

Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony

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Abstract

The population structure of the pseudo-metallophyte herb, *Arabidopsis halleri*, was studied using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) on chloroplast DNA (cpDNA). The history of metallicolous (M) populations showing increased zinc tolerance was investigated. Eight primer-enzyme combinations out of 72 tested were applied to a total of 625 individuals from 28 widespread populations, 14 of them being M. Eleven distinct chlorotypes were found: five were common to nonmetallicolous (NM) and M populations, whereas six were only observed in one edaphic type (five in NM and one in M). No difference in chlorotype diversity between edaphic types was detected. Computed on the basis of chlorotype frequencies, the level of population differentiation was high but remained the same when taking into account levels of molecular divergence between chlorotypes. Isolation by distance was largely responsible for population differentiation. Geographically isolated groups of M populations were more genetically related to their closest NM populations than to each other. Our results suggest that M populations have been founded separately from distinct NM populations without suffering founding events and that the evolution towards increased tolerance observed in the distinct M population groups occurred independently.

Keywords: *Arabidopsis halleri*, cpDNA, heavy metal pollution, local adaptation, PCR-RFLP, pseudometallophyte

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Introduction

Human activities can have acute effects on the dynamics of adaptation of natural plant populations. Industrial and mining activities, in particular, have left soils with very high levels of heavy metals which prove toxic to most living things (Antonovics *et al.* 1971). Such 'metalliferous' soils strongly differ from unpolluted ones with respect not only to heavy metal concentrations but also to numerous additional physical and biological factors (Macnair 1997). For most plant taxa, they constitute adaptative constraints that are too strong to be overcome. A few plant species (coined 'metallophytes') have nonetheless acquired the capacity to grow on them. Several of these species are

'pseudometallophytes' (also called facultative metallophytes), i.e. species having both metallicolous (M, that develop on metalliferous soils) and nonmetallicolous (NM, that do not) populations, sometimes only separated by a very sharp transition over a few metres (Jain & Bradshaw 1966). In pseudometallophytes, two edaphic types thus exist, sometimes referred to as ecotypes, which are exposed to very distinct ecological conditions. Pseudometallophytes therefore constitute highly relevant models for studying local adaptation in plants (Linhart & Grant 1996).

Pseudometallophytes have been the focus of many detailed studies for almost half a century (Baker 1987; Macnair 1987; Pollard *et al.* 2002) and the pseudometallophyte evolutionary framework has been largely discussed (Bradshaw & Mcneilly 1991; Macnair 1997). Overall, two hypotheses have been proposed to account for the origin and history of M populations. First, because the frequency

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of genotypes able to tolerate elevated heavy metal exposure is typically low in NM populations, substantial founder effects are expected to have occurred during the colonization of contaminated area (Lefèbvre & Vernet 1990; Wu 1990). As a result, the colonization of metalliferous soils from those populations may have caused strong genetic bottlenecks (Bradshaw 1984), thus leading to a reduction of gene diversity in M populations as compared to NM populations (Luikart *et al.* 1998). The second assumption concerns the origin of M populations. Considering that the distribution of metalliferous sites in Europe is highly disjunct (Buse *et al.* 2003), with site-to-site distances often too long to be easily overcome by natural dispersal and that the evolution of tolerance in NM populations suddenly exposed to heavy metal stress can occur rapidly and repeatedly (Al-Hiyaly *et al.* 1988), Schat *et al.* (1996) have argued that geographically distant conspecific M populations could have evolved independently and therefore constitute a polyphyletic group. In such a scenario, M and NM populations should not constitute distinct phylogenetic lineages and M populations should be more genetically related to geographically close NM populations than to more geographically distant M populations.

Antonovics *et al.* (1971) suggested that heavy metal tolerance is always populational or ecotypic in pseudometallophytes, i.e. it occurs with no within-population polymorphism in M populations but only at low frequencies (when present) in NM populations. As a result, major assumptions about the evolution of M populations in pseudometallophytes have focused on species exhibiting populational tolerance and have therefore failed to distinguish the colonization of metalliferous soil from the acquisition of heavy metal tolerance (see above). More recently, this initial view was challenged by the demonstration that several facultative metallophytes actually possess constitutive (species-wide) tolerance to heavy metals. In these species, individuals from any population have the capacity to grow on polluted soils, regardless of whether they currently grow on polluted or unpolluted soils (for a review, see Pollard *et al.* 2002). Intriguingly, however, recent studies have reported quantitative genetic variation for tolerance among edaphic types. For example, in the pseudometallophytes *Thlaspi caerulescens* (Meerts & van Isacker 1997) and *Arabidopsis halleri* (Bert *et al.* 2000), M populations were found to be more zinc-tolerant than NM populations in controlled conditions. This suggests that improved adaptation associated with metal exposure has influenced population genetics despite constitutive tolerance. Up to now, little is known about relationships between metal exposure and M population history in pseudometallophytes with constitutive tolerance.

Information from nonrecombinant and maternally inherited cpDNA markers has proven quite useful in population level studies in recent decades (McCauley 1995; Ennos *et al.* 1999). Several studies have investigated

cpDNA polymorphism to define intraspecific lineages and infer population history (for reviews, see Petit *et al.* 2003; Petit *et al.* 2005). Such an approach, which takes into account phylogenetic data from the organelle genome has recently been recommended to successfully detect the impact of environmental contamination on the genetic structure of a species (Staton *et al.* 2001). However, no study has yet attempted to determine M population history in pseudometallophytes using gene phylogeny. In this study, we used cpDNA to study the population genetic structure in the constitutively tolerant pseudometallophyte relative of *Arabidopsis thaliana*, *A. halleri*. We used the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique to investigate cpDNA polymorphism and to define genealogical lineages. In order to be representative of the intraspecific polymorphism over the whole species range, we sampled populations over a broad geographical range covering the main known polluted areas the species has colonized. We used our results to answer two questions that concern major assumptions about M population history in pseudometallophytes: did M populations suffer from genetic bottlenecks during the colonization of polluted areas? Were these populations founded from a single or from several independent events?

