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WITHIN-POPULATION GENETIC STRUCTURE AND CLONAL DIVERSITY OF A THREATENED ENDEMIC METALLOPHYTE, *VIOLA CALAMINARIA* (VIOLACEAE)[1]

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We studied the within-population genetic structure and the clonality extent of *Viola calaminaria*, a rare endemic species of calamine soils, by means of RAPD markers in two populations (one recent and one ancient) with expected harsh and heterogeneous heavy-metal stress. At a very local scale (0.2–3 m), clonal propagation was detected in both populations, but the levels of clonal diversity were high (number of genets/number of ramets sampled = 0.9 [recent] and 0.76 [ancient]) and the maximal observed extension of the clones was 0.4 m. This indicated that clonality is not, for the species, an important mode of propagation and that clonal growth cannot be interpreted as a strategy for propagating or perpetuating adapted genotypes under harsh ecological constraints. Spatial autocorrelations revealed a significant ($P < 0.001$) negative value of correlogram slope in the two populations even when a single individual per clone was considered (i.e., analysis at the genet level). We conclude that spatial genetic structure at a very local scale reflects limited gene flow due to restricted seed dispersal rather than variation in clonal pattern in response to environmental heterogeneity. At a larger scale (2–30 m), spatial autocorrelations revealed a positive ($P < 0.001$) correlation at < 3 m and a random pattern at larger distances for the two populations. This suggested a patchy distribution of the genetically linked individuals associated with a disrupted pattern at a longer distance probably due to gene flow by pollen dispersal and a seed bank effect. The implications for the conservation of *V. calaminaria* are discussed.

Key words: genetic structure; heavy metals; RAPD; spatial autocorrelation; *Viola calaminaria*.

Studies of within-population genetic structure are essential to the understanding of microevolutionary processes in plant populations. Spatial genetic structure within plant populations is influenced by various factors such as gene flow, clonal pattern, and microenvironmental selection (Wright, 1943; Heywood, 1991; Epperson, 1993; Kang and Chung, 2000; Vekemans and Hardy, 2004). Gene flow, mediated by pollen and seed dispersal, is a key factor per se, and also because it determines the scale of local adaptation and the role of population structure in the evolutionary process (Hamrick and Godt, 1996; Fenster et al., 1997, 2003). With spatially limited gene flow, populations should be more inbred and more likely to differentiate in response to local selective force or genetic drift (Kang and Chung, 2000). In addition to gene flow, within-population genetic structure may also be influenced by the balance between sexual and vegetative reproduction (clonal growth) (Chung and Chung, 1999; Van Rossum et al., 2004). Clonal multiplication has been hypothesized to result in a decrease in genetic variation and clonal diversity. However, several reviews have demonstrated that most clonal plants have high genetic diversity, similar to nonclonal plants and that a low rate of repeated sexually produced seedling recruitment may be sufficient to maintain genetic diversity (Eriksson, 1989, 1993; Watkinson and Powell, 1993; Hangelbroek et al., 2002).

Environmental conditions are generally heterogeneous in time and space even at a very fine scale (Price and Marshall, 1999). This means that the ramets of clonal plant are likely to experience environmental heterogeneity for various factors such as resource distribution patterns and exposure to disturbance or toxin. (Stuefer, 1996; Price and Marshall, 1999). The optimal balance between clonal and sexual recruitment may vary under variable growing conditions, each strategy being favored in different environmental circumstances (Eriksson, 1997; Eckert, 2002). The demographic balance between sexual and clonal recruitment is likely to affect genet and ramet dynamics and ultimately the genetic structure within a population (Ellstrand and Roose, 1987; Clark-Tapia et al., 2005). Population history, as well as environmental conditions, may modify the outcome of gene flow and equilibrium between sexual and vegetative reproduction (Leimu and Mutikainen, 2005). For example, Travis et al. (2004) found an increase of vegetative reproduction in recently founded populations compared to longer established populations, modifying the within-population genetic structure.

Linhart and Grant (1996) showed evidence from several studies that local adaptation to different environments (e.g., soils parameters) can occur on the scale of a few meters (even centimeters). This may also influence the genetic structure of a neutral genetic marker because environmental heterogeneity should promote the selection of a number of differently adapted genotypes perpetuated by clonality. This is supported by several studies on plant populations that revealed a fine scale intrapopulation genetic structure on the basis of a neutral genetic marker associated with habitat variation (Linhart and Grant, 1996; Lönn et al., 1996; Li et al., 2000; Prentice et al., 2000; Van Rossum et al., 2004). Examining jointly the genetic structure and the clonal pattern in plant populations representative of heterogeneous environmental conditions is thus a key feature to understanding the factors that shape equilibrium between vegetative and sexual regeneration.

