

Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*

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Summary

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- Genetic variation structure and breeding system were investigated in metallicolous (MET) and nonmetallicolous (NONMET) populations of the heavy-metal hyperaccumulator *Thlaspi caerulescens* from Belgium, Luxembourg and the French Mediterranean region.
- Allozyme variation showed a clear differentiation between the two ecotypes in Belgium and Luxembourg but not in southern France, in line with the lower degree of geographical isolation between the two ecotypes in this region.
- In both regions inbreeding coefficient (F_{IS}) of NONMET populations was significantly higher compared to MET populations. Pollen/Ovule ratios were significantly higher in MET compared with NONMET populations.
- These results suggest that NONMET populations of *T. caerulescens* are more self-fertile than their MET counterparts. This contrasts with earlier studies on other metal-tolerant species in which selfing rates were higher in MET populations. This pattern may be explained by the fact that both ecotypes are not in sympatry in the populations studied, and therefore reproductive isolation has not been selected to maintain the adaptations to metal-contaminated soils. In addition, higher selfing rates in NONMET populations may have evolved as a mechanism of reproductive assurance, because these populations generally are at low densities.

Key words: reproductive system, genetic differentiation, pollen : ovule ratios, gene flow, heterozygosity, heavy metals.

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Introduction

For almost half a century the biogeochemical, physiological, genetic and molecular aspects of metal tolerance, uptake and accumulation by plant species occurring in areas heavily polluted by toxic metals such as zinc, lead, copper, cadmium and nickel have received sustained attention (Baker, 1987; Macnair, 1987; Salt *et al.*, 1998; Hall, 2002). This is linked with the increasing interest in phytoremediation, an environmental technology using heavy-metal-tolerant plants either to clean up polluted soils or to limit dispersal of toxic elements (Salt *et al.*, 1998; Lasat, 2002). By contrast, the population genetics and biology of metal-tolerant plants have been relatively little investigated. If wild species are to be used and improved for phytoremediation, an accurate knowledge

of their life history traits and genetic structure (notably breeding system, gene flow and genetic diversity organisation) is needed to estimate the feasibility and efficiency of the process in time and space as well as for developing conservation strategies and maintaining the potential for breeding cultivars.

In this context, previous studies on population genetics and population biology revealed some general features of metal-tolerant populations. In particular, metal-tolerant ecotypes were generally found to be more self-fertile than their non-tolerant relatives. Self-fertility was assumed to be an adaptation to limit gene flow from nontolerant populations or to ensure offspring production to the first rare colonizers of the strongly selective metalliferous areas (Antonovics, 1968, 1972; Antonovics *et al.*, 1971; Lefèbvre, 1970, 1973; Macnair, 1987;

Ducouso *et al.*, 1990). Genetic differentiation among tolerant populations was higher compared with nonmetal-tolerant ones possibly owing to founder effects coupled with severe selection on toxic soils (Vekemans & Lefebvre, 1997). In most cases, metal tolerance evolved several times independently in time and space (Bush & Barrett, 1993, Vekemans & Lefebvre, 1997).

Thlaspi caerulescens is a heavy metal hyperaccumulator considered to be a promising species for phytoextraction (Baker *et al.*, 1994; Lasat *et al.*, 2001; Lombi *et al.*, 2002). It has a wide geographic distribution in Europe, from Scandinavia to the Mediterranean area and from central Europe to the Alps and the Pyrenées mountains (Koch *et al.*, 1998). Remarkably, *T. caerulescens* occurs on both metalliferous and nonmetalliferous soils. In the Cévennes (southern France), Belgium and Luxembourg, nonmetallicolous populations have lower tolerance and higher zinc accumulation capacity compared with metallicolous ones (Meerts & van Isacker, 1997, Escarré *et al.*, 2000, Reeves *et al.*, 2001, and references therein). Compared with the rapidly growing literature on biogeochemistry and physiology, there has been very little work on the population genetics and biology of *T. caerulescens*. Koch *et al.* (1998), studying populations from northern and central Europe and the British Isles, found low levels of allozymic variation. They also provide some evidence based on allozymic polymorphism, that outcrossing rate varies among populations, possibly in relation to population density.

