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Genetic structure among and within peripheral and central populations of three endangered floodplain violets

R. L. ECKSTEIN,* R. A. O'NEILL,† J. DANIHELKA,‡§ A. OTTE* and W. KÖHLER†

Department of Landscape Ecology and Resource Management, Interdisciplinary Research Centre, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, DE-35392 Giessen, Germany, †Institute for Agronomy and Plant Breeding, Division of Biometry and Population Genetics, Interdisciplinary Research Centre, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, DE-35392 Giessen, Germany, ‡Institute of Botany, Academy of Sciences of the Czech Republic, Department of Ecology Brno, Poříčí 3b, CZ-603 00 Brno, Czech Republic, §Institute of Botany and Zoology, Faculty of Science, Masaryk University, CZ-611 37 Brno, Czech Republic

Abstract

Understanding the partitioning of genetic variance in peripheral and central populations may shed more light on the effects of genetic drift and gene flow on population genetic structure and, thereby, improve attempts to conserve genetic diversity. We analysed genetic structure of peripheral and central populations of three insect-pollinated violets (*Viola elatior*, *Viola pumila*, *Viola stagnina*) to evaluate to what extent these patterns can be explained by gene flow and genetic drift. Amplified fragment length polymorphism was used to analyse 930 individuals of 50 populations. Consistent with theoretical predictions, peripheral populations were smaller and more isolated, differentiation was stronger, and genetic diversity and gene flow lower in peripheral populations of *V. pumila* and *V. stagnina*. In *V. elatior*, probably historic fragmentation effects linked to its specific habitat type were superimposed on the plant geographic (peripheral-central) patterns, resulting in lower relative importance of gene flow in central populations. Genetic variation between regions (3–6%), among (30–37%) and within populations (60–64%) was significant. Peripheral populations lacked markers that were rare and localized in central populations. Loss of widespread markers in peripheral *V. stagnina* populations indicated genetic erosion. Autocorrelation within populations was statistically significant up to a distance of 10–20 m. Higher average genetic similarity in peripheral populations than in central ones indicated higher local gene flow, probably owing to management practices. Peripheral populations contributed significantly to genetic variation and contained unique markers, which made them valuable for the conservation of genetic diversity.

Keywords: AFLP, conservation, genetic diversity, marginal populations, spatial genetic structure, *Viola*

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Introduction

The abundance and density of individuals and population frequency are not constant across a species range but usually decrease towards the range margin (Lawton 1993; Lesica & Allendorf 1995; '*abundant centre hypothesis*', cf. Sagarin & Gaines 2002). Range margin populations can be geographically or ecologically peripheral (Lesica & Allendorf 1995). In many cases the ecological conditions in peripheral populations will be different from those in central populations. Although the study of species ranges and the analysis of causes for distribution limits at the range margin have traditionally been a topic of plant geography, patterns and ecological processes at the range margin have also received attention from plant ecologists (e.g. Carter & Prince 1981; Nantel & Gagnon 1999; Kluth & Bruelheide 2005), conservationists and plant geneticists (e.g. Lesica & Allendorf 1995 and references therein; Durka 1999; Lammi *et al*. 1999; Van Rossum *et al*. 2003).

Low habitat quality at the range margin may affect species performance and reduce reproduction and dispersal (Pigott & Huntley 1981; García *et al*. 2000; Dorken & Eckert 2001). Additionally, the habitat type or safe-sites for

Correspondence: Lutz Eckstein. Fax: +49 641 99 37 169; E-mail: lutz.eckstein@agrar.uni-giessen.de

germination may be infrequent at the range margin (Dinsdale *et al*. 2000; Jump & Woodward 2003). Therefore, peripheral plant populations will often be (i) more isolated (Lawton 1993; Lesica & Allendorf 1995) and (ii) contain less individuals than central populations (Durka 1999; Lammi *et al*. 1999; but see Kluth & Bruelheide 2005). Small populations face an increased risk of extinction through environmental stochasticity or catastrophes (Lande 1993; Menges & Dolan 1998). The viability of these populations may also be reduced because of the increased chance of mating between relatives (Menges 1991; Fischer & Matthies 1998). Additionally, small isolated populations may suffer from pollinator limitation (Jennertsen 1988; Ågren 1996). Low habitat quality and/or small population size at the range margin lead to increased variability of demographic rates (Nantel & Gagnon 1999). This may lead to higher rates of extinction, while larger distances between suitable habitats and source populations reduce the rate of recolonization of empty habitats, lowering the overall proportion of occupied patches.

A higher degree of isolation at the range margin will have similar consequences as fragmentation (Young *et al*. 1996). Habitat destruction and land-use changes, which result in population isolation through fragmentation, may severely influence gene flow at the landscape scale (Manel *et al*. 2003). These anthropogenic effects also occur in the core of a species' distribution and may hence be superimposed on the plant geographic (core-periphery) patterns.

The expected population genetic consequences of small population size and isolation are (i) reduced genetic diversity of peripheral populations due to founder effects, bottlenecks, inbreeding, genetic drift, or directional selection and (ii) increased differentiation among populations through reduced rates of gene flow (Lesica & Allendorf 1995; Durka 1999; Hutchison & Templeton 1999; Lammi *et al*. 1999). Even under similar selection in central and peripheral populations, isolation will increase genetic divergence at the range margin (Cohan 1984). Consequently, geographically peripheral populations may differ considerably from core populations and hence contribute significantly to geographic variation (Durka 1999). Isolation and directional selection in peripheral populations, which support genetic divergence, may promote speciation at the boundaries of the species range (Lesica & Allendorf 1995). Peripheral populations may be especially important for the conservation of genetic variation *per se* (Lesica & Allendorf 1995; Van Rossum *et al*. 2003) and for conservation in the light of global change since they may contain genotypes evolved under variable, extreme, and/or suboptimal conditions (Safriel *et al*. 1994).