Soils with elevated concentrations of heavy metals (metal-

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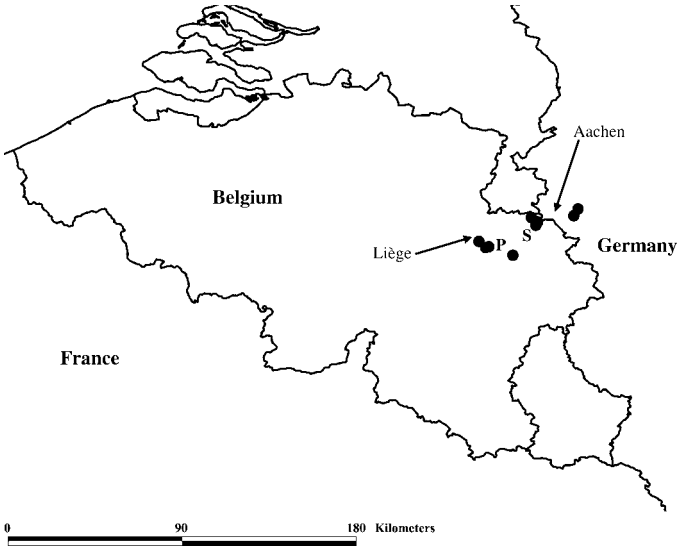

Fig. 1. Distribution of *Viola calaminaria*. Each point represents an area where *V. calaminaria* populations are found. The two sampled populations are represented by P for Prayon (recent population) and S for Schmalgraf (ancient population).

liferous soils), because of their phytotoxicity, represent very harsh and restrictive habitats for plants (Antonovics et al., 1971; Brown, 2001). This kind of environment is generally heterogeneous at a small spatial scale for several ecological factors (Linhart and Grant, 1996; Mattner et al., 2002). Metallophyte species, i.e., species restricted to sites with high level of heavy metals in soils, are thus good models to study variation of within-population structure and clonal growth in heterogeneous harsh environments. Up to now, only a few studies have analyzed spatial genetic structure within metallicolous populations (Van Rossum et al., 2004), and knowledge about the clonal structure and the relative importance of clonal vs. sexual recruitment in such species is poor. In eastern Belgium and western Germany, metalliferous sites consist of calamine soils with high concentrations of zinc, cadmium, and lead (Lambinon and Auquier, 1964). Metallophyte species historically occurred on natural metalliferous sites and on former mines (ancient populations). However, from the end of the 19th century to the 1970s, new populations (recent populations) appeared because of increasing habitat availability resulting from industrial pollution. *Viola calaminaria* (Gingins) Lej. is a rare threatened taxon, endemic to calamine soils with its main distribution limited to eastern Belgium and western Germany (Lambinon et al., 2004). The species is protected in Belgium, and its habitat is a target habitat for the maintenance of biodiversity at the European scale of Annex 1 in Habitats directive 92/43/EEc of the European Community (http://www.proact-campaigns.net/infoandlinks/id10.html). In regard to recent or potential partial destruction of calamine sites, population restoration is likely to be needed (Mattner et al., 2002). In this case, sampling methods for seed stocks and decisions about the size of the area to protect for a population may greatly benefit from spatial genetic information (Chung and Chung, 1999; Escudero et al., 2003; Torres et al., 2003) in order to maximize genetic diversity.

In this study, we assessed within population genetic structure in two populations of *V. calaminaria* with expected heterogeneous and different metal stresses and different demographic histories to contribute to the understanding of patterns of population genetic structure in clonal organisms. Our main aim was to examine the pattern of clonal diversity and within population genetic structure across a range of environmental conditions. As such, we were not explicitly interested in looking at the effect of the environmental differences but rather in examining clonal diversity and genetic structure under conditions where we might expect extreme differences. Additionally, our results are discussed in the context of conservation issues of this rare endemic taxon.

MATERIALS AND METHODS

Species—*Viola calaminaria*, a perennial, clonal pansy, is an ecological endemic of calamine sites (Bizoux et al., 2004). Reproduction occurs by seeds with clonal growth by means of rhizomes (Lambinon et al., 2004). The spatial extent of genets is unknown. The species is supposed to be mainly allogamous by comparison with closely related species, *V. lutea* and *V. tricolor* (Krahulcova et al., 1996), but self-incompatibility has not been reported in the genus. Therefore, low autogamy or geinotogamy cannot be excluded. The species flowers from April to November with the flowering peak in June and July. Flowers, generally yellow, are mainly visited by solitary bees, bumblebees and flies (Syrphideae) (J.-P. Bizoux, personal observation). The species distribution (Fig. 1) includes the east of Belgium (northeast of the Liège province), the southeast of Dutch Limburg and the vicinity of Aachen (west of Germany).

Viola calaminaria was first described by Lejeune (1811). The taxonomic status of the species is questionable, and a recent phylogenetic study (Hildebrandt et al., 2006) recommends classifying it as *Viola lutea* subsp. *calaminaria* (Gingins). In Belgium, *V. calaminaria* is considered as a rare calamine-endemic species (Lambinon et al., 2004), and undoubtedly, whatever its taxonomic position, *V. calaminaria* is considered as a threatened taxon that is worthy of conservation effort because of its ecological particularity.