Materials and methods

Arabidopsis halleri (syn. *Cardaminopsis halleri*, Brassicaceae) is a clonal, self-incompatible and highly outcrossing perennial weed. It is a close relative of the model organism *A. thaliana* which is supposed to have diverged from the other *Arabidopsis* species from about 5 million years ago (Ma) (Koch *et al.* 2000; Koch *et al.* 2001; Mitchell-Olds 2001). According to Al-Shehbaz & O’Kane (2002), three *A. halleri* subspecies can be distinguished: the montane subspecies *Arabidopsis halleri halleri* evolved from the subspecies *Arabidopsis halleri ovirensis*, an alpine plant of the southeastern Alps, the carpathians and the northern Balkan Peninsula and is ancestral to *Arabidopsis halleri gemmifera* distributed in eastern Asia. *Arabidopsis halleri* ssp. *halleri* (henceforth called *A. halleri* in this study) is a pseudometallophyte that occurs mainly in central Europe on both metalliferous soils (extractable [Zn] up to more than 30 000 µg/g, Bert *et al.* 2002; Van Rossum *et al.* 2004) and nonmetalliferous soils (Bert *et al.* 2002). To be more precise, almost all habitats suitable for *A. halleri* in western Europe and at the northern border of the species range are of anthropogenic origin and polluted. This suggests that the colonization of these regions has been greatly affected by humans and must be recent.

Sampling and DNA extraction

In 1999, 28 scattered populations of *A. halleri* were sampled in the northern half part of the species range (Table 1). The

Table 1 Locations of population samples of *Arabidopsis halleri* used for the cpDNA PCR-RFLP study

Population	References in previous studies	Edaphic type	Origin	Ecological background	GPS coordinates			
					E	N	n_i	N_i est.
D1	G1*	NM	Bohemian forest (Germany)	Nitrogenous Regen river's bank	12°09'88	49°10'64	7	< 50
D2	G2*	NM	Bohemian forest (Germany)	Regen river's bank, in front of G1	12°09'52	49°11'31	8	< 50
D3	G3*	NM	Bohemian forest (Germany)	Shady lawn near the Regen river bank	12°39'66	49°13'10	9	< 50
D4	G4*	NM	Bohemian forest (Germany)	Regen river bank	12°47'75	49°09'85	11	< 50
D6	G6*	NM	Bohemian forest (Germany)	Cham river bank	12°49'78	49°17'42	10	< 50
D8	G8*	M	Harz (Germany)	Old mine (19th century)	10°29'04	51°53'79	13	< 50
D9	G9*	M	Harz (Germany)	Roadside	10°25'16	51°53'46	18	< 50
D11	G11*	M	Harz (Germany)	Underwood	10°21'95	51°51'27	18	< 50
D12	G12*	M	Harz (Germany)	Mine rubble	10°17'90	51°51'91	18	> 500
D13	G13*	M	Harz (Germany)	Roadside and lawn	10°18'50	51°55'22	20	50–500
F1	Auby†	M	North (France)	Wood near a smelter plant	03°03'	50°25'	23	> 1000
F2	—	M	North (France)	Lawn near a smelter plant	03°01'	50°25'	43	> 1000
F3	—	M	North (France)	Metallicolous lawn	03°25'	50°30'	37	> 1000
B1	—	NM	Hautes Fagnes (Belgium)	Underwood	06°40'	50°29'63	29	500–1000
PL1	P1*	M	Silesia (Poland)	Wood in Katowice suburbs	18°57'04	50°14'80	63	> 500
PL2	P2*	M	Silesia (Poland)	Metallurgical factory	18°56'67	50°29'68	21	50–500
PL3	P3*	M	Silesia (Poland)	Metallurgical factory	18°57'58	50°29'50	15	50–500
PL4	P4*	M	Silesia (Poland)	Metallurgical factory	18°55'79	50°29'98	21	> 1000
PL6	P5*	M	Silesia (Poland)	Old mine	19°01'52	50°16'95	10	> 1000
SK2	SI2*	NM	Tatras (Slovakia)	Shady meadow	21°07'81	48°46'17	22	> 500
SK5	SI4*	NM	High Tatras (Slovakia)	Tatransla javorina (Nature reserve)	20°09'24	49°16'98	46	> 500
CZ4	Cz1*	NM	Sumava (Czech Republic)	Forest above a railway	13°45'841	49°02'87	15	50–500
CZ5	Cz2*	NM	Sumava (Czech Republic)	Meadow along a railway	13°46'39	48°59'25	24	50–500
CZ6	Cz5*	NM	Sumava (Czech Republic)	Beside road next to a hay meadow	13°46'	48°59'	12	> 500
CZ8	Cz7*	NM	Sumava (Czech Republic)	River bank next to a meadow	13°48'	48°57'	26	> 1000
CZ14	Cz8*	M	Sumava (Czech Republic)	Slope in a cool, shady wood	12°42'92	49°28'37	20	> 500
CZ16	Cz9*	NM	Sumava (Czech Republic)	Wet meadow	13°33'31	49°03'35	57	> 5000
CZ18	Cz11*	NM	Sumava (Czech Republic)	Woody footpath	13°32'18	49°05'70	9	50–500

M, metallicolous; NM, non metallicolous; n_i , number of genotyped individuals; N_i est., approximative population size; * in Bert *et al.* (2002); † in Bert *et al.* (2000).

scale of sampling ranged from a few kilometres between populations (e.g. D8 and D9) to more than 1300 kilometres for the most distant populations (e.g. F1 and SK2). Sampled M populations of *A. halleri* were discontinuously distributed in three geographically isolated groups (Poland, northern Germany and northern France) plus one isolated population in the western Czech Republic, i.e. at the border of the species range. Except for this isolated population where high metal concentration has been detected far from industrial activities, all M populations occurred in close proximity to zinc smelters or zinc mining sites (abandoned or still in activity). NM populations were sampled as close as possible to the M ones. The existence of closest NM populations can not be excluded but was not known at the moment of sampling. Finally, we sampled 14 M and 14 NM populations.