If differences in the rates of mutation and selection between core and peripheral populations can be ignored, the *relative* role of genetic drift and gene flow for shaping the regional population structure can be analysed through the relationships between genetic (F_{ST}) and geographic distances. This approach of Hutchison & Templeton (1999) is based on a stepping-stone model of population structure, i.e. a model in which gene flow is most likely between neighbouring populations. A pattern consistent with equilibrium between gene flow and drift, i.e. isolation by distance (Wright 1931), should be characterized by a positive monotonic relationship between genetic and geographic distance (cf. Fig. 1a, case I). Because of the homogenizing effect of gene flow, populations at closer distances should not only be separated by smaller genetic distance but also the variation in genetic distance should be lower. As geographic distances increase the relative effect of gene flow decreases and widely separated populations are both genetically more distant and genetic distance shows larger variation due to genetic stochasticity. The assumption of

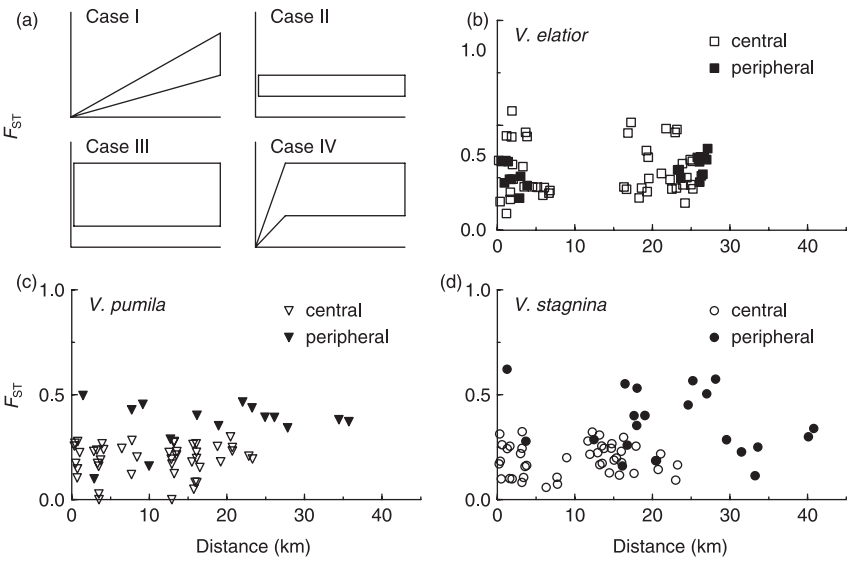

Fig. 1 Theoretical (a) and empirical relationships between genetic differentiation among pairs of populations (F_{ST}) and geographic distance (km) in *Viola elatior* (b), *Viola pumila* (c), and *Viola stagnina* (d). Panel (a) according to Hutchison & Templeton (1999), modified. Case I: equilibrium between genetic drift and gene flow; case II: nonequilibrium, gene flow relatively more important than genetic drift; case III: nonequilibrium, gene flow relatively less important than genetic drift; and case IV: lack of regional equilibrium, gene flow more important at shorter distances and drift more important at greater distances.

equilibrium conditions implicit in the isolation-by-distance model is often not met in natural populations. This may be because the populations and/or the required conditions have not been present long enough to achieve equilibrium patterns of isolation by distance (McCauley 1993). The expected patterns under nonequilibrium conditions are affected by (i) the time a region has been occupied (historical effects) and (ii) the degree to which regional dispersal is limited through fragmentation (contemporary effects). If, for example, a region has been colonized after the last glaciation from relatively homogeneous source populations in glacial refugia, the established populations will be genetically relatively similar and genetic and geographic distance will not be correlated (cf. Fig. 1a, case II). If gene flow remains relatively strong in comparison with random genetic drift this patterns will persist. Therefore, under nonequilibrium conditions small variation in F_{ST} indicates higher *relative* importance of gene flow over genetic drift (Hutchison & Templeton 1999). However, if environmental conditions lead to fragmentation and isolation of populations across the whole region genetic drift will become more influential and variation in F_{ST} will increase (Fig. 1a, case III).

Empirical data on the differential influence of genetic drift and gene flow on regional genetic structure in core and range margin populations are strongly biased towards wind-pollinated woody plants, mainly coniferous trees (Lesica & Allendorf 1995; Gapare *et al*. 2005, and references in these papers). However, relatively little is known about insect-pollinated perennial herbs (but see Dolan 1994; Durka 1999; Lammi *et al*. 1999; Van Rossum *et al*. 2003), which owing to their pollination and mating system — allowing gene flow only over relatively short distances — may suffer more from isolation and fragmentation than wind-pollinated plant species.

Viola elatior Fries, *Viola pumila* Chaix, and *Viola stagnina* Kit. (syn. *Viola persicifolia*) are very rare and endangered in Central Europe and red-listed in many European countries (Schnittler & Günther 1999). They have become rare and endangered through melioration and fragmentation of their habitats. Owing to their red-list status, populations of the study species have been the focus of intensive floristic inventories, monitoring programs and conservation biological studies in the two study regions (e.g. Hölzel 1999, 2003; Sumberová *et al*. 2000; Eckstein *et al*. 2004, 2006). Although a few populations may have been overlooked, the numbers and locations of all recently extant populations of the study species are exceptionally well documented. Populations in the floodplains of the lower Dyje River in the vicinity of Břeclav (Czech Republic) are within the main range of the species. Populations along the Upper Rhine south of Frankfurt (Germany) are situated at the periphery of the species ranges, separated from the main distribution area by about 600 km. Since the species occupy similar habitats as along the Dyje River, it is very likely that peripheral populations of the study species are geographically but not ecologically peripheral. These species belong to the section *Viola*, subsection *Rostratae* (Kirschner & Skalický 1990). They have continental distribution ranges with centres of occurrence in the temperate zone of Eastern Europe and Western Siberia (Hultén & Fries 1986). In Central Europe, the species reach their western range margin and show strong affinity to the valleys of large lowland rivers (Burkart 2001). These violets are iteroparous hemicryptophytes with a complex life cycle, a mixed mating system with chasmogamous and cleistogamous flowers ('true' cleistogamous species, Plitmann 1995) and a persistent seed bank (Hölzel & Otte 2004). *V. pumila* and *V. stagnina* occur mainly in species-rich, regularly managed floodplain meadows and wet grasslands, whereas *V. elatior* is typical of alluvial woodland fringes and other ecotonal habitats bordering floodplain meadows. Details on the taxonomy, habitat requirements, distribution, and population biology of the study species are given in Hölzel (2003) and Eckstein *et al*. (2004, 2006).

The main aims of our research were to analyse the local and regional genetic structure of these congeneric endangered insect-pollinated grassland herbs in order to evaluate the relative importance of genetic drift and gene flow in central and peripheral populations.