Plant material and population characterization—Plant material was collected from Prayon (recent population, latitude: 50°35′02″ N, longitude: 5°40′20″ E) and Schmalgraf (ancient population, latitude: 50°41′47″ N, longitude: 5°59′36″ E), two populations with different history. The population of Schmalgraf is established on an ancient mining site (exploited from 1862 to 1932) corresponding to a natural geological ore body. The site corresponds to a primary habitat of the species, and the presence of the species may be traced to the most ancient floristic surveys of the region dating from the 19th century (Duvigneaud et al., 1979). The area of the site is relatively limited, and the *V. calaminaria* population is one of the smallest known (150 m^2) (Bizoux et al., 2004). In contrast, heavy metals are found in excess in the site of Prayon as a result of atmospheric pollution from approximately 1829 to 1970 (Duvigneaud and Jortay, 1987). *Viola calaminaria* was observed at Prayon for the first time in 1972 (C. Lefèbvre, Free University of Brussel, personal communication). At this time, the species was limited to several scattered square meters. Subsequently, *V. calaminaria* rapidly extended, and the population at Prayon is now one of the largest in Belgium (3.22 hectares). The recent population was, because of its size, divided into subpopulations. The subpopulation selected for the sampling is the area of the first colonization of the site.

In each population, ramets were collected along two crossed transects at two sampling scales: a small scale (<3 m) and a large scale (<30 m). For the small scale structure, 29 ramets were sampled at 0.2-m intervals (Fig. 2a), whereas 30 ramets were sampled at 2-m intervals for the large scale within-population structure (Fig. 3a). Small scale sampling was primarily intended to detect the extent of the clonality. Large scale sampling was primarily intended to detect within-population structure resulting from limited gene flow. In the recent and largest population, 10 ramets were also sampled in a second subpopulation more than 200 m from the first one.

Soil analysis—The concentrations of heavy metals (Zn, Pb, Cd, and Cu), Ca, and K were estimated in the study area of each population to characterize the soil conditions and the spatial heterogeneity of the elements. Soil samples (5 cm depth) were collected at 2-m intervals along a 30-m transect. Concentrations of available elements were determined with the acid ammonium acetate–EDTA

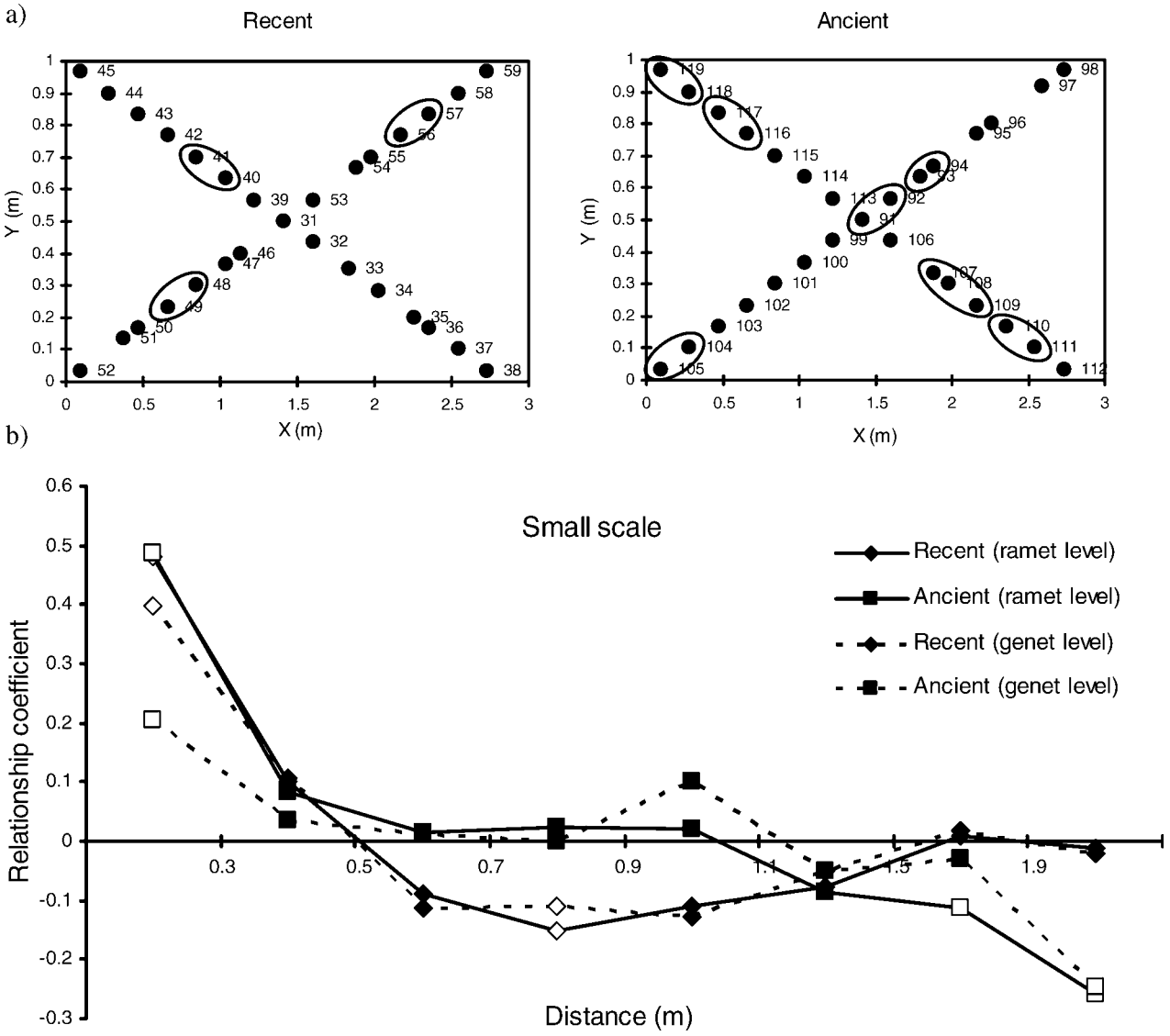

Fig. 2. Spatial genetic structure of *Viola calaminaria* at a small scale (0.2–3 m). (a) Maps of sampled individuals. Ramets of the same genet (RAPD phenotypes) are circled. (b) Relationship coefficient correlogram. Unfilled symbols represent significant ($P < 0.05$) relationship coefficient values.

method of Lakanen and Erviö (1971). The ratios of Zn/Ca and Pb/Ca were also calculated as a measure of soil toxicity (Simon, 1978; Brown, 2001).