In this paper we use codominant allozymic markers, allozyme variation and pollen : ovule ratios to investigate (1) population genetic structure and (2) breeding system in metallicolous and nonmetallicolous populations from South France, Luxembourg and Belgium. The ratio of the number of pollen grains to the number of ovules per flower (P/O) was shown to decrease with selfing (Cruden, 1977) as predicted by theoretical models (Charlesworth & Charlesworth, 1981). In previous studies, P/O ranges from an average of 28 for strictly autogamous species to an average of 5860 for strictly allogamous species with intermediate values for mixed mating systems (Cruden, 1977).

We examine if the patterns of genetic variation are consistent with the hypothesis that metallicolous populations are more selfing than their nonmetallicolous relatives.

Materials and Methods

The species

Thlaspi caerulescens J. & C. Presl, an annual, biennial or short-lived perennial self-compatible species of the family Brassicaceae, is widely distributed in Europe (Koch *et al.*, 1998). The species is found on heavy metal (mainly zinc (Zn), lead (Pb) and cadmium (Cd)) contaminated soils, on serpentine outcrops, and on 'normal' calcareous or acidic soils, and is highly polymorphic (Ingrouille & Smirnoff,

1986). In north-western and central Europe, metallicolous populations are given formal taxonomic recognition by some authors on the basis of subtle morphological characteristics (*T. caerulescens* subsp. *calaminare* (Lej.) Dvorakova; Lambinon *et al.*, 1992). However, a recent study using allozyme markers did not support such a view (Koch *et al.*, 1998). In this work, the two types of populations will thus be referred to ecotypes rather than to subspecies.

Study sites

The French populations were sampled from the Cévennes region (North of Montpellier). In this region, metalliferous and nonmetalliferous sites (hereafter MET and NONMET populations, respectively) are only a few kilometres apart. Heavy metal contaminated populations were sampled on abandoned Zn and Pb mine wastes near Saint-Laurent-le-Minier (five populations: Avinières (AV), Malines (MA), Middle Age (MG), Pommiers (PO) and St Bresson (SB)). Three of these mine sites were abandoned 10 yr ago (MA, PO and SB), the AV site 100 yr ago and the MG site for about 700 yr ago. The five uncontaminated populations were sampled from calcareous areas in the Causse du Larzac (Charnier (CH), Infruts (IN), Navacelles (NA), St Michel (SM) and Séranne Mountain (SE)). See Escarré *et al.* (2000) for further details on these populations.

The two Belgian metallicolous populations were from Plombières (PL), a Pb and Zn mine abandoned for 50 yr, and from Prayon (PR), which has been contaminated for about 150 yr by dust from a Pb–Zn smelter. In the Grand Duchy of Luxembourg, *c.* 80 km in the south of the two Belgian contaminated sites, two populations were collected from uncontaminated soils on Devonian shale near Wilwerwiltz (WL) and Winseler (WN). See Meerts & van Isacker (1997) for further details on these populations.

Soil chemical characteristics of the different sites are given in Table 1. It is notable that soils from polluted sites contained on the order of 100 times more Zn and Pb and 10 times more Cd than normal soils.

Allozyme variation

Studies in France and in Belgium were performed independently using distinct experimental processes. For this reason, data from the two experiments cannot be pooled.

The French metallicolous and nonmetallicolous plants for the allozymes analysis were collected in the field as seedlings and grown in the experimental garden of the CEFÉ. Plants were collected at least 1 m apart one from each other to avoid collecting genetically related individuals. The same plants were used for the analysis of the pollen : ovule ratios. Two nonmetallicolous populations (IN and CH) had very low population size. For these two populations, we also used individuals from seeds collected the preceding year in the field.