We tested the following predictions of the abundant centre hypothesis (Lawton 1993; Sagarin & Gaines 2002):

1 Peripheral populations of these species are smaller and more isolated than central ones.
2 Owing to larger effects of random genetic drift in peripheral populations these are characterized by lower genetic diversity and stronger genetic divergence among populations.

We further asked whether there is spatial genetic structure in central and peripheral populations at the local scale, and whether peripheral populations contain unique genetic markers not present in core populations, which would make them valuable for conservation from a population genetic point of view.

Materials and methods

Study regions

The study was carried out in two regions that represent two strongholds of the study species in Central Europe (Hölzel 2003). The Upper Rhine region is densely populated and the landscape fragmented through settlements, roads, and large areas of intensive farming. Similarly, also in the Dyje region northwest of Břeclav, intensive crop fields prevail outside the floodplain, whereas directly in the floodplain,

forests and relatively un-intensively managed meadows have been preserved. The region south of Bøeclav is only very sparsely populated, and alluvial forests, surrounding large and middle-size patches of floodplain meadows, dominate the landscape (Grulich *et al*. 2000).

Field sampling and data collection

Within each region we selected 7 (Germany) to 10 (Czech Republic) populations that occurred in the characteristic vegetation types and covered the main regional distribution of the species. A population consisted of those conspecific individuals that occurred within the same grassland allotment as the basic units within the cultural landscape receiving identical land-use management. Populations were separated by at least 150 m. *Viola pumila* and *Viola stagnina* occur mostly as patches of scattered individuals within the floodplain meadows. Although their populations may consist of hundreds or thousands of individuals, their spatial extent within a habitat is restricted. This is also true for *Viola elatior*, which often grows in linear habitats (Eckstein *et al*. 2006). Since populations of the species (except for *V. stagnina* in Germany) were aggregated in two areas, we adopted a stratified sampling design to assure the inclusion of populations from both areas (Appendix); because of technical problems one German population of *V. pumila* had to be omitted. We collected samples from about 37% (*V. elatior*, *V. stagnina*) and 17% (*V. pumila*) of all known extant populations in both study regions.

From digital maps, we obtained the geographic coordinates of all extant populations to calculate (for each population) the distance to the nearest conspecific population as a measure of isolation. This measure of isolation has two advantages over, e.g. the average pairwise distance. First, it nicely matches the stepping-stone model of Hutchison & Templeton (1999), where gene flow is most likely between neighbouring populations and therefore distance to the nearest population is more important than the average distance of all pairs of populations. Second, we obtained an independent value for each population, which makes this measure more appropriate for univariate inferential statistics (e.g. analysis of variance) than pairwise distances.

Additionally, we estimated population size (number of plants except seedlings) of all sampled populations on a logarithmic scale, i.e. 1–100 (= 1), 101–1000 (= 2), and 1001–10 000 (= 3) individuals by walking line transects across populations.

Within each population a line transect was laid out, along which a maximum of 20 plants were randomly selected and their coordinates (x: distance along the transect, and y: distance perpendicular to the line, distance between two sampled plants: > 0.1 m) recorded. In populations with < 20 individuals, tissue samples of all individuals were taken. The youngest shoot tip of each individual was sampled, stored in a paper bag, and dried at room temperature. Samples were brought to the laboratory for DNA extractions as fast as possible. During DNA extraction and further processing a few samples were lost, but in total we analysed 930 individuals from 50 populations (Appendix).

DNA extraction and AFLP analyses

Protocols for DNA extraction and amplified fragment length polymorphism (AFLP) analyses followed those described in detail by O'Neil (2005) for *Viola arvensis*. Briefly, DNA was extracted according to Doyle & Doyle (1987). Dried leaf material frozen with liquid nitrogen was crushed before being transferred into cetyltrimethyl ammonium bromide (CTAB) extraction buffer and incubated at 65 °C for 30 min. After two washes with a chloroform : isoamylalcohol mix (24:1) and centrifugation, sodium acetate and ammonium acetate were added to the supernatant. Isopropyl alcohol was added to precipitate the DNA, and after further centrifugation, the remaining pellet was washed with 70% ethanol containing ammonium acetate. After drying, the pellet was resuspended in 100 µL TE buffer with added RNase.

AFLP analysis was performed essentially as described by Vos *et al*. (1995), using AFLP Core Reagent Kits (Gibco Life Technologies). The DNA of each sample was digested with the restriction enzymes *Mse*I and *Eco*RI in a volume of 25 µL containing reaction buffer at 37 °C for 2 h, followed by a final step of 70 °C for 15 min. Both the +1 and +3 selective amplification began with a 3-min 94 °C denaturation and ended with a 5-min 72 °C polymerization. For the +1 amplification, the denaturation (94 °C, 30 s), annealing (56 °C, 1 min), and polymerization (72 °C, 1 min) cycle was repeated 20 times. For the +3 amplification, two sets of cycles were carried out. The first consisted of 12 cycles of denaturation (94 °C, 30 s), annealing (65 °C, 30 s), and polymerization (72 °C, 1 min), with the annealing temperature decreasing by –0.7 °C per cycle. Following these cycles, the polymerase chain reaction (PCR) process continued with a denaturation (94 °C, 30 s), annealing (56 °C, 30 s), and polymerization (72 °C, 1 min) cycle repeated 23 times.

The following four +3-primer combinations were used for all species: (i) *Eco*RI-ACT/*Mse*I-ACG, (ii) *Eco*RI-ACT/*Mse*I-ACT, (iii) *Eco*RI-ATC/*Mse*I-AGG, and (iv) *Eco*RI-ATC/*Mse*I-ATT.

Gel electrophoresis

The amplification products from AFLP analyses were visualized through the use of a 0.2 mm thick, 25 cm long polyacrylamide gel (based on an 8% Long Ranger Gel Solution) in a LI-COR Gene Readir 4200 DNA sequencer (LI-COR). All products were mixed with a STOP loading buffer at a 1:1 ratio before being denatured at 94 °C for

3 min. Reverse primers of +3 *Eco*RI primers for AFLP analysis were fluorescently labelled with IRD800.