DNA extraction and RAPD amplification—DNA was extracted from leaves using the DNeasy (Qiagen, Germany) extraction kit. DNA amplification reactions were performed in a total volume of 25 μl containing: 1× buffer, 1.5 mM $MgCl_2$, 200 μm each DNTP (Fermentas Gmbh), 200 $\mu g \cdot ml^{-1}$ BSA (Fermentas Gmbh), 1 U of *Taq* DNA polymerase (New England Biolabs, Inc.), 10 pmol of each primer (Operon Technologies), and 20–30 ng of template DNA. Amplifications were performed in a PTC 200 (MJ Research: Biozym) for an initial denaturing step of 2 min at 95°C, 44 cycles of 20 s at 94°C, 60 s at 36°C, 60 s at 72°C and a final step of 10 min at 72°C before cooling at 4°C. Controls were included to detect any possible contamination. Amplification products (12 μl) were subjected to electrophoresis in 1.8 % (w/v) agarose gels (containing ethidium bromide) in 1× TAE buffer, then detected under UV light and photographed. Molecular weights were estimated by reference to a 100 base-pair DNA Ladder (GeneRuler, Fermentas Gmbh). Seven primers were selected: OPA-2, OPA-10, OPB-12, OPL-1, OPL-2, OPM-15 and OPT-15. Band selection was based on a repeatability test. DNA amplifications for 23 individuals were repeated twice, and only bands reproducible in both runs were considered for analysis.

Data analysis—Mean soil elements concentrations and the two ratios (Pb/Ca and Zn/Ca) were compared among the two sites with *t* tests using the software MINITAB 13.20 (Minitab Inc. 2000). When data normality or variance equality could not be reached following data transformation, nonparametric Mann–Whitney tests were used. The level of spatial variation in element concentration was estimated with the coefficient of variation (ratio standard deviation/mean). The pattern of spatial variation was further described by a Mantel correlation test between pairwise Euclidean distance of element concentration and geographic distance matrices using the program Passage version 1.1 (Rossenberg, 2001).

The amplification products for the different samples were screened for presence/absence (0/1) of the 57 selected bands. Bands of identical size amplified with the same primer were considered as homologous.

Clonal diversity was measured at the small scale (<3 m) in two ways: (1) the proportion of distinguishable genets ($PD = G/N$, where G is the number of genets and N is the total number of ramets sampled) (Ellstrand and Roose, 1987) and (2) Simpson's index of diversity ($D = 1 - \Sigma n_i(n_i - 1)/N(N - 1)$, where n_i is the number of ramets with RAPD phenotype i and N is the total number of ramets sampled) in each population. The probability that two unrelated individuals share the same banding pattern at locus i is $2p_i + (1 - p_i)2 = 1 - 2p_i(1 - p_i)$, where p_i is the frequency of the band at that locus in the

