colony (PLS) were similar for 90–97% of the offspring in each area, irrespective of the assumptions about the distance of pollen transfer, that is whether the calculations were made within the populations or within the areas.

Finally, *S* estimated with Mltr, Rmes or manually, was significantly higher in HP population 3 and/or 2 than in the other populations (Fig. 5a–c; Table S18). By contrast, *S* derived from Colony (PLS) significantly decreased in the gradient of pollution

Table 3 Genetic differentiation of *Lychnis flos-cuculi* (AMOVA, the parental generation)

Molecular variance Source of variation	df	SS	Var.	% of Total	Fixation indices Fixation index	Value	*P*-value
Between areas	2	31.47	0.15	8.6	F_{RT} (between areas)	**0.086**	**0.003**
Between populations	6	14.41	0.03	1.7	F_{ST} (between populations)	**0.104**	**0.001**
Between individuals	126	192.43	1.53	0	F_{SR} (betw. pop. within areas)	**0.019**	**0.004**
Within individuals	135	208.50	1.54	89.7	F_{IS} (within populations)	−0.006	0.601
Total	269	446.82	1.71	100	F_{IT} (individual variation)	**0.098**	**0.001**

Significant values are in bold.

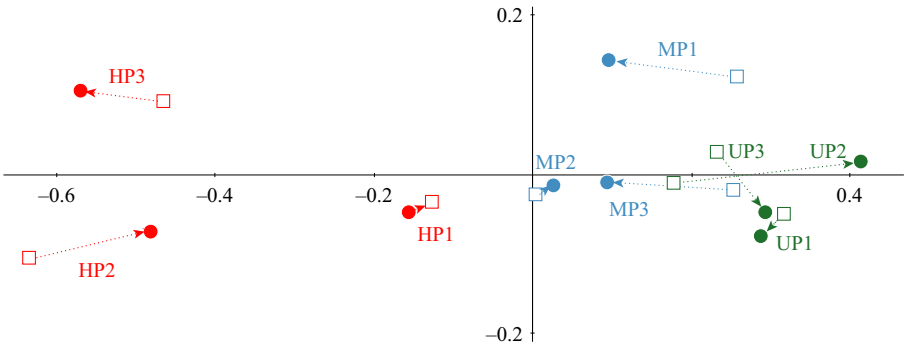

Fig. 4 Genetic differentiation of *Lychnis flos-cuculi* populations from different areas in the parental generation (empty squares) and progeny (solid circles). Pairwise F_{ST} visualized with a nonmetric multidimensional scaling (NMDS) technique. Arrows illustrate changes in the relative differentiation of populations between generations. UP, unpolluted area; MP, moderately polluted area; HP, heavily polluted area.

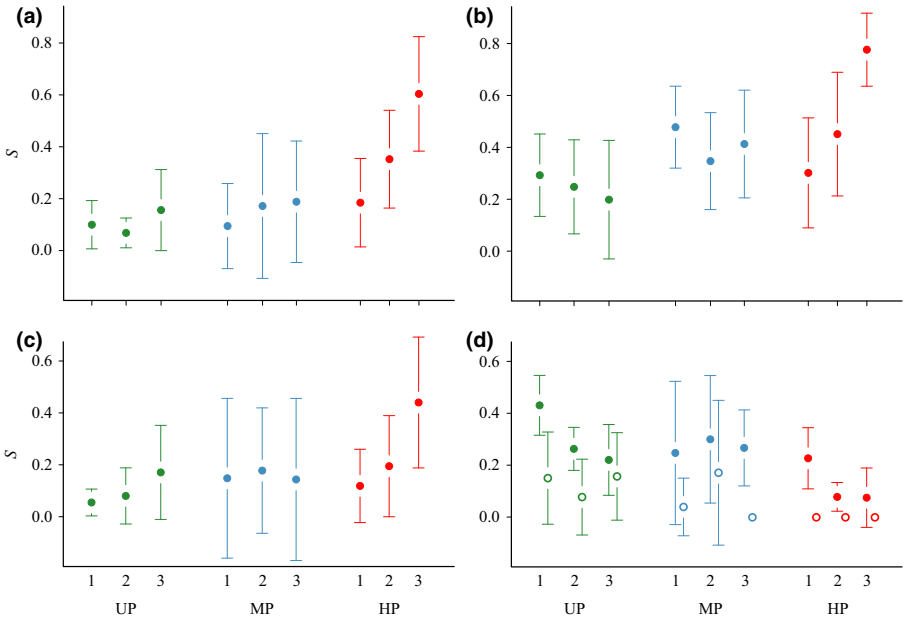

Fig. 5 Mean selfing rates (*S*) in *Lychnis flos-cuculi*, calculated (a) manually, (b) with RMES, (c) MLTR and (d) COLONY PLS (solid circles) and FPLS (empty circles). UP, unpolluted area; MP, moderately polluted area; HP, heavily polluted area. 95% confidence interval is shown. The pairwise comparisons are presented in Table S18.