With a laser emitting a wavelength of 800 nm, this dye is excited and fluoresces, allowing the DNA to be detected. A 1× TBE buffer was used for running the gels. Standard parameters were used for the separation of fragments: 1500 V, 50 W, 35 mA, 48 °C. The size (in base pairs) of bands appearing on the gel were determined by comparison to a 50–350 bp molecular size standard (LI-COR) run on both edges of the gel. AFLP products were scored visually as the presence (1) or absence (0) of unambiguous AFLP bands. All samples were scored by the same person.

Calculations and data analyses

The effects of species, region, and their interactions on the population size and on isolation (i.e. distance to the nearest conspecific population) were tested in a two-way fixed effect permutation analysis of variance (Quinn & Keough 2002).

We used two estimates of genetic diversity. One was based on allele frequencies, i.e. an estimator of expected heterozygosity (H_E) according to Lynch & Milligan (1994, *gene diversity*). The other was based on the number of pairwise differences in banding patterns within populations divided by $n - 1$ (where n is the number of samples, cf. Fischer & Matthies 1998; *molecular variance*). Gene diversity was calculated after the estimation of allelic frequencies using a Bayesian method with nonuniform prior distribution of allele frequencies (Zhivotovsky 1999) implemented in the program AFLP-SURV (Vekemans *et al*. 2002). This method gives accurate unbiased estimates of null allele frequencies in dominant marker systems (Zhivotovsky 1999; Kraus 2000). To account for the mixed mating system of the study species (Eckstein & Otte 2005) and because selfing rates in violets may vary dramatically between years (Culley 2002), we assumed a selfing rate of 0.5, i.e. an F_{IS} of 0.33, in all calculations. However, assuming either Hardy–Weinberg equilibrium for the populations (i.e. using $F_{IS} = 0$ in the calculations) or highly selfing populations ($F_{IS} = 0.9$) had very little effect on the results (a difference of 6% and 13%, respectively, as compared with $F_{IS} = 0.33$) and did not change the general conclusions.

First, a three-level hierarchical analysis of molecular variance (AMOVA) was calculated with the program ARLEQUIN version 3.0 (Excoffier *et al*. 2005). We analysed the following levels for each species-region combination: (i) region, (ii) populations within region, and (iii) individuals within populations within regions. Additionally, a two-level AMOVA was calculated for each species-region combination to analyse the partitioning of molecular variance among and within populations.

Since AFLP markers are dominant, of the two 'phenotypic' states of a polymorphic marker (0/1) only one produces a band on the gel. We classified the visible bands of the AFLP markers (i.e. only the dominant state) according to their occurrence in populations as widespread (occurring in ≥ 25% of the populations) or localized (< 25%), and according to their average frequency across populations as common (average frequency ≥ 0.05) or rare (< 0.05). This approach is similar to the two-way classification of alleles developed by Marshall & Brown (1975). By restricting the analysis to only one state the estimated occurrences and frequencies produced unbiased independent data. We obtained contingency tables for core and peripheral regions of each species that could be compared using a χ^2 test.

We calculated a Mantel test (Legendre & Legendre 1998) for each species-region combination, using F_{ST} values obtained from AFLP-SURV to test for significant correlation between pairwise genetic and geographic distances. Monotonic increasing genetic distance with geographic distance would indicate case I (Fig. 1a), i.e. an equilibrium between genetic drift and gene flow. For each species and region, we analysed the scattergram of F_{ST} vs. geographic distance to infer the relative influences of gene flow and drift on the distribution of genetic variability following Hutchison & Templeton (1999). We explicitly tested, whether average pairwise F_{ST} was higher in peripheral than in central populations using two-sample Monte Carlo tests (Manly 2001). Additionally, we resampled the variance ratio in F_{ST} between peripheral and central populations to test whether the scatter in F_{ST} values was significantly higher in peripheral populations.

To test for the presence of small-scale genetic structure within populations, we used geographic distances and Sørensen similarity index, which is similar to the Dice index (Legendre & Legendre 1998), based on the presence/absence of AFLP markers among pairs of individuals within populations. For each species and region we constructed a matrix **S** that contained the pairwise genetic similarity between all individuals in all populations and a matrix **D** with the respective geographic distances. We computed multivariate Mantel correlograms (Legendre & Legendre 1998: 736ff) by coding in **D** all distances of a certain predefined distance class by 1 and all other distances (of all within- and among-populations pairs) by 0 to obtain the model matrix $\mathbf{X_1}$. Different model matrices $\mathbf{X_d}$ were prepared for all distance classes **d**. Distance classes were chosen to comprise roughly similar numbers of pairs. Then a Mantel test was calculated between **S** and each of the model matrices using the normalized Mantel statistic (r_M). This approach is the multivariate pendant to autocorrelation analysis on univariate quantitative data using, e.g. Moran's *I* (Legendre & Legendre 1998). Significant deviation of r_M from zero was tested for each distance class by a permutation approach. We applied progressive Bonferroni correction (Legendre & Legendre 1998) to account for multiple testing. Mantel tests were calculated with the program PC-ORD 4 (McCune & Mefford 1999) using 9999 permutations.

Table 1 Results of a permutation analysis of variance on the effects of species and the location of populations with respect to the species range (peripheral vs. central) on the size of the populations sampled for the genetic study and on the isolation (distance to the nearest conspecific population) of all known extant populations. Abbreviations: d.f., degrees of freedom; MS, mean square; *P*, proportion of permutations, where the randomized *F* value was larger than the original *F* value (number of permutations: 10 000)

	Population size			Isolation		
Source of variation	d.f.	MS	*P*	d.f.	MS	*P*
Species (S)	2	3.61	0.0001	2	58.31	0.0001
Location (L)	1	2.34	0.0094	1	72.27	0.0001
S * L	2	0.21	0.5040	2	70.75	0.0001
Model	5	2.15	0.0001	5	43.83	0.0001
Error	46	0.30		177	1.18	
Total	51	0.48		182	2.35	

Table 2 Average distance to the nearest conspecific population for *Viola elatior*, *Viola pumila*, and *Viola stagnina* located at the periphery (Upper Rhine, Germany) or at the centre (Dyje, Czech Republic) of their ranges

Species	Location	Mean (km)	*n*	*P*
Viola elatior	central	1.07 ± 0.22	26	0.0077
	peripheral	0.42 ± 0.07	19	
Viola pumila	central	0.52 ± 0.06	57	0.0092
	peripheral	0.97 ± 0.21	35	
Viola stagnina	central	0.57 ± 0.10	34	0.0001
	peripheral	5.01 ± 0.88	12	

Data are means ± SE, *n* is the number of known extant populations in the study areas and *P* gives the proportion of permutations, where the randomized *F* value was larger than the original *F* value (one-way permutation GLM ANOVA, number of permutations: 10 000).