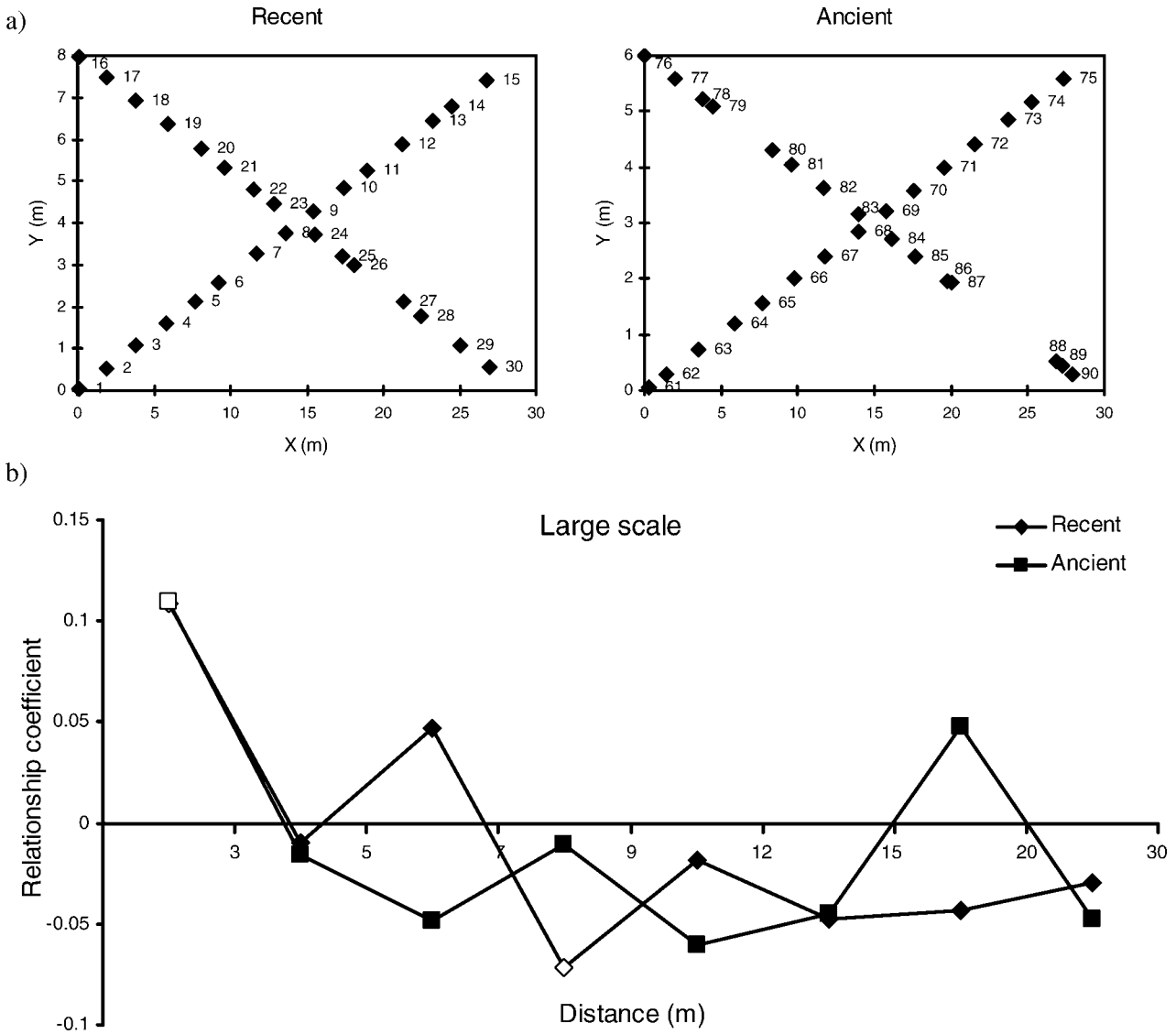

Fig. 3. Within-population spatial genetic structure of *Viola calaminaria* at a large scale (2–30 m). (a) Maps of sampled individuals. (b) Relationship coefficient correlogram. Unfilled symbols represent significant ($P < 0.05$) coefficient values.

population. Therefore, assuming that the 57 loci are independent (unlinked), the probability that two individuals share the same RAPD profile just by chance (i.e., without being replicates of a clone) was estimated as $\Pi\,[1 - 2p_i(1 - p_i)]$, where the product is taken over all loci.

In order to characterize the spatial genetic structure, we used two related approaches based upon analysis of kinship. First, spatial autocorrelation statistics were applied using a relationship correlogram calculated with SPAGEDI version 1.2 (Hardy and Vekemans, 2002) to characterize spatial genetic structure without assumption of its pattern. The relationship coefficient developed by Hardy (2003) is a measure of pairwise genetic similarity between individuals. Distance classes were chosen to obtain at least 30 pairs of ramets in each one and a number of pairs as similar as possible among distance classes. The significance of relationship coefficient in each class was tested by permutation test (10 000 iterations). For the small scale structure, eight distance classes were defined: 0–0.3 m, >0.3–0.5 m, >0.5–0.7 m, >0.7–0.9 m, >0.9–1.1 m, >1.1–1.5 m, >1.5–1.9 m and >1.9–3.0 m. At this scale, spatial autocorrelations were also performed at the genet level. When several ramets had the same genotype, only one (putative) genet was taken into account for the analysis considering the mean geographic position of the identical ramets. For the large scale within-population structure, distance classes were: 0–3 m, >3–5 m, >5–7 m, >7–9 m, >9–12 m, >15–20 m and >20–30 m. Because the relationship coefficient requires an assumption about the individual inbreeding coefficient, we used the estimation of $F_{is} = 0.16$ derived with the program Hickory version 1.0 (Holsinger et al., 2002) from a previous study on 12 populations in Belgium (J.-P. Bizoux, unpublished data). Using a null value of inbreeding did not substantially modify the results (not shown). Second, the regression slopes (b) of the pairwise relationship coefficients on the logarithm (ln) of pairwise geographical distances between individuals were estimated. Under the isolation by distance model in a two-dimensional space, kinship is expected to decrease approximately linearly with the logarithm of the spatial distance (Rousset, 1997; Hardy and Vekemans, 1999; Fenster et al., 2003). In this approach, we a priori assumed that the spatial genetic structure resulted from isolation by distance.

The level of differentiation between the two subpopulations of the recent population was evaluated by F_{st} estimated with Hickory version 1.0 (Holsinger et al., 2002).

RESULTS

Comparisons of soil conditions—The variation in the concentrations of heavy metals in the soil of the study sites

TABLE 1. Characterization of soil conditions in the recent and ancient populations of *Viola calaminaria*. Mean differences between populations based on Mann–Whitney test, unless indicated. The coefficient of correlation, *r*, refers to Mantel test correlation between matrices of euclidean concentrations distance and geographic distance.