Table 1 Soil characteristics from the populations sites

Country/population	pH	Calcium	Zinc	Lead	Cadmium
Metallicolous					
France					
AV	7.2	4550	4700	5000	60
MA	8	3700	3500	253	16.5
MG	5.9	600	1250	1660	11.3
PO	7.3	500	6000	4350	17
SB	7.1	800	2650	3800	23
Belgium					
PL	5.9	1730	7580	7800	ND
PR	6.3	2020	21290	3210	ND
Nonmetallicolous					
France					
IN	7.2	6000	9	17	1.4
NA	7.5	8000	18	38	1.2
SM	7.3	7500	50	28	1.4
CH	7.1	8500	14	55	1.3
SE	7.1	4300	7	5	1.9
Luxembourg					
WL	5.9	2460	36	34	ND
WN	4.0	140	13	46	ND

AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson; CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Séranne Mountain; PL, Plombières; PR, Prayon; WL, Wilwerwiltz; WN, Winseler. Concentrations are expressed as $\mu\text{g g}^{-1}$ ammonium acetate-ethylenediaminetetraacetic acid (EDTA) extractable element. ND, Not determined.

Starch gel electrophoresis of French populations was performed on fresh young leaves ground with sand in 0.1 M Tris-HCl buffer (pH 7.5), 5% sucrose (w : w), 0.6% mercaptoethanol and polyvinylpyrrolidone (PVPP). Protocols for electrophoreses were from Soltis & Soltis (1989). The Four polymorphic enzymatic systems were obtained: phosphogluco-isomerase with two loci (PGI-a, three alleles and PGI-b, two alleles), leucine-aminopeptidase (LAP, four alleles) and esterase (ESTE, two alleles), for a total of 11 alleles for four loci. A total of 331 individuals from 10 populations were sampled.

The Belgium and Luxembourg populations were collected as seeds in the field and grown in controlled conditions in an experimental garden. A total of 275 individuals from 15 families for four populations were sampled.

For the Belgium and Luxembourg populations, the enzymatic systems were resolved in polyacrylamide gels with a Tris-glycine buffer at pH 8.3. The grinding buffer was as follows: Tris pH 7.5, Polyvinylpyrrolidone 2% (PVP), polyethylene glycol (PEG) 1%, dithiothreitol (DTT) 0.08% and mercaptoethanol 0.1%. Four polymorphic loci locus were detected with nine alleles: esterase (ESTE, three alleles), leucine-aminopeptidase 2 (LAP-2, two alleles), acid phosphatase 1 (ACPH, two alleles) and isocitrate deshydrogenase 1 (IDH-1, two alleles).

Pollen : ovule ratios

Seedlings raised from seeds collected in each site were grown in glasshouses, on compost soil for NONMET and in contaminated soil for MET, for the assessment of P/O. Pollen : ovule ratios were obtained from floral buds kept in ethanol 70%. One floral bud was crushed in a micropipette tip in 100 ml of a lactophenol solution (Cruden, 1977) and ultrasonicated for 1 min. Pollen grain number was scored from an aliquot of 1 ml in a Malassez slide (a slide engraved with little squares of 0.05×0.05 mm in size) under the light microscope. Ten individuals were scored per population with generally three flower buds per plant. Ovules were counted in immature ovaries.

Pollen fertility was estimated by acetic carmine, which stains the fertile pollen grains red whereas empty grains remains uncoloured. Pollen fertility ranged from 90 to 100% and there was no significant differences between MET and NONMET populations (data not shown).

Data analysis

Genetic data Two statistics of genetic variation were computed with the program GENE SURVEY (Vekemans & Lefèbvre, 1997): the observed heterozygosity (H_o) and the expected heterozygosity (H_e) corrected for small sample size (Nei, 1978).

Tests of significance of the deviation from Hardy-Weinberg genotypic proportions were performed by comparing the observed coefficient of inbreeding values F_{IS} with values obtained after random permutations of genes among individuals using the program FSTAT (Goudet, 2000). Assuming that populations are not structured and at equilibrium, F_{IS} can directly be related to the selfing rate, s , by the formula $F_{IS} = s/(2 - s)$ (Crow & Kimura, 1970).

The F_{ST} statistic of Wright (1977) was used to estimate differentiation among populations separately for each ecotype (MET or NONMET), between the two ecotypes, and for each pair of populations. Significance of the differentiation was assessed with Fisher exact tests with a sequential Bonferroni correction. All genetic parameters were estimated with TFPGA (Miller, 1997) and FSTAT (Goudet, 2000) freeware. To test if populations genetic differences were due to the model of isolation by distance, we regressed the relationship between the genetic difference between pairs of populations (by the formula $F_{ST}/(1 - F_{ST})$ Rousset, 1997) and on the logarithm of their geographical distance. Statistical significance was assessed by random permutation of population location.