Results

Consistent with our expectation peripheral populations were significantly smaller than central populations, and this pattern did not differ among species (Table 1). Across species, peripheral populations were significantly more isolated than central ones. The largest average distance to the nearest population was found in peripheral populations of *Viola stagnina* (Table 2). In contrast to our working hypothesis, peripheral populations of *Viola elatior* were significantly less isolated than core populations (significant species–region interaction).

AFLP analyses produced 160 scorable bands, 75% of which were polymorphic. We recorded a total of 90, 155, and 162 AFLP phenotypes in *V. elatior*, *V. pumila*, and *V. stagnina*, respectively. For further analyses, we prepared three data sets, one for each species, based on the AFLP markers present in each of the three species (*V. elatior*: 152 in total/97 polymorphic markers; *V. pumila*: 155/108; *V. stagnina*: 156/113). Percentage polymorphism across regions ranged from 63% to 72%. There was no indication of size homoplasy in the data sets because no significant negative correlation between fragment size and frequency was found (Vekemans *et al*. 2002). The numbers of markers and percentage of polymorphic markers per population were significantly higher in central populations of *V. pumila* and *V. stagnina* but did not differ in *V. elatior* (Table 3). Similarly, gene diversity and molecular variance, both measures of genetic diversity, were higher in central than in peripheral populations in *V. pumila* and *V. stagnina* (Table 3). There was no significant difference in *V. elatior*.

Three-level analysis of molecular variance revealed that all variance components were significant and that partitioning of molecular variance was similar in the three study species (data not shown). Three to 6% of the variation was found between regions, 30–37% among populations within regions and 60–64% among individuals within populations. Two-level analyses for each species-region

Table 3 Gene diversity, molecular variance, number of markers (# markers) and percentage polymorphic markers within central (Dyje floodplains, Czech Republic) and peripheral (Upper Rhine, Germany) populations of the three studied violets. For estimation of gene diversity a selfing rate (*s*) of 0.5*, i.e. a F_{IS} of 0.33, was assumed (see Methods). *P* values are from a permutation *t*-test (10 000 permutations). Data are mean ± SE

Species	Location	*n*	Gene diversity*	*P*	Molecular variance	*P*	# markers	*P*	Polymorphic markers (%)	*P*
Viola elatior	central	10	0.1146 ± 0.0066	0.4189	5.03 ± 0.69	0.7939	122.9 ± 2.0	0.3657	19.6 ± 2.8	0.3936
	peripheral	7	0.1121 ± 0.0141		5.35 ± 1.02		126.1 ± 2.9		20.8 ± 3.0	
Viola pumila	central	10	0.1735 ± 0.0059	0.0002	10.59 ± 0.56	0.0001	129.0 ± 0.8	0.0185	42.2 ± 1.4	0.0001
	peripheral	6	0.1164 ± 0.0072		6.20 ± 0.56		124.3 ± 2.0		25.9 ± 1.2	
Viola stagnina	central	10	0.1364 ± 0.0070	0.0276	8.06 ± 0.54	0.0160	113.2 ± 1.4	0.0260	40.5 ± 2.8	0.0151
	peripheral	7	0.1129 ± 0.0083		6.11 ± 0.56		108.9 ± 1.1		30.5 ± 3.0	

*When assuming either Hardy–Weinberg equilibrium ($s = 0.0$) or highly selfing populations ($s = 0.95$) average gene diversity differed by 6% and 13%, respectively, as compared with $s = 0.5$. The outcome of the comparison between regions was not affected by the assumptions.

Table 4 Percentage of four classes of AFLP markers and number of unique markers in *Viola elatior*, *Viola pumila*, and *Viola stagnina* from the central (Dyje-floodplains, Czech Republic) and peripheral populations (Upper Rhine, Germany). *P* values are from a χ^2 test for differences between regions (d.f. = 3)

Species	# markers	Location	Classes of markers				*P*	# unique markers
			% cw	% rw	% cl	% rl		
		central	85.53	0.66	5.92	7.89	0.0178	1
Viola elatior	152	peripheral	86.84	5.26	1.32	6.58		6
		central	87.10	2.58	0.00	10.32	0.3533	2
Viola pumila	155	peripheral	85.81	3.23	1.94	9.03		1
		central	75.64	4.49	0.64	19.23	0.0006	12
Viola stagnina	156	peripheral	73.72	0.00	8.33	17.95		4

Classes were based on occurrence (marker found in ≥ 25% of populations per region: widespread, **w**; < 25%: localized, **l**) and frequency of markers (average frequency ≥ 0.05: common, **c**; < 0.05: rare, **r**). Classes: common widespread, **cw**; rare widespread, **rw**; common localized, **cl**; rare localized, **rl**.

Table 5 Bootstrapped average F_{ST} for each species region combination, ratio of the variance in F_{ST} between central (Dyje floodplains, Czech republic) and peripheral populations (Upper Rhine, Germany). Bootstrapped 95% confidence limits in brackets

Species	Location	F_{ST} (average)	F_{ST} (variance ratio)
Viola elatior	central	0.2817 ns (0.2469–0.3185)	0.2229 ns† (0.1116–0.3987)
	peripheral	0.2849 (0.2599–0.3089)	
Viola pumila	central	0.1916*** (0.1683–0.2129)	2.0362* (0.3553–4.5117)
	peripheral	0.3637 (0.3064–0.4129)	
Viola stagnina	central	0.1924*** (0.1712–0.2134)	4.1957*** (2.2430–6.7428)
	peripheral	0.3635 (0.3014–0.4267)	

F_{ST} values were calculated with AFLP-SURV (Vekemans *et al*. 2002). Significantly higher average F_{ST} and significantly higher variance in peripheral than in central populations indicate a stronger *relative* influence of genetic drift vs. gene flow on genetic structure (case III, cf. Fig. 1a). Significance limits were obtained from Monte Carlo permutations, testing for the hypothesis that F_{ST} (peripheral populations) > F_{ST} (central populations), variance F_{ST} (peripheral populations) > variance F_{ST} (central populations). Bootstrap sample size was 10 000. Significance levels: NS, not significant ($P > 0.05$); *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).
†The variance ratio was significantly lower than the observed ratio, i.e. F_{ST} values show stronger scatter in central than in peripheral populations.

combination showed that partitioning of molecular variance between and within populations was similar in peripheral and core populations (37.0% vs. 39.1%, respectively) of *V. elatior*, while in *V. pumila* (48.8% vs. 24.3%) and *V. stagnina* (50.7% vs. 25.9%) divergence was higher in peripheral than in core populations.