(Fig. 5d), while *S* estimated in COLONY (FPLS) did not differ between areas or populations.

Assessment of the 'flower position' effect

To examine the FPE in the populations studied, a set of initial GLMMs (Table S9) was tested for data obtained from the different methods (except for RMES, for which *S* was assessed only for full progeny arrays, but not for separate flowers). The best models (based on AIC) included 'population' and 'flower position' as the fixed factor and 'family' or 'flower × family', as the random factors. The marginal R^2 (the proportion of variance explained by the fixed factors alone; Johnson, 2014) was 0.02–0.18, while the conditional R^2 (for both the fixed and random factors) was found to be 0.23–0.75. Therefore, the variability of *S* explained by maternal plants comprised 11–72%, depending on the method of *S* estimation used.

Significant FPE (with *S* being higher in earlier-opening flowers at the top of the inflorescence) was registered with the best model only for the data obtained in COLONY (FPLS) after exclusion of populations where all progeny have been assigned to outcrossing and COLONY (PLS) (Fig. 6). According to AIC and R^2, the interaction of 'flower position' with 'population' or 'area' brings almost no new information to the model. Therefore, we consider FPE equal in all populations, irrespective of the method of *S* assessment.

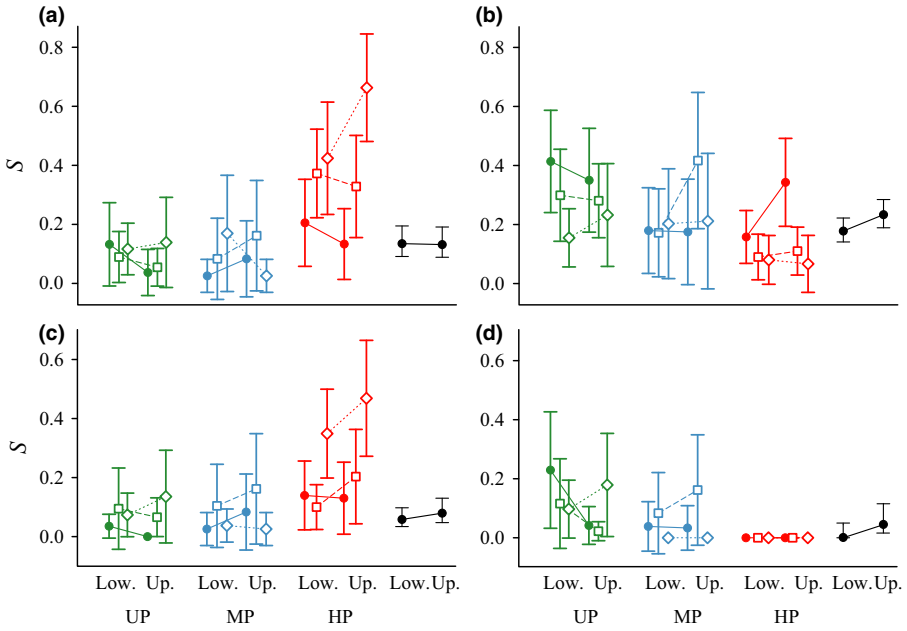

Fig. 6 Mean selfing rates (*S*) in *Lychnis flos-cuculi* flowers from different positions in the inflorescence (Up., early-opening upper flowers; Low., later-opening lower flowers) estimated (a) manually, (c) with MLTR, (b) COLONY (PLS) and (d) COLONY (FPLS). Black circles denote FPE estimated with GLMM. UP, unpolluted area; MP, moderately polluted area; HP, heavily polluted area; circles, squares and diamonds refer to population identification numbers 1, 2 and 3, respectively, within the areas. 95% confidence interval is shown.

Discussion

Low genetic variation and high differentiation of the HP populations from each other and from the MP and UP populations imply that *L. flos-cuculi* in the HP area have experienced founder effect or genetic bottleneck during the recolonization of heavily contaminated sites or elimination of a considerable part of the populations under toxic load.