Pollen : ovule ratios Data on pollen : ovule ratios were analysed by nested ANOVAs with populations (as a random factor) nested within ecotype (MET vs NONMET). Comparisons of means were made by a LSMEANS test of SAS (1996).

Table 2 Allelic frequency per locus and origin

France											
	PGI-a			PGI-b		LAP				ESTE	
	1	2	3	1	2	1	2	3	4	1	2
Metallicolous											
AV	0.031	0.938	0.031		1.000	0.029	0.059	0.5	0.412	0.609	0.391
MA		1.000		0.091	0.909	0.015	0.045	0.848	0.091	0.266	0.734
MG		1.000		0.265	0.735		0.929	0.071			1.000
PO	0.024	0.976		0.524	0.476			1.000			1.000
SB		1.000		0.097	0.903	0.014	0.108	0.851	0.027		1.000
Mean	0.011	0.983	0.006	0.195	0.805	0.012	0.228	0.654	0.106	0.175	0.825
Nonmetallicolous											
CH	0.058	0.942		0.692	0.308	0.019	0.192	0.673	0.115	0.058	0.942
IN		1.000		0.031	0.969	0.531		0.469			1.000
NA	0.014	0.986		0.819	0.181		0.214	0.743	0.043	0.229	0.771
SE	0.017	0.983		0.500	0.500	0.038	0.058	0.788	0.115	0.500	0.500
SM		1.000		0.464	0.536		0.019	0.981		0.056	0.944
Mean	0.018	0.982	0.000	0.501	0.499	0.118	0.097	0.731	0.054	0.169	0.831
Belgium and Luxembourg											
	LAP-2		ESTE			ACPH		IDH-1			
	1	2	1	2	3	1	2	1	2		
Metallicolous											
PL	0.119	0.881	0.044	0.811	0.144	0.705	0.295	0.333	0.667		
PR	0.075	0.925	0.097	0.792	0.111	0.424	0.576	0.287	0.713		
Mean	0.097	0.903	0.071	0.802	0.127	0.565	0.435	0.310	0.690		
Nonmetallicolous											
WL	0.354	0.646	0.273	0.473	0.255	0.955	0.045	0.355	0.645		
WN	0.477	0.523	0.553	0.298	0.149	0.942	0.058	0.254	0.746		
Mean	0.415	0.585	0.413	0.385	0.202	0.949	0.051	0.304	0.696		

PGI-a and PGI-b, phosphoglucosyl isomerase a and b; LAP, leucine-aminopeptidase; ESTE, esterase; LAP-2, leucine-aminopeptidase 2; ACPH, acid phosphatase 1; IDH-1, isocitrate dehydrogenase 1. AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson; CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Sérane Mountain; PL, Plombières; PR, Prayon; WL, Wilwerwiltz; WN, Winseler.

Results

Genetic variation

Allelic frequencies per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e) are shown in Tables 2 and 3.

With the unique exception of St Bresson, all populations showed a statistically significant deficit in heterozygotes. However, within the French populations the NONMET populations showed a significantly higher deficit in heterozygotes ($F_{IS} = 0.578$) compared with the NONMET populations ($F_{IS} = 0.217$) (Student's t -test: $P < 0.01$) (Table 4).

Similarly, a higher deficit in heterozygotes was found for the two NONMET populations from Luxembourg ($F_{IS} = 0.704$) compared with the Belgian MET populations ($F_{IS} = 0.501$) (Table 4). Owing the low number of populations (two for each ecotype) no statistical test was applied to these data.

Pairwise differentiation between all French populations and between populations within each ecotype (MET or NONMET), were all significant (Table 5); F_{ST} values are very high, indicating a strong genetic differentiation. However the estimated F_{ST} between the two origins was comparatively weak (although significantly different from zero), indicating overlap between genetic variation of the two ecotypes.