The distributions of markers into different classes based on occurrence and frequency differed significantly between core and peripheral regions in *V. elatior* and *V. stagnina* (Table 4). Peripheral populations of *V. elatior* had fewer common but localized markers, while they contained more rare widespread ones than central populations. In *V. stagnina* these differences between regions were diametrically opposed. Similarly, more unique AFLP markers were present in peripheral populations of *V. elatior* than in core populations but the pattern was reversed in *V. stagnina*. In peripheral populations of *V. elatior*, *V. pumila*, and *V. stagnina* one, two, and eight rare localized markers were absent, respectively. Additionally, peripheral populations of *V. stagnina* lacked one rare widespread marker and three common widespread markers that occurred in ≥ 40% of the core populations and had an average frequency of ≥ 0.1120. Peripheral populations contained one unique common widespread marker in *V. elatior* (frequency 0.0563/occurrence 28.6%), one in *V. pumila* (0.0515/33.3%), and one common localized marker in *V. stagnina* (0.1612/16.7%).

Based on Mantel tests between genetic and geographic distances among pairs of populations we rejected the null hypothesis of regional equilibrium between gene flow and genetic drift for all species–region combinations. The scatterplots suggested that gene flow is relatively more important than genetic drift for the regional distribution of genetic variability in *V. elatior* from the Upper Rhine, *V. pumila* from Rhine and Dyje, and *V. stagnina* from the Dyje floodplains (Fig. 1). This interpretation was supported by a comparison of variance in F_{ST} among regions (Table 5). The variance ratio of resampled F_{ST} values between Rhine and Dyje was significantly lower than the observed variance

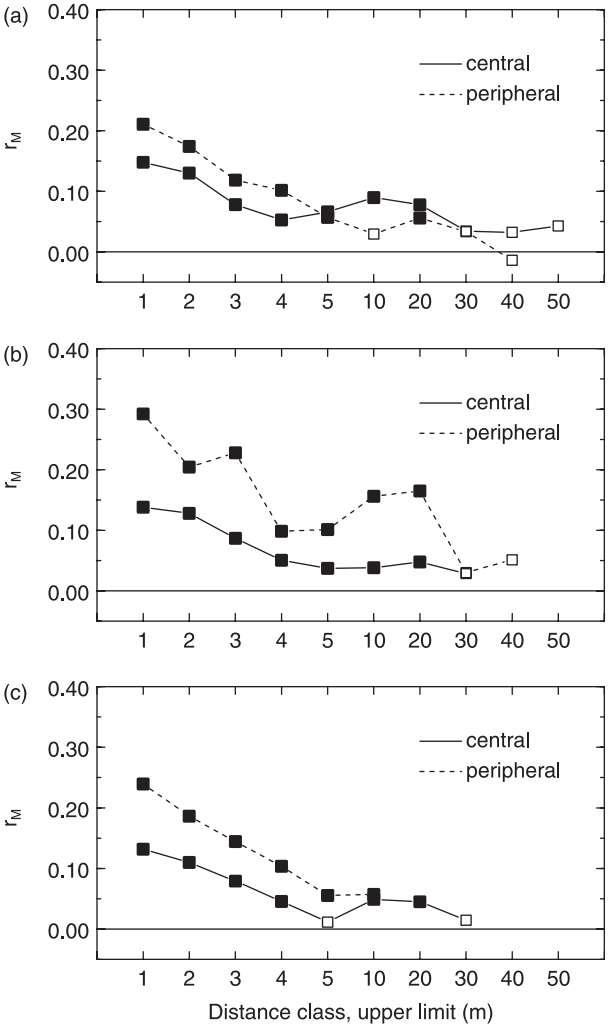

Fig. 2 Multivariate Mantel correlogram showing spatial autocorrelation between genetic and geographic distance classes within populations of *Viola elatior* (a), *Viola pumila* (b), and *Viola stagnina* (c). Filled symbols denote normalized Mantel statistics (r_M) that are significantly different from zero after progressive Bonferroni correction. Positive values of r_M show that individuals within a distance group are more genetically similar than pair of individuals at all other distances.

ratio for *V. elatior* (i.e. significantly stronger scatter of central populations) and significantly higher for *V. stagnina* and *V. pumila* (stronger scatter of peripheral populations). Average resampled F_{ST} values did not differ in *V. elatior*, but population differentiation was significantly larger in peripheral populations and gene flow higher in central populations of the other two species.

Within populations multivariate Mantel correlograms showed that there was significant spatial genetic structure up to about 10–20 m in populations of *V. elatior* and *V. pumila* from both Rhine and Dyje (Fig. 2), and in *V. stagnina* from the Dyje floodplains. In peripheral populations of the latter species, there was significant spatial genetic structure up to

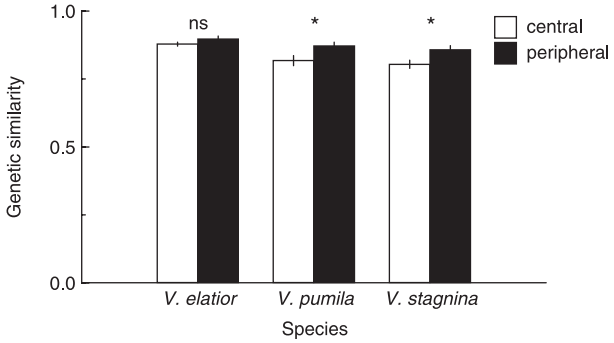

Fig. 3 Average genetic similarity (Sørensen index) of pairs of individuals within populations of *Viola elatior*, *Viola pumila* and *Viola stagnina* in central (Dyje floodplains, Czech Republic) and peripheral populations (Upper Rhine, Germany) for distance classes from 0 to 20 m (mean ± SE, $n = 7$ classes, cf. Fig. 2). Asterisks indicate significant difference between groups at $P < 0.05$ (permutation *t*-test, 10 000 permutations).

a distance of 10 m. Higher distance classes were only represented by very few individuals in the data set. For the first seven distance classes, where the Mantel correlograms revealed positive autocorrelation (i.e. 1–20 m), average genetic similarity was higher within peripheral populations than in core populations (Fig. 3). Differences between regions were significant in *V. pumila* and *V. stagnina*.