		Concentrations (ppm)				
Element	Population	Range	Mean	*P* (Mean differences)	CV (%)	*r*
Ca	Recent	87–1181	301	<0.001	91	0.05 ^{NS}
	Ancient	39–246	93		59	−0.25 ^{NS}
K	Recent	4.6–29	17	0.011 (*t* test)	35	−0.17 ^{NS}
	Ancient	3–31.5	10		75	0.08 ^{NS}
Zn	Recent	1188–14 250	7693	0.003	51	0.02 ^{NS}
	Ancient	913–5500	3403		40	0.24 ^{NS}
Pb	Recent	250–2900	1020	<0.001	72	−0.04 ^{NS}
	Ancient	220–12 850	3996		88	−0.01 ^{NS}
Cd	Recent	25–303	179	<0.001	43	0.22 ^{NS}
	Ancient	1.8–42	8		53	0.62 ***
Cu	Recent	53–1623	542	<0.001	90	−0.02 ^{NS}
	Ancient	4.3–18.9	11		40	−0.16 ^{NS}
Zn/Ca	Recent	5.4–122.8 [a]	38	0.602 (*t* test)	84	−0.01 ^{NS}
	Ancient	20.4–65.5 [a]	42		35	0.03 ^{NS}
Pb/Ca	Recent	0.3–25 [a]	5	<0.001	112	−0.08 ^{NS}
	Ancient	8.7–309 [a]	67		123	0.07 ^{NS}

[a] Values represent ratios of concentrations and are not in ppm.

(Pb, Zn, Cd, Cu) was quite important (up to two orders of magnitude; Table 1). For example, Pb concentrations varied from 220 ppm to 12 850 ppm and from 250 ppm to 2900 ppm and Zn concentrations varied from 913 ppm to 5500 ppm and from 1188 ppm to 14 250 ppm for the ancient and the recent populations, respectively. Significant mean differences were found for soil concentrations of all heavy metals (Zn, Pb, Cd and Cu) between the two populations. The recent population always had higher concentrations of heavy metals in the soil except for Pb (Table 1). Soil Ca and K concentrations of the recent population were significantly higher compared to the ancient one. The ratio Pb/Ca was significantly higher in the ancient population than in the recent one, and no significant difference was found for the Zn/Ca ratio. The coefficients of variation (CV) within sites for heavy metal concentrations varied from 39.5% to 89.7% (Table 1). They were quite similar between the two sites except for Cu (twice as high in the recent population). Pb had the highest concentration variation in the two populations as did Cu in the recent population (CV > 70%). Other heavy metals had a CV of ~50%. The examination of the spatial variation with a Mantel test, followed by Bonferroni corrections for multiple comparisons, showed that only one correlation between pairwise heavy metal concentrations differences and pairwise spatial distances was significant: Cd ($r = 0.6178$, $P < 0.001$) in the ancient population. The two ratios (Zn/Ca and Pb/Ca) also appeared to be very variable in the two populations particularly for Pb/Ca (Table 1).

Clonality and genetic structure at the small spatial scale—From the 58 ramets sampled, 47 different putative genets were found. The proportion of distinguishable genets (PD) within the populations was 0.9 for the recent population and 0.76 for the ancient. The Simpson index of diversity (*D*) was 0.99 for the recent and 0.98 for the ancient population. Most of the shared RAPD profiles were in two copies. Only one RAPD profile was shared by three ramets (Fig. 2a). All ramets belonging to the same genet were located in each other's direct vicinity (20 cm) except one at 40 cm. The probabilities that two ramets shared the same RAPD phenotype by chance only were only 5.51×10^{-5} in the recent population and 1.39×10^{-4} in the ancient population. These values indicated that clonality is the most parsimonious explanation for shared RAPD phenotypes.

The mean slope (*b*) of the regression between the pairwise relationship coefficient and the logarithmic spatial distance was significantly negative in the two studied populations: −0.244 ($P < 0.001$) and −0.138 ($P < 0.001$) for the ancient and the recent population, respectively (Table 2). Those slopes were not significantly different between the two populations ($P = 0.095$; Student *t* test with polymorphic loci as replicates). The variance explained by the regression line was 17.8% and 5.8% for the ancient and the recent population respectively. Spatial autocorrelation at very short distances revealed positive correlation extending to 0.4 m in the recent population and 1 m in the ancient one (Fig. 2b). Only the first distance class values were significantly positive ($P < 0.001$) for the two populations. Negative significant autocorrelation was present between 0.7 and 0.9 m in the recent population, while it only appeared only after 1.5 m in the ancient population.

Spatial autocorrelation (Fig. 2b, Table 2) also showed that differences appeared in the first distance class between ramet and genet correlograms for the ancient population but not for the recent one. In the ancient population, the relationship coefficient value in the first distance class decreased from 0.486 (ramet level, $P < 0.001$) to 0.206 (genet level, $P = 0.016$), but the difference did not prove to be significant ($t = 1.71$, $P = 0.097$). The mean slope (*b*) of the regression (genet level) between pairwise relationship coefficient and spatial distance was significantly negative: $b = -0.140$ ($P < 0.001$) for the ancient population and $b = -0.113$ ($P = 0.001$) for the recent. These slopes did not significantly differ from the slopes at the ramet level ($P = 0.250$ for the ancient population, $P = 0.628$ for the recent; Student *t* test with polymorphic loci as replicates). The variance explained by the regression line at the genet level was equal to 7.3% and 4.4% for the ancient and the recent population, respectively.