Pairwise comparisons between populations showed that high F_{ST} values can be obtained whatever the pairs considered (i.e. from a same origin or between the two origins) (Table 6). The highest F_{ST} values were found for pairs of MET populations and not for the comparisons between ecotypes, as one could expect. The MG population was responsible for this discrepancy in showing the highest F_{ST} values with the four other French MET populations. By contrast, F_{ST} was very low (0.0078) for the pair PO (MET)/SM (NON MET).

Table 3 Mean sample size (n), observed heterozygosity (H_o) and unbiased gene diversity (expected heterozygosity, H_e)

Country/population	n	H_o	H_e
France			
Metallicolous			
AV	30.00	0.182	0.297
MA	32.75	0.162	0.210
MG	49.00	0.097	0.132
PO	41.75	0.096	0.138
SB	36.25	0.123	0.111
Nonmetallicolous			
CH	26.00	0.154	0.291
IN	16.00	0.031	0.144
NA	35.50	0.142	0.273
SE	29.00	0.104	0.354
SM	27.50	0.091	0.163
Belgium and Luxembourg			
Metallicolous			
PL	48.75	0.057	0.100
PR	40.75	0.051	0.100
Nonmetallicolous			
WL	55.75	0.045	0.118
WN	50.25	0.033	0.113

AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson; CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Séranne Mountain; PL, Plombières; PR, Prayon; WL, Wilwerwiltz; WN, Winseler.

A test of these data against the isolation by distance model applied to the French populations did not reveal any pattern when all population pairs were considered ($r = -0.18$; $n = 20$; $P = 0.21$). In addition, the model did not work with MET populations only ($r = -0.23$; $n = 10$; $P = 0.48$), mostly due to the high genetic differentiation of population MG vs all

Table 5 Fixation index (F_{ST}) and associated exact tests for French populations

	F_{ST}	Exact test
All populations	0.364	$P < 0.0001$
Metallicolous vs nonmetallicolous	0.097	$P < 0.0001$
Metallicolous	0.453	$P < 0.0001$
Nonmetallicolous	0.210	$P < 0.0001$

other MET populations. If MG is excluded from the analysis the correlation increases but remains nonsignificant ($r = 0.26$, $n = 6$; $P = 0.31$). By contrast, the correlation was significant with the NONMET ($r = 0.64$; $n = 10$; $P = 0.03$) (Fig. 1) indicating that the genetic differentiation among these populations increased with geographical distance.

Low F_{ST} values not statistically different from zero were obtained within MET populations from Belgium and NONMET populations from Luxembourg (0.042 and 0.036, respectively). By contrast, comparisons between the two ecotypes revealed an F_{ST} statistically different from zero ($F_{ST} = 0.191$, $P < 0.051$). The MET and NONMET populations are thus genetically differentiated in this geographic region.

Pollen : ovule ratio

Nested ANOVAS on French populations showed a significant effect of the ecotype ($F = 20.25$, $P = 0.002$, d.f. = 1,8) and nonsignificant mean differences among populations within ecotype ($F = 1.11$, $P = 0.36$, df = 8,82). The MET populations had significantly higher P/O values compared with NONMET ones (Fig. 2a). The results do not change after discarding the SM populations which have the lowest P/O values. For the Belgian and Luxembourg populations the

Table 4 Inbreeding coefficient (F_{IS}) values per population and ecotype

	Metallicolous							
	France					Belgium		
	AV	MA	MG	PO	SB	PL	PR	
F_{IS}	0.388	0.229	0.265	0.310	0.107	0.492	0.510	
Means (SD)	0.217 (0.191)						0.501	
	Nonmetallicolous							
	France					Luxembourg		
	CH	IN	NA	SE	SM	WL	WN	
F_{IS}	0.471	0.783	0.481	0.708	0.446	0.669	0.739	
Means (SD)	0.578 (0.156)						0.704	

AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson; CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Séranne Mountain; PL, Plombières; PR, Prayon; WL, Wilwerwiltz; WN, Winseler.