In each of these populations, leaves were collected from 7 to 63 individuals separated by at least 3 m to avoid clone sampling (Van Rossum *et al.* 2004). Overall, 623 genotypes were collected. Leaves were immediately dried in silica gel prior to molecular analysis. DNA from each genotype was extracted from 15 to 20 mg dry material using QIAGEN DNeasy® Kit and PCR amplification was performed on 1/100 dilutions.

PCR–RFLP analysis of chloroplast intergenic regions

Choice of intergenic regions. To assess cpDNA polymorphism in *A. halleri*, we first screen a subset of 12 individuals using PCR–RFLP. Individuals were randomly chosen in 12 scattered populations so as to be representative of the sample range. Sampled populations included populations D2, D5, D9, D12, F2, PL4, PL6, SK2, SK5, CZ5, CZ14 and CZ16 (Table 1). From these individuals, nine noncoding cpDNA regions were amplified and restricted using eight 4 bp cutter restriction endonucleases. Amplified regions included eight intergenic regions (AS, CD, CS, DT, K2Q, SfM, TC, VL) and the *trnK* region containing the *matK* intron (K1K2). The primer pairs used were those defined by Demesure *et al.* (1995) and Dumolin-Lapegue *et al.* (1997). Only the CD primer pair was modified to match the *A. thaliana* cpDNA sequence and improve PCR yield (*TrnC7F*: CCAGTTCAAATCCGGGTGCC and *trnD7R*: GGGATTGTAGTTCAATTGGT). Amplification products were digested with the 4-bp cutter restriction endonucleases: *AluI*, *HinfI*, *Tru9I*, *HpaII*, *DpnII*, *Sau96I*, *AseI*, *AcsI*. Overall, 72 different primer-enzyme combinations (PEC) were tested. Based on this survey, PECs that gave good PCR yield, nonambiguous and nonredundant polymorphisms were selected to be used over the whole sample. We qualified the polymorphisms as ‘redundant’, when the variation detected by distinct enzymes in the same amplified region clustered the individuals in the same way.

PCR and restriction conditions. The following PCR conditions were utilized: a total volume of 15 µL consisting of 5 µL (~5 ng) of template DNA, 3.5 mM MgCl₂, 200 µg/mL BSA, 200 µM of each of the four dNTPs, 0.2 µM of each primer, 0.625 U of *AmpliTaq* Standard (Perkin Elmer®) and 1× *AmpliTaq* Standard buffer (Perkin Elmer). PCRs were carried out in Perkin Elmer thermocyclers (model 2400 or 9700), using one cycle of 5 min at 95 °C, 36 cycles of 45 s at 92 °C, 45 s at 52.5–62 °C (depending on the primers sequences) and 2–4 min at 72 °C (depending on the size of the amplified fragment, precise protocols available upon request), followed by one cycle of 10 min at 72 °C. Restriction enzyme reactions were performed on a total volume of 10 µL consisting of 5 µL of PCR product, 200 µM of spermidine, 1× of restriction buffer (provided by supplier) and 1–1.5 U of restriction enzyme. Reagent mixtures were incubated at 37 °C (except for *AcsI*, 50 °C) for 2–12 h.

RFLP analysis. Restriction fragments were separated by electrophoresis on either 1.5–2% agarose gels (to detect variation in fragments larger than 300 bp) or 8–9% polyacrylamide gels (prepared with a 19 : 1 acrylamide-bisacrylamide solution; for fragments sized between 75 and 300 bp). Agarose gels were stained with ethidium bromide and photographed with the BioImage system (Bioprobe) under UV light, whereas polyacrylamide gels were silver stained. Restriction fragments were labelled by decreasing order of molecular weight as described by Demesure *et al.* (1996). Length polymorphisms (indel) and restriction polymorphism were encoded as in Dumolin-Lapegue *et al.* (1997). Fragment size polymorphisms were interpreted as insertion/deletions and were encoded as ‘1’ or ‘2’, according to decreasing size of the variable fragment. Polymorphisms corresponding to replacement of a large fragment by two smaller fragments of complementary size were interpreted as the absence or presence of a restriction site and encoded by ‘1’ or ‘9’, respectively.

Statistical analysis

Due to the nonrecombining nature of the chloroplast genome, alleles observed at all nine loci were combined into chlorotypes (cpDNA haplotypes).

Relationship among chlorotypes. A minimum-spanning tree (MST) of the chlorotypes was computed from a distance matrix containing the number of differences between each pair of chlorotypes using MINSNET, a program implemented within the software ARLEQUIN (Schneider *et al.* 2000). The same mutational weight was given to substitutions and to insertions/deletions. MST construction assumes that each chlorotype is linked by a single or a series of mutational events to all other chlorotypes through

a unique pathway and the construction method minimizes the number of such events (Excoffier & Smouse 1994).