Discussion

As predicted by the theory of range margins (Lawton 1993; Sagarin & Gaines 2002) population size (numbers of individuals) was consistently and significantly smaller in peripheral populations of the study species. Larger central populations have also been found in *Lychnis viscaria* (Lammi *et al*. 1999) and *Corrigiola litoralis* (Durka 1999), but size differences between peripheral and central populations were not evident in *Silene nutans* from Western Europe (Van Rossum *et al*. 2003) and *Silene regia* along an east–west gradient in North America (Dolan 1994). Contrary, adult *density* was higher in peripheral than in central populations of *Hornungia petraea* (Kluth & Bruelheide 2005). The same holds true for total adult density in permanent plots of the study species within central (25 adults m^{-2}) and peripheral (40 adults m^{-2}) populations (Eckstein & Otte, unpublished data), although densities of single life-cycle stages (except seedlings) did not differ significantly between regions owing to large within-group variation (Eckstein *et al*. 2004).

Isolation was larger in peripheral than in central populations of *Viola pumila* and *Viola stagnina*, while the opposite relationships were found in *Viola elatior*. It seems improbable that this was caused by differences in the intensity of population inventories between regions, since the study species are targets of intensive floristic inventories and monitoring programs in both regions (Eckstein *et al*. 2006).

Unlike the other two species, habitats of *V. elatior* are prone to changes in species composition and environmental conditions in the course of forest succession. Analysis of herbarium collections suggests that there have been considerably more populations of this species present around the town of Lednice (CZ, northwest of Bøeclav) during the 1950s (J.D., unpublished). In response to habitat deterioration the species develops a 'remnant'-type of population dynamics (*sensu* Eriksson 1996), i.e. above-ground plant density slowly decreases as adult plants die until populations only persist as seeds in the soil. After major disturbance by logging, the populations recur through germination from the seed bank (Eckstein *et al*. 2006). The percentage cover of alluvial forests is much higher along the Dyje River than at the Upper Rhine, and in the former region, many populations occurred in late-successional habitats within forest stands or along forest tracks. Therefore, we suggest that regional differences in availability of suitable, early- or mid-successional habitats may be responsible for the stronger isolation of population in the core region. Fragmentation effects are hence superimposed on plant geographical patterns (core–periphery) in this species.

Peripheral populations of *V. elatior* and core populations of *V. pumila* and *V. stagnina* were situated within an average distance of about 0.5 km to the nearest conspecific population, while central populations of *V. elatior* and peripheral populations of *V. pumila* were separated by about 1 km from their nearest neighbour. This is well within the average flight distance of bees and bumblebees (Hymenoptera, Apidae) for pollen and nectar, which is about 2.5–5 km (Eckert 1933; Araújo *et al*. 2004). However, since most bees feed at a distance of about 1 km or less around their colony (Eckert 1933), this distance may represent a limit for regular gene flow through pollen (cf. Kwak *et al*. 1998 and references therein). This is supported by our analysis of the *relative* importance of gene flow and genetic drift. There was no equilibrium between these processes in the study species. The relationships between genetic and geographic distances suggested a stronger effect of gene flow for all species-region combinations with an average distance to the nearest conspecific population of about 0.5 km or less. The influence of drift was larger than that of gene flow for those species-region combinations separated by about 1 km and especially in peripheral populations of *V. stagnina* that were situated, on average, 5 km apart from the nearest population. Stronger genetic divergence among peripheral populations of *V. pumila* and *V. stagnina* (F_{ST} values) further supported this view. Similarly, Culley & Grubb (2003) found nonequilibrium conditions and a very similar scatter between genetic and geographic distance than in the present study, indicating that in fragmented populations of *Viola pubescens* (pairwise distances ranged from 0.3 to 45 km) genetic drift had much stronger influence than gene flow on genetic population structure.

Divergence among populations tended to be higher in peripheral than in central populations of *S. nutans* (Van Rossum *et al*. 2003). Similar differentiation between populations as in the present study has been reported from other cleistogamous (Auge *et al*. 2001; Culley & Grubb 2003), selfing (Durka 1999), or rare plant species (Travis *et al*. 1996; Lammi *et al*. 1999; Schmidt & Jensen 2000). Genetic divergence among populations was high in three rare species of the genera *Silene* and *Lychnis* (Dolan 1994; Lammi *et al*. 1999; Van Rossum *et al*. 2003), whereas gene flow was still high in the common *Lychnis flos-cuculi* (Galeuchet *et al*. 2005). In a study on eight populations of *V. elatior* from Austria, Germany, Italy, and Switzerland, 82% of the genetic variance rested among populations, while the remaining 18% were found within populations (Gygax 2001). The differences to the present study are most probably due to the much larger geographic range sampled in that study which results in a larger among population genetic differences.

Assuming constant ecological niches over time, the species have most probably been more widely distributed and more frequent at the end of the last glaciation when climatic conditions in Central Europe were more continental (Younger Dryas period, 12 000 years BP; Frenzel 1968; Burkart 2001). With the development of the current climate and the increase of human influence on the landscape, they retreated to floodplains, which provide regionally subcontinental conditions, flood disturbances, and only low to moderate human land use. The analysis of the past distribution suggests that all three species have undergone a severe decline during the last decades (Eckstein *et al*. 2006), which caused strong fragmentation and isolation of populations. Nonequilibrium metapopulations with few or no recolonizations provide ideal conditions for population divergence (Harrison & Hastings 1996), which is supported by the present data. The large proportion of genetic variance still present within populations may be a result of the perennial nature of the species and/or the presence of a persistent seed bank. The conservation of genotypes through time in a soil seed bank reduces genetic divergence among populations and may increase genetic diversity (Cabin *et al*. 1998; McCue & Holtsford 1998; Morris *et al*. 2002). Therefore, populations of perennials with a persistent seed bank may be a patchwork of genotypes from a spatial as well as from a temporal point of view.