Table 6 Fixation index (F_{ST}) between among French populations (above the diagonal) and statistical significance (below the diagonal) after a sequential Bonferroni correction

	Metallicolous					Nonmetallicolous				
	AV	MA	MG	PO	SB	CH	IN	NA	SE	SM
AV		0.175	0.542	0.487	0.382	0.401	0.356	0.434	0.185	0.410
MA	***		0.547	0.280	0.092	0.295	0.256	0.354	0.150	0.192
MG	***	***		0.630	0.573	0.458	0.560	0.520	0.519	0.600
PO	***	***	***		0.276	0.107	0.474	0.185	0.224	-0.008
SB	***	**	***	***		0.331	0.297	0.426	0.309	0.207
CH	***	***	***	***	***		0.380	0.022	0.138	0.099
IN	***	***	***	***	*	***		0.472	0.343	0.408
NA	***	***	***	***	***	NS	***		0.114	0.168
SE	***	***	***	***	***	***	***	***		0.156
SM	***	***	***	NS	***	***	***	***	***	

*, **, *** $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; NS, nonsignificant. AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson; CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Séranne Mountain.

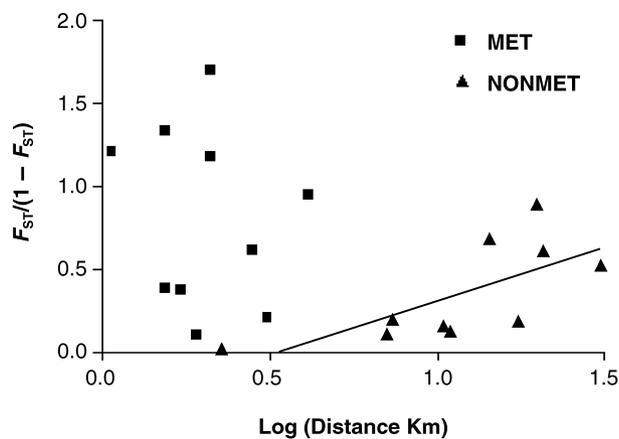


Fig. 1 Relationship between the geographical distances and the genetic distances between pairs of five metallicolous (squares) and five nonmetallicolous (triangles) French populations. Comparisons among pairs of metallicolous (MET) and nonmetallicolous populations (NONMET) are not shown. Only the significant regression line for nonmetallicolous populations is represented. The four highest values of $F_{ST}/(1 - F_{ST})$ correspond to the comparisons of the Middle Age (MG) population against the four other metallicolous populations.

results were similar, with an a significant effect of ecotype ($F = 469.82$, $P = 0.0021$, $df = 1,2$) and a nonsignificant mean effect of population within ecotype ($F = 0.12$, $P = 0.88$, $df = 2,42$). The MET Belgium populations showed a P/O two times higher than the NONMET populations from Luxembourg (Fig. 2b). In the French populations the differences in P/O were due only to the number of pollen grains per anther (MET = 10472.5 ± 520 , NONMET = 6994 ± 508) as there were no differences in the number of ovules per flower (MET = 8.06 ± 0.11 , NONMET = 8.15 ± 0.11). In Belgium and Luxembourg both the number of pollen grains and the number of ovules differed between MET and NONMET populations.