Chlorotype diversity. Allelic richness (El Mousadik & Petit 1996; Kalinowski 2004) was estimated for each population i using the rarefaction method in FSTAT version 2.9.3 (Goudet 2001). Given that the smallest sample concerned population D1 with $n = 7$, estimates of a_g^i were standardized to a standard sample size $g = 7$. Chlorotypic diversity and its sampling variance (Nei 1987) were estimated within each population (H_{Si}), and over the whole sample (H_T) using ARLEQUIN (Schneider *et al.* 2000). To test for differences among edaphic types in allelic richness (a_7^{NM} vs. a_7^M) and chlorotype diversity (H_{SNM} vs. H_{SM}) permutation procedures (1000 permutations of populations between edaphic types) were carried out using FSTAT version 2.9.3 (Goudet 2001).

Genetic structure. Genetic differentiation was estimated using Weir and Cockerham's estimators in ARLEQUIN (Schneider *et al.* 2000): (i) between all populations (F_{ST}); (ii) by hierarchical analysis of variance among populations within edaphic type (F_{SC}) and between edaphic types (F_{CT}); (iii) between populations within each edaphic type separately (F_{STM} and F_{STNM}). Estimations were conducted using either allele frequencies only (F -statistics) or both allele frequencies and molecular information as given by the distance matrix among chlorotypes in an AMOVA framework (Φ -statistics). The difference between the two estimators with greatest value of Φ_{ST} compared to F_{ST} is an indication that the species presents some degree of phylogenetic subdivision (Excoffier *et al.* 1992). The statistical significance of estimators was computed by nonparametric permutational procedures of chlorotypes between populations within edaphic type, and between populations among edaphic type. To test for differences in population differentiation between edaphic types (F_{STM} vs. F_{STNM}) 1000 permutations of populations between edaphic types were carried out using FSTAT version 2.9.3. A neighbour-joining (NJ) population tree was drawn from Cavalli-Sforza and Edwards distances based on allele frequencies using the POPULATIONS software (O. Langella, UMR de Génétique Végétale, Ferme du Moulon, Gif/Yvette, France). To test for node robustness, bootstrapping was performed on individuals using 10 000 resamplings. Isolation-by-distance patterns between populations were tested considering all populations and then considering metallicolous and nonmetallicolous populations separately. The Mantel correlation coefficient between geographic and genetic distances was compared with its random distribution obtained from Mantel-like permutations of the genetic [$F_{ST}/(1 - F_{ST})$] and geographic [$\ln(\text{geographical distance})$] matrices as described in Rousset (1997), and implemented in the GENEPOP 3.4 software (Raymond & Rousset 1995).

Results

cpDNA polymorphism

Screening cpDNA of the subset of individuals using 72 different primer-enzyme combinations (PEC) revealed low polymorphism. Most intergenic regions were either monomorphic (SfM) or presented polymorphism for a single restriction reaction only (AS-AcsI; DT-AcsI; K2Q-HinfI; TC-AluI; VL-HinfI). In these cases, the revealed polymorphism was always unique to a particular population (singleton). Three amplification products gave at least two nonredundant polymorphisms. They concerned the CD, CS and *trnK* (K1K2) fragments differentially restricted by *AluI*, *HinfI*, *Tru9I*, *HpaII* or *AcsI*. Overall, eight non-redundant PECs involving these fragments were retained for use over the whole sample. These PECs generated 13 independent polymorphic sites, nine of which consisted in gain/loss of a restriction site whereas four were indels from 5 pb to 34 pb (Table 2). By sequencing three individuals representative of the variations detected, we confirmed the substitutive nature of the four restriction-site polymorphisms identified in the K1K2 region (GenBank Accession nos: DQ149105, DQ149106 and DQ149107).

Chlorotype relationship and diversity

Overall, 11 distinct chlorotypes labelled from A to K were defined (Tables 2 and 3). The MST revealed that they were linked by 13 nonhomoplastic mutation events (Fig. 1). In three cases (chlorotypes E-G, G-J and G-H), chlorotypes were indeed separated by more than a single mutation. Although they were not represented in our sampling, we considered that intermediate chlorotypes exist but are missing because of very low frequencies in the sampled regions. Anyway, these infrequent chlorotypes should have no influence on the estimation of the genetic structure. Most chlorotypes that were shared between edaphic types (four out of five) were interior chlorotypes. In contrast, chlorotypes that were specific to a given edaphic type were spread at the tips of the MST and did not cluster together.

Chlorotypes were not equally frequent overall (Table 3). Whereas chlorotypes A to F, that clustered together in the MST, were widely represented (78.7% of individuals), chlorotypes G to K were much less widespread (21.3% of individuals). Twenty-two out of the 28 populations were polymorphic and allelic richness varied from 1 to 4.983 across populations. Within-populations genetic diversity H_{Si} varied from 0 to 0.893 (± 0.086) with a high level of overall chlorotype diversity ($H_T = 0.853$, see Table 3). Since either monomorphy or a high level of diversity could be found for both large (F3 and SK5, respectively) and small sample sizes (Cz18 and D2, respectively), allelic richness and genetic diversity did not appear to be correlated with

Table 2 Description of the 11 PCR-RFLP chlorotypes identified in 28 populations of *Arabidopsis halleri*. The length variants are noted from 1 to 2; 9 correspond to restriction mutation site (Demesure *et al.* 1996)