Higher F_{ST} values and higher average similarity within peripheral populations indicated that gene flow at the local scale may be higher in peripheral than in central populations for two of the three species. This may be owing to differences in agricultural management between regions (Eckstein *et al*. 2004). Peripheral populations are situated exclusively in nature conservation areas or managed under conservation contracts, whereas populations in the

Czech Republic are found mainly in irregularly managed sites. Regular management improves stand and population stage structure of the species and increases densities of seedlings (Eckstein *et al*. 2004) and total adult plants, as well as fecundity in peripheral populations (Eckstein *et al*., unpublished). Higher densities of mature (flowering) plants are linked to shorter pollinator flight distances but higher percentage interplant flights in *Viola* (Beattie 1976). The latter enhances interplant gene exchange, and percent cross-pollination. Leptokurtic distributions of pollinator flight distances will restrict gene flow and enhance the divergence of subpopulations at a very localized scale (Beattie 1976). In contrast, in wind-pollinated Sitka spruce strong spatial genetic structure in peripheral but not core populations was probably caused by overlapping seed shadows owing to higher density of adults in core populations (Gapare & Aitken 2005).

Absence of rare localized genetic markers in peripheral populations demonstrates possible effects of genetic drift. Absence of some widespread markers in peripheral populations of the most isolated species (*V. stagnina*) may even indicate genetic erosion. However, the existence of *V. stagnina* var. *lactaeoides* in the Netherlands (Weeda 2001), although with uncertain taxonomic status, suggests that isolation and genetic divergence from the original taxon may promote speciation at the boundaries of the species range (Lesica & Allendorf 1995). Owing to postglacial range contraction and recent habitat fragmentation there is no equilibrium between gene flow and genetic drift in the study species. Despite this, the balance between gene flow and genetic drift still shapes genetic diversity. In metapopulations of these insect-pollinated plants with an average distance of < 1 km to the nearest neighbour, gene flow is sufficiently large to outweigh the effects of random genetic drift and retain relatively high levels of genetic similarity between populations. Larger average distances to the nearest population, as is often the case at the margin of species ranges, will reduce gene flow to levels that are no longer capable to counterbalance genetic drift. Consequently, random loss of alleles will lead to increasing genetic differentiation between populations and loss of regional genetic diversity. However, colonization history and responses to habitat fragmentation are species specific. We suggest that conservation of the floodplain violets should include peripheral populations since they contained a number markers lacking in central populations and thus contributed to genetic diversity at the species level (cf. Safriel *et al*. 1994; Lesica & Allendorf 1995; Durka 1999).

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Lutz Eckstein is interested in the ecology, genetics and conservation of plants within the research group of Annette Otte, whose work is concerned with biodiversity analysis and management in the cultural landscape from the molecular to the regional scale. Rob O'Neill has done his PhD on the genetic diversity of arable weeds. Wolfgang Köhler leads a research group that works on genetic diversity of crop plants and biodiversity modelling. Jiři Danihelka is lecturer and researcher, interested in the taxonomy, ecology, and genetics of violets.

Appendix

Geographic location of the study populations [northern latitude (N) and eastern longitude (E)] and number of sampled individuals (*n*) in the Czech Republic (Morava-Dyje floodplains) and in Germany (Upper Rhine)

	Czech Republic				Germany			
Species	ID	N	E	*n*	ID	N	E	*n*
Viola elatior	EC1	48°49′24″	16°46′27″	20	ED1	49°35′59″	08°25′44″	19
	EC2	48°48′43″	16°49′05″	19	ED2	49°35′54″	08°26′53″	16
	EC3	48°49′25″	16°46′36″	11	ED3	49°49′51″	08°24′18″	20
	EC4	48°49′16″	16°49′35″	8	ED4	49°50′03″	08°25′34″	20
	EC5	48°49′33″	16°48′06″	14	ED5	49°50′20″	08°24′09″	20
	EC6	48°37′58″	16°57′11″	20	ED6	49°48′31″	08°25′42″	20
	EC7	48°41′33″	16°56′50″	16	ED7	49°35′46″	08°25′59″	20
	EC8	48°41′04″	16°56′13″	20				
	EC9	48°37′55″	16°57′28″	20				
	EC10	48°38′49″	16°57′39″	20				
			Σ	**168**			**Σ**	**135**
Viola pumila	PC1	48°48′43″	16°49′05″	20	PD1	49°36′03″	08°27′01″	20
	PC2	48°48′43″	16°49′39″	20	PD2	49°36′41″	08°26′19″	20
	PC3	48°49′24″	16°46′27″	19	PD3	49°40′05″	08°22′37″	19
	PC4	48°49′08″	16°46′31″	20	PD4	49°48′33″	08°25′43″	20
	PC5	48°48′47″	16°49′20″	20	PD5	49°50′06″	08°25′36″	18
	PC6	48°43′01″	16°55′08″	20	PD6	49°55′05″	08°22′30″	18
	PC7	48°41′53″	16°57′14″	20				
	PC8	48°42′36″	16°54′36″	20				
	PC9	48°42′38″	16°55′13″	20				
	PC10	48°38′31″	16°55′57″	20				
			Σ	**199**			**Σ**	**115**
Viola stagnina	SC1	48°48′44″	16°49′39″	19	SD1	49°51′33″	08°23′29″	18
	SC2	48°49′26″	16°46′44″	18	SD2	50°01′16″	08°54′09″	18
	SC3	48°48′47″	16°49′20″	20	SD3	49°53′03″	08°49′43″	18
	SC4	48°49′20″	16°46′52″	20	SD4	49°59′28″	08°31′40″	20
	SC5	48°48′57″	16°49′16″	19	SD5	50°03′29″	08°44′16″	20
	SC6	48°43′09″	16°54′04″	20	SD6	49°59′05″	08°30′47″	15
	SC7	48°42′37″	16°55′08″	19	SD7	49°50′01″	08°25′27″	11
	SC8	48°42′36″	16°54′43″	20				
	SC9	48°38′30″	16°65′01″	20				
	SC10	48°41′54″	16°55′52″	18				
			Σ	**193**			**Σ**	**120**
			Total	**560**			**Total**	**370**