However, in the tests of the mutation-drift equilibrium, for the closest to the smelter population we revealed a recent increase in the effective population size which may illustrate the first stages of *L. flos-cuculi* populations' dynamics after colonization of the HP area, and can be explained considering habitat transformation under the pollution effect and the recent reduction of the smelter emissions. In particular, for the period of the smelter functioning the high level of soil toxicity and acid gases in the HP area has caused the thinning of forest stands (Vorobeichik *et al.*, 2014), which are known to impede gene flow between *L. flos-cuculi* populations (Aavik *et al.*, 2014). Consequently, in the HP area, habitats suitable for *L. flos-cuculi* have recently expanded and become less isolated from each other compared with those in the MP and UP areas, and the gene flow between neighbouring HP populations has increased. Moreover, a 45-fold decline of acid fallouts for the past two decades could enhance the growth of *L. flos-cuculi* population density in the HP area. However, the content of heavy metals in the soil remains high (see Table 1) and intolerable for other herb species in this territory (Vorobeichik *et al.*, 2014) providing the lack of interspecific competition.

The F_{IS} revealed for *L. flos-cuculi* in the present work ranged from −0.26 to 0.13, although they did not differ significantly from zero. These values are lower than those reported for the species in the work of Galeuchet *et al.* (2005a), though close to the F_{IS} documented by Aavik *et al.* (2012). The lowest F_{IS} found for the parental generation in three populations of the HP and MP areas may result from inbreeding depression. This suggestion is favoured by the negative ΔF_{IS} between parental and offspring generations reported in this work and the decline in fitness of selfed or inbred individuals registered earlier in experiments (Hauser & Loeschcke, 1995, 1996) or in natural *L. flos-cuculi* populations (Galeuchet *et al.*, 2005b). Another source of low F_{IS} may be the increased outcrossing following active pollen flow to populations in the HP area from adjacent populations, which resulted from forest thinning, as discussed earlier. Moreover, in previous experiments, the fitness of *L. flos-cuculi* offspring sired by multiple pollen donors was higher than of offspring sired by one pollen donor, and this effect was more pronounced in less heterozygous populations (Vergnerie, 2006).

Despite the fact that the methods used for parentage inference and estimation of mating systems using genetic marker data have become more precise in recent years (Ritland, 2002; David *et al.*, 2007; Sefc & Koblmuller, 2009; Wang *et al.*, 2012; Whitehead *et al.*, 2015), the disparate estimates of *S* obtained in this work may be subject to bias. For example, Rmes apparently tended to overestimate *S* in all populations, while Colony (PLS) did so in populations with a higher genetic variation. However, *S* estimated for the UP, MP areas and HP population 1 manually or with Mltr and Colony (FPLS) agreed quite well (0–0.23). These low values of *S* correspond to that suggested by Witt (2003) and Jürgens *et al.* (2002), and conform with the low F_{IS} reported for the species in this work and work by Aavik *et al.* (2012).

The most pronounced discrepancy between the methods used was found for populations with low numbers of alleles, in particular, in HP populations 2 and 3 characterized by many genetically identical parental plants, that is 8–10 genotypes per 15 sampled individuals. In these populations, *S* estimated manually and with Mltr comprised 0.35–0.60 and 0.19–0.44, respectively, while Colony (both PLS and FPLS) inferred *S* of 0–0.07 through overestimation of the minimum number of sires required to explain a progeny array. This sort of bias had already been documented for the latter approach earlier (Sefc & Koblmuller, 2009; Whitehead *et al.*, 2015). The obtained results show that even for markers with high levels of polymorphism (sufficient for the identification of genotypes), a high proportion of genetically identical individuals in a population suffering from a strong genetic bottleneck may bring uncertainty to the estimation of mating system parameters based on genetic data.

Finally, we failed to verify our second hypothesis on the full set of populations. However, *S* estimates for HP population 1 obtained with different approaches are congruent and similar to those in populations of the UP and MP areas (Fig. 5). This conclusion corresponds to the results obtained for *T. caerulescens* (see Introduction) (Dubois *et al.*, 2003) and contradicts the hypothesis that selection for more self-fertile individuals in polluted habitats may promote allele fixation and isolation from nontolerant relatives (Antonovics *et al.*, 1971). Moreover, the frequencies of pollinator visits to *L. flos-cuculi* flowers in polluted areas exceed the UP level, and other herbaceous species (*Geranium sylvaticum*, *Polygonum bistorta*, *Galium boreale*, *Potentilla erecta*, *Alhemilla* sp., *Geum rivale*, *Ranunculus* spp., *Stellaria* spp., *Myosotis arvensis*, *Bupleurum longifolium*) are abundant in the UP and MP areas (Dulya *et al.*, 2013), where they bloom simultaneously with *L. flos-cuculi*. Therefore, interspecific competition for pollinators in these areas should theoretically be higher than that in the HP area, where *L. flos-cuculi* is the sole pollinator attractor. Although visiting pollinators were counted during a short period relative to the flowering time and may differ between years, our findings correspond well to the results of entomological surveys in the studied sites performed in 1989 and 2006–2008. According to them, the abundance of *L. flos-cuculi* potential pollinators does not decrease (or even increase for some taxa) in the pollution gradient (Table S11).