Mutation number	1	2	3	4	5	6	7	8	9	10	11	12	13
PCR fragment	K1K2	K1K2	K1K2	CS	CS	CD	CD	CD	K1K2	K1K2	K1K2	CD	CD
Endonuclease	<i>HpaII</i>	<i>AcsI</i>	<i>Tru9I</i>	<i>AluI</i>	<i>HinfI</i>	<i>AcsI</i>	<i>AcsI</i>	<i>AcsI</i>	<i>HinfI</i>	<i>HinfI</i>	<i>Tru9I</i>	<i>HinfI</i>	<i>HinfI</i>
Polymorphic fragment	3	3	5	1	4	1	1	2	6	5	8	7	8
Haplotype													
A	9	9	9	1	1	1	1	9	1	1	1	1	2
B	9	9	9	9	1	1	1	9	1	1	1	1	2
C	9	9	9	1	1	1	9	9	1	1	1	1	2
D	9	1	9	1	1	1	1	9	1	1	1	1	2
E	1	9	9	1	1	1	1	9	1	1	1	1	2
F	1	9	9	1	1	1	1	9	1	1	2	1	2
G	1	9	9	1	1	1	1	1	1	2	1	1	2
H	1	9	1	1	9	1	1	1	1	2	1	1	2
I	1	9	9	1	1	1	1	1	9	2	1	1	2
J	1	9	9	1	1	9	1	1	1	2	1	2	2
K	1	9	9	1	1	9	1	1	1	2	1	2	1

Table 3 Distribution of chlorotypes among populations of *Arabidopsis halleri*

Pop.	Edaphic type	n_i	Chlorotype											a_7^i	H_{Si} (\pm SE)
			A	B	C	D	E	F	G	H	I	J	K		
D1	NM	7	.	.	2	.	4	1	.	3	0.667 (\pm 0.160)
D2	NM	8	2	.	2	.	1	.	.	1	.	2	.	4.983	0.893 (\pm 0.086)
D3	NM	9	2	.	.	.	6	.	.	1	.	.	.	2.960	0.556 (\pm 0.165)
D4	NM	11	5	.	.	.	2	.	.	4	.	.	.	2.990	0.691 (\pm 0.086)
D6	NM	10	10	1	0
D8	M	13	1	.	.	10	1	2.674	0.318 (\pm 0.164)
D9	M	18	3	.	.	15	1.962	0.294 (\pm 0.119)
D11	M	18	5	.	.	9	4	2.987	0.660 (\pm 0.069)
D12	M	18	4	.	.	6	8	2.989	0.680 (\pm 0.056)
D13	M	20	.	.	.	17	3	1.940	0.268 (\pm 0.113)
B1	NM	29	2	27	1.680	0.133 (\pm 0.081)
F1	M	23	.	.	.	21	2	1.780	0.166 (\pm 0.097)
F2	M	43	.	.	.	27	16	1.999	0.478 (\pm 0.041)
F3	M	37	.	.	.	37	1	0
P11	M	63	40	1	.	.	22	.	2.209	0.482 (\pm 0.040)
P12	M	21	14	.	.	.	7	.	1.999	0.467 (\pm 0.075)
P13	M	15	13	.	.	.	2	.	1.934	0.248 (\pm 0.131)
P14	M	21	7	1	.	.	13	.	2.560	0.529 (\pm 0.079)
P16	M	10	10	1	0
SK2	NM	22	4	18	1.967	0.312 (\pm 0.106)
SK5	NM	46	19	1	.	25	1	.	2.565	0.545 (\pm 0.037)
CZ4	NM	15	.	13	1	.	.	1.759	0.143 (\pm 0.119)
CZ5	NM	24	.	22	.	.	.	2	1.762	0.159 (\pm 0.094)
CZ6	NM	12	.	12	1	0
CZ8	NM	26	.	26	1	0
CZ14	NM	20	20	.	1	0
CZ16	NM	57	56	1	.	1.231	0.035 (\pm 0.034)
CZ18	NM	9	9	1	0
Total		623	32	73	4	142	116	123	3	6	26	71	27	6.237	

NM, non metallicolous; M, metallicolous; n_i , number of genotyped individuals per population; a_7^i , standardized allelic richness; H_{Si} (\pm SE), chlorotype diversity (\pm SE).

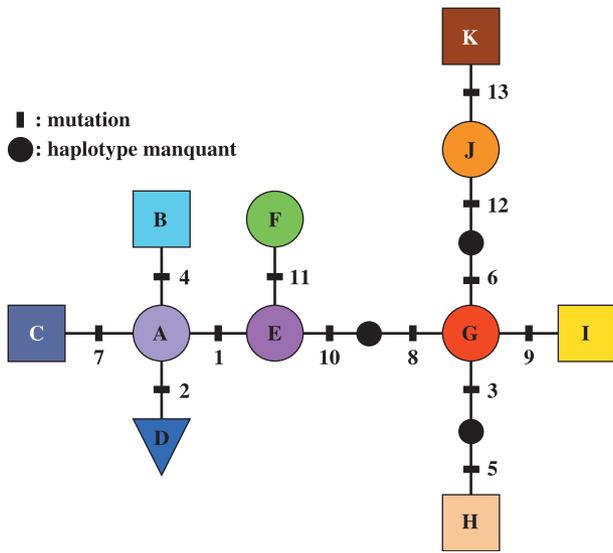


Fig. 1 Minimum-spanning tree representing the relationship between haplotypes inferred by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of *Arabidopsis halleri* cpDNA fragments. Triangles and squares represent haplotypes private to M and NM populations, respectively. Haplotypes occurring in both M and NM populations are indicated by circles. Numbers refer to mutation numbers as presented in Table 2.

sample size (respectively, Pearson’s $r = -0.195$, $P = 0.320$; $r = -0.078$, $P = 0.693$). Interestingly, 6 chlorotypes out of 11 (B, C, D, H, I, K) were not shared between NM and M populations but were specific to one edaphic type (Fig. 2). Most of them (B, C, H, I, K) were NM specific, thus providing partial support to the hypothesis of a founder effect in M populations. However, neither allelic richness nor chlorotype diversity differed significantly between NM and M populations ($a_7^{NM} = 2.064$, $a_7^M = 2.002$, $P = 0.42$; $H_{SNM} = 0.237$, $H_{SM} = 0.349$, $P = 0.31$). NM-specific chlorotypes were among the least represented chlorotypes and

probably did not contribute much in terms of gene diversity estimates.