TABLE 2. Synthesis of the different results obtained in the spatial genetic structure analysis. *b* represents the mean slope value of the correlogram; patch size was estimated according to Sokal (1979).

	Small scale structure (0.2 m – 3 m)		Large scale structure (2 m – 30 m)	
	Ancient population	Recent population	Ancient population	Recent population
Correlogram	$b = -0.244$ $P < 0.001$	$b = -0.138$ $P < 0.001$	$b = -0.039$ $P = 0.027$	$b = -0.050$ $P = 0.002$
Patch size (m)	1	0.5	4	4
Ramet/Genet difference	Diminution of the relationship coefficient in the first class distance but not significant	No		

Large scale within-population structure—At the large spatial scale, all the ramets sampled had different RAPD phenotypes (Fig. 3a). The slopes (*b*) of the regression between the pairwise kinship coefficients and the logarithm of the distances were significantly negative (Table 2): $b = -0.05$ ($P = 0.002$) for the recent population and -0.039 ($P = 0.027$) for the ancient population, with no significant difference between the two population means *b* ($P = 0.893$, Student *t* test with polymorphic loci as replicates). The variance explained by the regression line was lower than at the small spatial scale: 2.3% for the recent and 1% for the ancient population.

Spatial autocorrelation revealed a positive and significant ($P < 0.01$) correlation in the first distance class (0–3 m) for both populations (Fig. 3b) and a negative correlation in the second distance class. Subsequent correlogram patterns were random for the two populations with only one negative significant value for the recent population between 7 and 9 m. Positive correlations were found at 5–7 m for recent population and at 15–20 m for the ancient population.

The F_{st} value between the two subpopulations of the recent population was 0.136 and was highly significant ($P < 0.001$).

DISCUSSION

Our characterization of soil conditions indicated a high variation in the heavy metals both within and among sites, a situation leading to high spatial heterogeneity in environmental stress as measured by the ratios Zn/Ca and Pb/Ca. The range of variation coefficients within sites for metal concentrations was similar to those reported by Paz-Gonzales et al. (2001) in serpentine soils and are indicated to be of medium to high variability. With one exception, metal concentrations were not autocorrelated, indicating that pairwise heavy metal differences at a short distance (2 m) could be as high as those at long distances.

In addition, at the site level, the two examined sites differed in a number of ways. The ancient population presented a higher Pb/Ca ratio than the recent one and may thus be a more toxic environment. However, this must be tempered with the fact that, in addition to Zn and Pb, two other heavy metals (Cd and Cu) were in excess in regard to phytotoxicity thresholds (De Temmerman et al., 1984) in the recent population and not in the ancient one. The presence of these heavy metal in excess (Cd and Cu) could lead to a harsher environment for *V. calaminaria* growth. As expected, the two sites were representative for examining the pattern of clonal diversity and genetic structure in heterogeneous conditions of heavy metal stress.

Clonality and genetic structure at the small spatial scale—In comparison with other clonal plants, *Viola calaminaria* had high clonal diversity. Our mean values of PD (0.83) and *D* (0.985) were superior to means reported by Hangelbroek et al. (2002) and based on nine studies ($D = 0.74$ and $PD = 0.44$) and were similar to the nonmetallophyte-related *Viola riviana* ($D = 0.99$ and $PD = 0.93$; Auge et al., 2001). In general, ramets sharing the same genotype were spatially aggregated (0.20 m) with only one genet spreading over larger distances (0.4 m). We did not detect any spatial mixing of ramets from different genets. The results indicated that clonality is not an important mean of propagation, even in populations having undergone a recent rapid demographic extension such as in the recent population. Our results suggest that after colonization, *V. calaminaria* follows the repeated seedling recruitment (RSR) model (Eriksson and Fröborg, 1996).

Balance between sexual and vegetative reproduction could also be modified by environmental stresses. For instance, for different species, clonal growth was reported to be higher under harsh ecological conditions, e.g., at range margins (Eckert et al., 1996), increasing latitude (Stenström et al., 2001) and altitude (Young et al., 2002), or on a copper–nickel-polluted soil (Salemaa et al., 1999; Salemaa and Sievanen, 2002). Clonal growth can contribute to the propagation or perpetuation of well-adapted genotypes (Salemaa and Sievanen, 2002; Van Rossum et al., 2004) in harsh environments. In our study, the ancient population was a bit more prone to clonality, but no important clonal growth patterns were observed between the two populations. The little difference observed could simply be the result of better reproduction success in the recent population (fructification rate: 68%, 28%; and seed production: 20.6, 13.9 seeds per fruit, respectively, for the recent and ancient populations) (J.-P. Bizoux, unpublished data), which may promote higher sexual recruitment (Van Rossum et al., 2004). In summary, in *V. calaminaria*, clonal growth cannot be interpreted as a strategy for propagating or perpetuating adapted genotypes under the harshest ecological constraints (soil conditions).