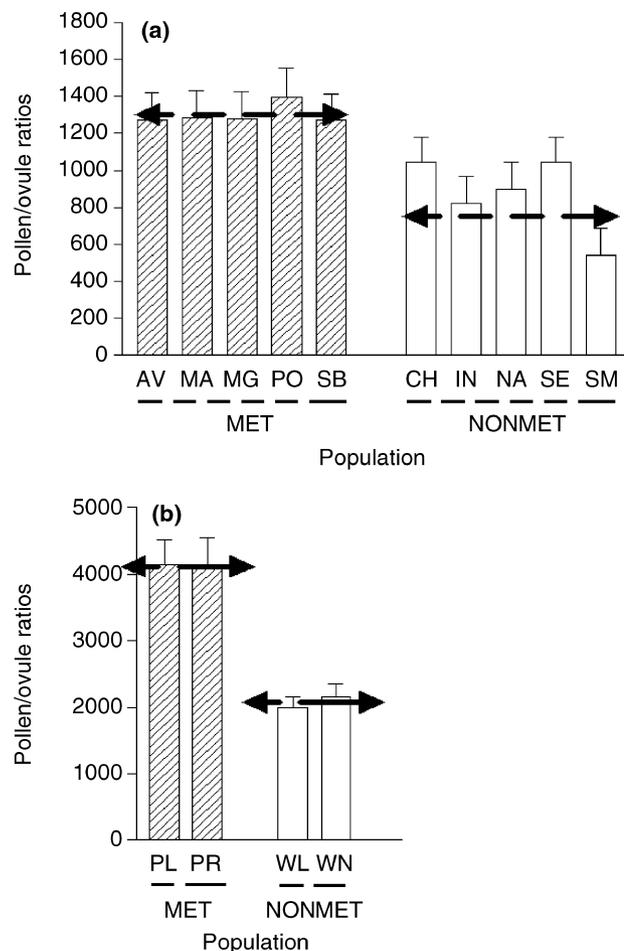


Fig. 2 Mean values of pollen : ovule ratios (\pm SE) for the (a) five metallicolous (MET: AV, Avinières; MA, Malines; MG, Middle Age; PO, Pommiers; SB, St Bresson) and five nonmetallicolous (NONMET: CH, Charnier; IN, Infruts; NA, Navacelles; SM, St Michel; SE, Séranne Mountain) French populations and (b) from the four Belgian and Luxembourg populations (PL, Plombières; PR, Prayon; WL, Wilwerwiltz; WN, Winseler). Black dotted arrows are the means of the populations.

The NONMET populations had higher numbers of ovules (9.25 ± 0.61) and lower numbers of pollen grains (18931 ± 4824) compared with MET populations (ovules 6.41 ± 1.1 ; pollen grains 25774 ± 7300). Both types of populations from Belgium/Luxembourg had two to three times more pollen grains per flower than populations from southern France.

Discussion

Two independent indirect methods sets of data (within-population allozymic variation analysis (F_{IS}) and pollen : ovule ratios) provide circumstantial evidence that nonmetallicolous populations of *T. caerulea* may have higher selfing rates compared with metallicolous populations, although we cannot exclude the possibility that individuals may be issued of biparental inbreeding. This observation is consistent in the two geographical regions investigated. This observation is consistent in the two geographical regions investigated.

The P/O, as a measure of the relative allocation to male and female functions, is an indicator of the type of reproductive systems of plant species (Cruden, 1977), being lower in autogamous breeding systems compared to allogamous ones. The markedly lower P/O in the NONMET populations thus suggests that they are more selfing than their MET counterparts. Interestingly, the difference in P/O values is in the same direction for the two geographical regions, despite much lower P/O values in southern France. The P/O values of MET populations from Belgium (4000) are typical of strictly allogamous species following Cruden (1977), while the values for the populations from Luxembourg and from South of France (800–2000) are in the range of the 'alternative allogamous' category, a mixed mating system where allogamy predominates over autogamy. Koch *et al.* (1998) provide estimations of outcrossing rates for a few populations, ranging from 0 to 0.881, with widely overlapping ranges for the two ecotypes.

These results are surprising, considering that metallicolous populations are generally found to have higher selfing rates than their nonmetallicolous counterparts; see, for example, *Anthoxanthum odoratum* and *Agrostis capillaris*, (Antonovics, 1968, 1972; McNeilly & Antonovics, 1968), *Armeria maritima* (Lefèbre, 1970, 1973) and *Arrhenatherum elatius* (Cuguen *et al.*, 1989). Different mutually nonexclusive hypotheses have been put forward to explain these results. Antonovics (1968) suggested that selfing might evolve in metallicolous populations as a selected mechanism preventing breakdown of coadapted characters through gene flow from adjacent nontolerant populations. From a study on metallicolous isolated populations of *A. maritima*, Lefèbre (1970) inferred that self-fertility in this species was more probably selected as a mechanism ensuring a reliable seed production in colonizing situations when individuals are at low density (Herlihy & Eckert, 2002). In a recent study, Vekemans &

Lefèbre (1997) concluded that the evolution of metal tolerance was not associated with reproductive barriers, as the pattern of partial reproductive barriers was related to gene flow and was similar for MET and NONMET populations. In *Mimulus guttatus*, Macnair & Christie (1983) found that reproductive barriers evolved as a pleiotropic effect of metal tolerance.