Genetic structure

Considering the whole sample, estimates of population differentiation revealed a high level of population structure ($F_{ST} = 0.678$, $P < 10^{-5}$). Taking into account molecular distances only slightly modified the inferred population genetic structure ($\Phi_{ST} = 0.677$, $P < 10^{-5}$). A Mantel test between the matrices of pairwise F_{ST} and Φ_{ST} values showed that weighting the estimates of genetic differentiation by molecular distances among chlorotypes did not affect the pattern of population differentiation ($P < 10^{-3}$). Population differentiation within each edaphic type was also high but did not differ significantly between NM and M populations ($F_{STM} = 0.538$, $F_{STNM} = 0.731$, $P = 0.123$).

Differentiation between edaphic types was found to be significant in terms of chlorotype frequencies only ($F_{CT} = 0.124$, $P = 4.9 \times 10^{-3}$, Table 4). Nevertheless, the level of differentiation due to variation between edaphic types dropped strongly when molecular distances between chlorotypes were taken into account ($\Phi_{CT} = 0.036^{ns}$, Table 4). This result suggests that, although edaphic types differed in chlorotype frequencies, chlorotypes in M populations were not more closely related to each other than randomly chosen chlorotypes. The distribution of chlorotypes in populations (Fig. 2) showed that chlorotypes present in eastern and western M populations (A, D, E and F, G, J, respectively) were indeed among the most distant in the MST (Fig. 1).

The NJ tree further confirmed that M and NM populations did not constitute distinct genetic groups (Fig. 3). Despite low bootstrap values, which might be explained by monolocus data combined with a relatively low sample size, M populations clustered in three distinct groups dispersed among NM populations. Each of these three groups

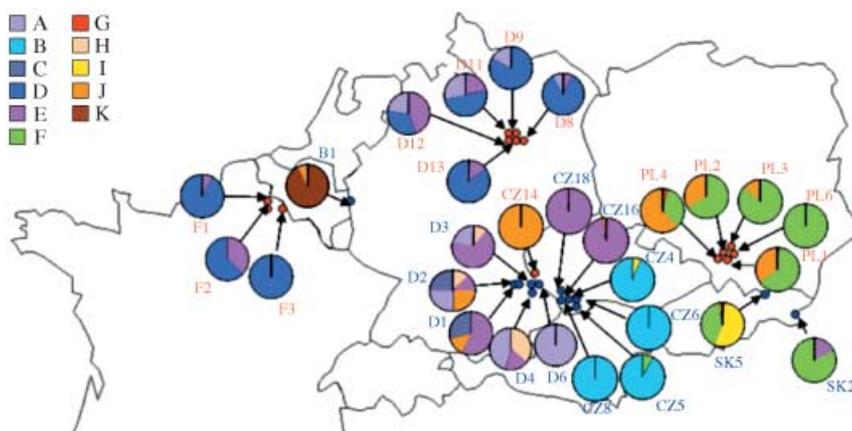


Fig. 2 Geographic distribution of *Arabidopsis halleri* chlorotypes. Metallicolous populations are noted in red, nonmetallicolous ones in blue.

Table 4 Analysis of molecular variance (AMOVA) for 28 populations of *Arabidopsis halleri* using (a) haplotype frequencies only (F -statistics) or (b) both haplotype frequencies and molecular information (Φ -statistics). Statistics include degrees of freedom (d.f.); sums of squares deviations (SSD), variance component (VC), and percentages of total variance contributed by each component (%TV)

(a) Source of variation	d.f.	SSD	VC	%TV	P
Between edaphic types	1	25.890	0.057	12.37	**
Between populations within edaphic type	26	147.523	0.256	55.43	***
Within populations	595	88.641	0.149	32.20	***
Total	622	262.055	0.463		
(b) Source of variation	d.f.	SSD	VC	%TV	P
Between edaphic types	1	46.267	0.05306	3.63	NS
Between populations within edaphic type	26	536.717	0.93632	64.08	***
Within populations	595	280.673	0.47172	32.29	***
Total	622	863.657	1.46111		

NS, nonsignificant; ** $P < 0.01$; *** $P < 0.001$.

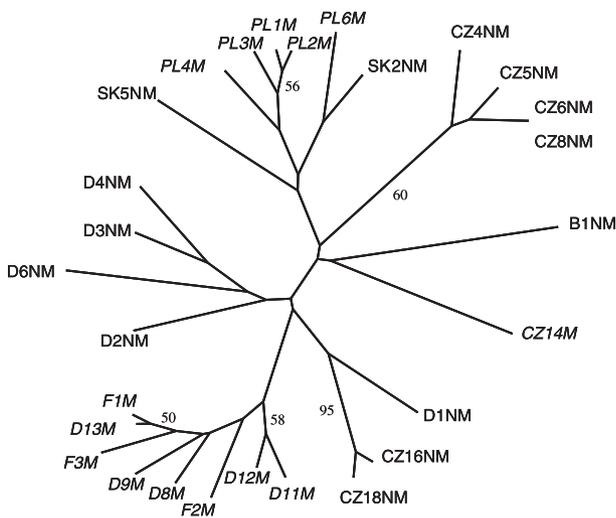


Fig. 3 Neighbour-joining population tree using Cavalli-Sforza and Edwards distances. Only bootstrap values greater than 50% are indicated. NM, nonmetallicolous; M, metallicolous.

clustered together geographically close M populations and was more genetically distant from the other ones than from geographically close NM populations. Thus, French and German M populations clustered with German NM ones (with which they shared two out of three chlorotypes: chlorotypes A and E, chlorotype D being M-specific), Polish M populations clustered with Slovak NM ones (mainly due to the presence of chlorotype F) and the CZ14 population clustered with the Belgian one. Inspection of Fig. 3 indicated that Cavalli-Sforza genetic distances were generally related to geographic distance.