For spatial structure at the small scale, we found a strong pattern of isolation by distance with highly significant negative values of the regression slopes (*b*) and a large part of the variation of the pairwise relationship coefficients explained by geographic distance. In the ancient population, this is corroborated by the examination of the ramet correlogram that had significant positive correlation in the first distance class and significant negative autocorrelation in the largest distance class with a more or less regular decrease of relationship coefficients with increasing distance. In the recent population, the pattern was less clear because significant negative relationship coefficients appeared at intermediate distance

classes and were no more present at the higher distance classes. In this population, the isolation by distance pattern seemed to be restricted to a lower spatial scale. Nevertheless, those results indicated that, on average, spatially close ramets were more likely to be genetically related than ramets that were separated by a larger distances. These results highlighted a spatial genetic structure at very short distance that resulted from a combination of limited gene flow and a low level of clonality. However, correlograms obtained at the genet level were also significant, and no significant difference appeared with the ramet level in the two populations, indicating that clonality did not have a major impact on the spatial genetic structure at the small scale. Nonrandom gene dispersal is thus the key factor in establishing the internal spatial genetic structure observed. Although gene movement in seed plants involves both pollen and seed, a variety of arguments and empirical data indicate that the development of spatial genetic structure within populations is more strongly influenced by seed dispersal than pollen dispersal (Fenster, 1991a; Nason and Hamrick, 1997; Kalisz et al., 2001; Chung et al., 2004). The shape of the regression between the relationship coefficient and the logarithm of the distance obtained for the spatial autocorrelation (genet level, small scale) was found to be concave ($k > 0$ for cubic regression), suggesting that seed dispersal was more restricted than pollen dispersal (Heuertz et al., 2003; Vekemans and Hardy, 2004). Beattie and Lyons (1975) found that the mean distance of dispersal obtained by capsule explosion of diverse *Viola* species ranged from 0.8 to 2.1 m. In addition, *Viola* seeds are known to be dispersed by ants. Studies on seed dispersal by ants show that the distance of transport is very limited. For example, in a review, Gomez and Espadaler (1998) found a mean distance of 0.87 m for northern hemispheric myrmecochorous species, and for *Viola* species, some authors reported that the dispersal distance probably does not exceed 2–3 m (Beattie and Lyons, 1975; Oostermeijer, 1989; Ohkawara and Higashu, 1994). Thus, the spatial structure observed at the small scale was probably due to limited seed dispersal by means of capsule explosion and possible ant transport. According to Sokal (1979) and based on correlograms, patch size of genetically linked individuals (genets) observed at the small spatial scale can be estimated to be ~1 m. This distance corresponds well to the two distances of seed dispersion (capsule explosion and ant transport).

Overall, the available evidence indicates that the small scale spatial genetic structure observed resulted from limited gene flow and weak clonal propagation rather than from heterogeneity of soil conditions or population history.

Large scale within-population structure—At the large scale, we also found significant negative regression slopes (b), but the variation in the relationship coefficients explained by geographical distances was much lower than at the small scale, suggesting a less pronounced pattern of isolation by distance. This was corroborated by the shape of the correlograms that did not have the typical more or less continuous decrease in the relationship coefficients with distance. Those correlograms revealed positive autocorrelation at very short distance, but we did not observe any trend at greater distances. In other words, the spatial genetic autocorrelation was limited to very short distances. This probably reflects the occurrence of patches of genetically more similar individuals resulting from limited seed dispersal (as described for the small scale structure). Subsequent correlogram patterns were random for the two populations. The pattern of genetic similarity found at a small distance may be disrupted at a larger distance by long-distance pollen flow. *Viola calaminaria* is mainly pollinated by bumblebees, solitary bees, and syrphidae (J.-P. Bizoux, personal observation) that can transport pollen at distances farther than a few meters. Fenster (1991b) showed how increased fitness progeny from crosses outside the neighborhood has the effect of opening the population structure resulting in larger neighborhood areas and lowering the degree to which genetic drift will cause local differentiation. In addition, *V. calaminaria* develops a short-term, persistent seed bank containing about 1000 seeds·m^{-2} (J.-P. Bizoux, unpublished data). The seed bank may also significantly modify the outcome of limited spatial seed and pollen dispersal on the genetic structure because the seed bank could have a different spatial genetic structure or a different genetic composition than does the adult stage (e.g., Cabin, 1996; England et al., 2003; Shimono et al., 2006) and can influence the effective number of individuals (Templeton and Levin, 1979). However, as suggested by Mahy et al. (1999), genetic consequences of a seed bank may vary among species and/or populations, and Fenster (1991b) demonstrated that a seed bank with low generation time has a small effect on the effective number of individuals. More detailed studies are needed to assess the importance of the seed bank in shaping genetic structure in *V. calaminaria*.

Again, we found no clear differences in the genetic structure between the two studied populations. This suggests that population history and heavy metal toxicity differences did not influence within-population structure of *Viola calaminaria*.

Conservation implications—The study of spatial genetic structure helps provide guidelines for selecting individuals to transplant during restoration after population destruction and defines the meaningful within-population conservation unit. Our estimation of the *V. calaminaria* patch size that results from the local genetic structure suggests that it is not worth collecting individuals that are closer than 1 m from each other. Individuals within this distance class are more closely related than the average for the population, and collecting individuals within this area may result in a lower level of genetic diversity among the sampled individuals (Tero et al., 2005).

Our results also showed that at distances greater than 200 m, subpopulations had significant genetic differentiation. This implies that seeds must also be collected in different subpopulations. Partial destruction of a site can lead to a loss in genetic variation and implies that different subpopulations must be conserved to maintain differentiated genetic pools of *Viola calaminaria*.

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