Significant FPE was registered only for the data obtained in Colony, and is in agreement with the concept that protandry combined with basipetal blooming may result in a more pronounced deficiency of outcross pollen for early-opening than for later-opening flowers (Brunet & Charlesworth, 1995). These results confirm the suggestions of high levels of geitonogamous and cross pollinations in the species (Galeuchet *et al.*, 2005a). Nevertheless, we found no difference between FPE in populations with a different selfing rate or pollinator abundance,

supposed in our third hypothesis. Possibly, the expected positive relation between FPE and the selfing rate in a population could have been revealed by taking into account the difference in the selfing rate, not only between flowers on different nodal branches (as in this study), but also between flowers of different orders on branches of the same node, since their sequential opening is even more extended in time (Biere *et al.*, 1989). However, such an analysis would require larger samples and a more detailed sampling scheme. Moreover, capsule collection in the field is labour-intensive due to the high density of the herbaceous layer in the UP and MP areas (Fig. S2) and extensive damage of capsules by the abundant phytophagous insects in the HP area (Nesterkov & Vorobeichik, 2009).

In conclusion, the results present a direct *in situ* estimation of *S* for *L. flos-cuculi* in populations with high genetic variation from undisturbed and moderately altered habitats. However, considering the relatively high discrepancy between the estimates of *S* obtained with the different approaches for populations with low genetic variation, the employment of different methods appears to be necessary to prevent mistaken conclusions, though not a guarantee of their reliability.

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Author contributions

O.V.D. planned and designed the research. O.V.D. and V.S.M. conducted fieldwork, analysed data, and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Scheme of the seed collection from *L. flos-cuculi* inflorescence.

Fig. S2 Photographs of the studied *L. flos-cuculi* habitats.

Fig. S3 Flowchart of the simulation algorithm used to assess the accuracy of the selfing inference.

Fig. S4 Boxplot of the true positive and false positive rate in COLONY simulation; accuracy of the selfing inference.

Fig. S5 *L. flos-cuculi* pollinators spectra in the study areas.

Fig. S6 Difference in spectra of pollinators visiting *L. flos-cuculi* in different areas and populations.

Fig. S7 Probability of identity for increasing locus combinations.

Table S1 Pairwise distances between *L. flos-cuculi* populations within pollution zones

Table S2 Spatial distribution of genotyped parental plants within *L. flos-cuculi* populations

Table S3 Primer sequences and fluorescent labelling information for genetic analysis of *L. flos-cuculi*

Table S4 PCR conditions for genetic analysis of *L. flos-cuculi*

Table S5 Estimated null allele frequencies at each locus in *L. flos-cuculi* populations

Table S6 Structure of data used for analysis with COLONY

Table S7 Parameters specified for the COLONY analysis

Table S8 Example of the mating matrix that was generated according to the data structure of HP population 1

Table S9 Set of the tested models that were used to estimate the relationship of selfing rate with flower position in the inflorescence and pollution level

Table S10 The results of pairwise comparisons between diversity of *L. flos-cuculi* pollinators in different populations

Table S11 Abundance of invertebrates (ind. m^{-2}) in the herb layer of the *L. flos-cuculi* habitats

Table S12 Genetic diversity statistics for *L. flos-cuculi* microsatellite loci

Table S13 Difference between F_{IS} (ΔF_{IS}) values in parental generation and progeny

Table S14 Summary of Mantel tests between Queller-Goodnight relatedness coefficient and log-transformed geographic distances in each population

Table S15 Pairwise F_{ST} values between *L. flos-cuculi* populations in parental generation and in offspring

Table S16 Difference between pairwise F_{ST} estimated in parental generation and in offspring

Table S17 Pearson's correlation between the estimates of selfing rate produced by different methods

Table S18 Results of Tukey's multiple comparison test for the difference of selfing rate between study areas

Table S19 The results of pairwise comparisons in the parameters of within–population variation between *L. flos-cuculi* populations in the parental generation and offspring

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