This pattern was confirmed by a significant isolation-by-distance (IBD) pattern for either all populations

($P = 0.003$) or each edaphic type separately ($P < 10^{-5}$ for M sites and $P = 0.022$ for NM sites). The chlorotype distribution confirmed that chlorotype D was dominant in northern Germany and northern France (74.7% of individuals), closely related chlorotypes A and E were dominant in southern Germany and western Czech (74% of individuals), eastern Czech populations were almost exclusively composed of chlorotype B (94.8% of individuals), whereas Polish and Slovak populations were composed of chlorotype F (61.7% of individuals).

Discussion

Level of cpDNA polymorphism and population structure in *Arabidopsis halleri*

Few studies have explored population genetic structure in metalloicolous plant populations. To our knowledge, this is the first study in pseudometallophytes using PCR-RFLP on the maternally inherited genome over a broad geographical range. Despite our exhaustive initial screening effort, which involved scattered populations and numerous PECs, we found low overall polymorphism in *Arabidopsis halleri*. Nevertheless, as usually observed (King & Ferris 1998; Raspé *et al.* 2000; Rendell & Ennos 2002; Magni *et al.* 2005), fragments K1K2 and CD were among the most variable cpDNA regions. Contrary to more general observations in the chloroplast genome (Clegg *et al.* 1994) and to results obtained in other species with the same approach (Raspé *et al.* 2000; Grivet & Petit 2002; Palme *et al.* 2003a, b), most polymorphisms revealed in *A. halleri* were due to point mutations rather than to size polymorphism. Although our data are not appropriate for rigorously discussing the molecular evolution of cpDNA (sequencing analysis should be more relevant), these results may

suggest divergence of molecular evolutionary processes in the chloroplast genome between taxa.

Most studies that have analysed population structure and history at a large geographic scale using PCR-RFLP on cpDNA have been performed on woody species (Petit *et al.* 2005), and similar reports on herb species are scarce (Stehlik 2002; Stehlik *et al.* 2002; McCauley *et al.* 2003). In comparison to the detected number of cpDNA chlorotypes and level of population structure typically reported in woody species [respectively mean = 16.9 (min: 4 – max: 50) and 0.54 (min: 0.09 – max: 1) for 22 European trees and shrubs studied over Europe, in Petit *et al.* 2003], values in *A. halleri* (11 chlorotypes, $F_{ST} = 0.678$) can be considered typically average. The level of population differentiation we observed could be due to the relatively recent colonization of industrially polluted sites or to metapopulation dynamics of the species in the sampled area (McCauley 1995; Ouborg *et al.* 1999). However, since we did not find any significant difference in the level of population differentiation between edaphic types, high values of the estimators cannot be linked to a particular feature of pseudometallophyte species.

For most studies which have compared estimators of population differentiation either taking into account or not the molecular distance between haplotypes in European woody species (for a review see Petit *et al.* 2003), the existence of a 'phylogeographic' structure (Schaal *et al.* 1998) has been revealed by greater value of the estimator when the molecular distance is taken into account. Overall patterns of postglacial colonization of Europe have thus been defined which have confirmed that the southern peninsulas of Europe acted as major ice age refugia where species were restricted during the last glacial maximum (LGM; Hewitt 1999). In our study, sampling was adapted to the study of M population history and thus did not cover southern parts of the species range. However, the striking similarity of F_{ST} and Φ_{ST} strongly suggests that *A. halleri* shows a different pattern than would be expected in a species that was confined to southern refugia during the LGM. Further sampling, including populations from southern Europe, will be needed to test the existence of refugia at higher latitudes in *A. halleri*.

Colonization of polluted areas and genetic bottleneck

The founder-effect hypothesis in M populations initially relied on the assumptions that tolerant genotypes have low frequency in founder populations and that evolution towards heavy metal tolerance causes a strong genetic bottleneck during colonization of polluted areas (Bradshaw 1984). Many studies of pseudometallophytes exhibiting populational tolerance have therefore attempted to detect reduced genetic variation in M populations as compared to NM ones. Surprisingly, most of them failed to detect strong founder effect using allozymes (for a review, see Vekemans &

Lefèbvre 1997; Mengoni *et al.* 2001). Recently, however, Mengoni *et al.* (2001) compared studies of eight geographically closed populations of the pseudometallophyte *Silene paradoxa* using either random amplified polymorphic DNA (RAPD) or chloroplast microsatellite loci (cpSSR) and highlighted the greater relevance of cpDNA markers for successfully detecting reduction in genetic diversity in M populations. Using cpSSR data, the authors detected a founder effect in tolerant populations of *Silene vulgaris* from copper-contaminated soils in comparison to copper-intolerant populations from serpentine soils or uncontaminated sites that were not detected from RAPD data. The founder effect at nuclear loci may indeed be eroded by subsequent pollen flow from populations neighbouring contaminated sites which could increase genetic variation in M populations (Vekemans & Lefèbvre 1997; Mengoni *et al.* 2001).