Our results for *T. caerulea* are not well explained by Antonovics' (1968) hypothesis for three main reasons. First, it is worth noting that MET and NONMET populations are not closely adjacent, being a few kilometres apart from each other in France and up to 80 km in Belgium and Luxembourg. Therefore, barriers to gene flow are not clearly advantageous at the present time for MET populations although this could have been the case at the time of their foundation. Second, Koch *et al.* (1998) found some evidence for a positive correlation between outcrossing rates and population density in *T. caerulea*. The NONMET populations of *T. caerulea* in South France mostly do consist of very few, sparse individuals (from 20 to less than 100 individuals in the five populations studied). In the same region, mine populations are generally much larger and denser (> 50 individuals m^{-2}). Relatively small population size is also typical of NONMET populations from Luxembourg. By contrast, the MET population of Prayon is very large ($> 100\,000$ individuals) and dense (> 10 individuals m^{-2}). Plombières is also a large population, although it recently decreased in size because of reclamation of the old mine. Although it is not demonstrated in this study, these observations suggest that inbreeding selfing rate may conceivably have evolved in NONMET populations as a response to low density and/or small population size. Finally, NONMET populations typically occur in unstable habitats such as road verges while MET populations probably occur in habitat which that have remained unchanged since mining works ceased.

Very high F_{ST} values (compare our data with those compiled by Hamrick & Godt, 1989), indicative of restricted gene flow, were found for pairwise comparisons among the 10 French populations. Remarkably, this differentiation cannot be accounted for by interecotypic divergence, since F_{ST} values for interecotypic comparisons were generally low ($F_{ST} = 0.097$). This indicates that the two ecotypes, although differentiated for metal tolerance and accumulation (Escarré *et al.*, 2000) are not distinct for allozymes which are assumed to represent neutral alleles. This may indicate that the ecotypic differentiation is relatively recent and/or that gene flows are still effective and reproductive isolation between MET and NONMET populations is not achieved.

In French populations, the positive correlation between the F_{ST} and the geographical distance is significant for the NONMET origin, in agreement with the isolation by distance model. This is not the case with for the MET populations. The relatively strong differentiation of the metallicolous population MG can be related to the long

period of time elapsed since the mine was abandoned (fifteenth century; Bailly Maître, 1989) so that MG has evolved in isolation for several centuries. Populations from mining sites abandoned in the nineteenth and twentieth century are less differentiated from each other.

Seed dispersal by sheep may be the main factor of gene flow between the NONMET populations from the flat Causses plateaus, where *T. caerulescens* is most often found along the verges of paths and roads. Despite their geographical vicinity (less than 5 km in a straight line), MET populations are separated by landscape discontinuities such as steep hills and deep valleys, even if the landscape at the beginning of the twentieth century was open, mainly comprising cultivated terraces (Debussche *et al.*, 1999). In addition, mine sites probably were not frequently visited by cattle and men because of the well-known toxicity of the plants and the soil. For this reason, gene flow among MET populations has probably been very limited and increased the isolation among the different mine sites.

The pattern of genetic differentiation is quite distinct in Belgium and Luxembourg. There, most of the variation can be ascribed to the ecotypic differentiation, most likely owing to the large geographic distance between MET and NON-MET populations (80 km). The weak differentiation between Plombières and Prayon may be surprising, considering the relatively large distance between them (28 km). It is possible that population of Prayon, which started to be heavily polluted 150 yr ago, was founded by seeds transported from Plombières, a mining site known since the Roman period.

In conclusion, based on indirect evidence we have shown that metallicolous populations of *T. caerulescens* have a more allogamous reproductive system than nonmetallicolous populations. A more autogamous breeding system may have evolved in nonmetallicolous populations as a reproductive assurance mechanism, because these populations most often have a lower number of individuals and a lower density compared with metallicolous populations in the same region.

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