The study of Mengoni *et al.* (2001) provided some evidence that, as expected from the founder-effect hypothesis, adaptation to metal-contaminated environments is associated with a genetic bottleneck in species with populational tolerance. Conversely, our large-scale survey suggests that it is not the case for species with constitutive tolerance. Although the number of PCR-RFLP chlorotypes is overall lower in *A. halleri* M populations than in NM ones, we did not observe any significant difference between edaphic groups in allelic richness estimated by the rarefaction method or in gene diversity. Chlorotypes that are specific to NM populations are tip-chlorotypes in the MST and are among the less geographically widespread, as expected (Crandall & Templeton 1996). Although they were probably not represented in the pool of migrants that colonized polluted sites, their absence in M populations does not appear to be a consequence of strong gene diversity reduction. Moreover, only one chlorotype is specific to the M populations (chlorotype D, private to French and German M populations), suggesting that the diversity observed in M populations cannot be explained only by new chlorotypes produced by mutation events after colonization. Our results differ from a previous survey using 42 nuclear microsatellites loci to study population genetic structure in *Arabidopsis* species (Clauss *et al.* 2002). The survey revealed an excess of heterozygotes in one M population of *A. halleri* from Thuringia, Germany, that could coincide with a hypothesized founder event during the expansion of mining in Thuringia. However, as highlighted by the authors, analysis of variation in additional populations and comparisons with further M or NM populations were lacking. As a result, the possibility that it revealed a general feature of *A. halleri* populations, or, conversely, a particular feature of the sampled population independently of its edaphic type cannot be excluded from this study.

Recently, Macnair (1997) demonstrated that polluted areas differ from unpolluted ones in many components

apart from higher metal contamination (soil structure, organic and mineral content, dryness, etc.). He suggested that the evolution of metal tolerance could be only a part of the evolution of M populations. In this context, the founder-effect hypothesis could be extended to other adaptive characters required for the colonization of polluted areas and apply to species with constitutive tolerance. Nevertheless, our study suggests that when the capacity to grow on polluted soils exists in founder populations, it could be sufficient to prevent a founder effect in M populations. In consequence, founder effects in pseudometallophyte M populations may only be due to adaptation for heavy metal tolerance and may not be detected in species exhibiting constitutive tolerance despite the numerous ecological constraints encountered in metal-polluted areas.

The independent origin of metalicolous populations

The hypothesis of multiple independent origins of M populations within pseudometallophytes has already been validated for species showing tolerance in M populations only (Westerbergh & Saura 1992; Vekemans & Lefèbvre 1997; Koch *et al.* 1998; Mengoni *et al.* 2001), or in both M and NM populations as for *Thlaspi caerulescens* (Koch *et al.* 1998). Our results strongly suggest that in *A. halleri* the foundation of M populations also occurred several times and that the increased tolerance observed in M populations has a polyphyletic origin. Hierarchical AMOVA clearly showed that M populations are not distinct from NM ones when phylogenetic information is taken into account and no correspondence between edaphic type and chlorotype lineage was observed. Population structure was instead due to isolation by distance rather than to zinc exposure; population haplotypic composition rather depended on geographical location than on edaphic origin. As a result, M populations did not cluster together in the NJ tree but in three separated groups dispersed among NM populations and corresponding to the different polluted areas we sampled. Except for French M populations, genetic relationships between groups of M populations and their spatially closest NM populations allow us to draw assumptions about foundation events: German M populations may have been founded by German NM populations (haplotypes A), Polish M populations may come from the Slovakian NM population (haplotype F), and sharing of haplotype J by CZ14 and the geographically closest D1 and D2 strongly suggests the foundation of CZ14 from D1 or D2. In such a scenario, almost all M populations have been founded either from distinct NM population groups (e.g. D8-13 and PL1-6) or from the same group but separately in time (e.g. D8-13 and CZ14). Accordingly, both historical (Berton 1946) and genealogical data (Fig. 2) suggest that *A. halleri* was introduced into northern France during the first part of

the 20th century from metalicolous sites located in the Harz (Germany), where its distribution was associated with mining activities since the Middle Ages (Liessmann 1997; Brooks 1998).

The fact that different independently founded M populations could present different levels of tolerance has led several authors to consider that locally selected tolerance mechanisms could differ between M populations (for a review, see Schat *et al.* 1996). In *S. vulgaris*, Schat *et al.* (1996) found identical major zinc-tolerance genes in tolerant populations which were supposed to have evolved independently from intolerant ones and suggested that variation in their tolerance abilities should rather be due to allelic variation between major loci or to the presence of 'modifiers' (Smith & Macnair 1998). In *A. halleri*, tolerance is species-wide but quantitative variation exists among and within edaphic type (Bert *et al.* 2000; Pauwels *et al.* in prep.). Parsimony suggests that fixed tolerance has occurred only once, probably early in the species' history, and that major gene(s) that produce constitutive tolerance are identical for all individuals. However, secondary mechanisms (allelic variation or presence of modifiers) may exist that confer enhanced tolerance to M populations. According to Schat *et al.* (1996), the genetic make-up of such mechanisms may differ in separately founded M populations. Mapping QTL for tolerance in recombinant populations from multiple crosses between a NM population and independently founded M populations should provide further support for this hypothesis (Symonds *et al.* 2005).

The findings of this study clearly demonstrate that integrating the phylogenetic component of the spatial distribution of gene lineages and relocating the colonization of recently polluted sites in the overall background of the species' history makes it easier to identify the impact of contaminant exposure on the genetic structure of a species (Staton *et al.* 2001). Such an approach should therefore be successful in investigating genealogical relationships between M populations in pseudometallophytes from both ecological categories (i.e. presenting either constitutive or populational metal tolerance). It has to be noticed that some of the results from intraspecific studies could partially be due to the sharing of alleles with other subspecies or close relatives. In our study, for example, region-specific results could result from a 'swamping out' of adaptive lineage divergence. In this case, a comparative approach including other subspecies or close relatives should be successful in determining how other underlying divergence processes may have accelerated or possibly impeded divergence of chlorotypes. Moreover, in all pseudometallophytes such as *A. halleri* with species-wide tolerance, the evolutionary origin of the tolerance character cannot be determined from intraspecific sampling and a comparative approach covering the closest nontolerant relatives is necessary.

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