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Cadmium hyperaccumulation and genetic differentiation of *Thlaspi caerulescens* populations

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Abstract

Although the knowledge on heavy metal hyperaccumulation mechanisms is increasing, the genetic basis of cadmium (Cd) hyperaccumulation remains to be elucidated. *Thlaspi caerulescens* is an attractive model since Cd accumulation polymorphism observed in this species suggests genetic differences between populations with low versus high Cd hyperaccumulation capacities. In our study, a methodology is proposed to analyse at a regional scale the genetic differentiation of *T. caerulescens* natural populations in relation to Cd hyperaccumulation capacity while controlling for different environmental, soil, plant parameters and geographic origins of populations. Twenty-two populations were characterised with AFLP markers and cpDNA polymorphism. Over all loci, a partial Mantel test showed no significant genetic structure with regard to the Cd hyperaccumulation capacity. Nevertheless, when comparing the marker variation to a neutral model, seven AFLP fragments (9% of markers) were identified as presenting particularly high genetic differentiation between populations with low and high Cd hyperaccumulation capacity. Using simulations, the number of outlier loci was showed to be significantly higher than expected at random. These loci presented a genetic structure linked to Cd hyperaccumulation capacity independently of the geography, environment, soil parameters and Zn, Pb, Fe and Cu concentrations in plants. Using a canonical correspondence analysis, we identified three of them as particularly related to the Cd hyperaccumulation capacity. This study demonstrates that populations with low and high hyperaccumulation capacities can be significantly distinguished based on molecular data. Further investigations with candidate genes and mapped markers may allow identification and characterization of genomic regions linked to factors involved in Cd hyperaccumulation.

Keywords: Amplified fragment length polymorphism (AFLP); Cadmium (Cd); Genetic differentiation; Hyperaccumulation; Outlier loci; *Thlaspi caerulescens*

1. Introduction

Heavy metals are naturally occurring elements in soils but their amount can be increased by pollution of anthropogenic origin (Prasad, 2004). They can be a source of environmental and health hazards since they will be

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accumulated along the food chain (Sanita di Toppi and Gabbrielli, 1999). Many of them, like zinc (Zn) and copper (Cu), have physiological functions in plants and animals but become toxic at high concentrations. Others, such as cadmium (Cd) and lead (Pb), are toxic for both plants and animals even when present in small amounts (Prasad, 2004), Cd being one of the most ecotoxic metals (Sanita di Toppi and Gabbrielli, 1999). The major source of heavy metals in plants is the direct uptake from contaminated soil. Plants called accumulators, take up metals in their roots and shoots probably as a way of detoxification and resistance to high metal concentrations present in soils (Baker, 1981). Hyperaccumulators have the ability to accumulate a limited number of heavy metals in amounts of at least 100 times superior to those expected for non-accumulating plants (Brooks, 1998). For instance, a foliar Cd concentration above 100 mg kg^{-1} d. wt (0.01%) is considered exceptional and is used as threshold value for Cd hyperaccumulation (Baker et al., 2000). Those hyperaccumulating plants are of special interest for biologists and soil scientists because of their potential use in soil decontamination through phytoextraction (Krämer, 2005).

Thlaspi caerulescens J. Presl & C. Presl (Brassicaceae) is a model plant to study Cd hyperaccumulation (Assunçao et al., 2003a). It grows on soils with large variation of Cd concentrations (Reeves and Brooks, 1983) and presents a range of capacities for its accumulation (Baker et al., 1994; Reeves et al., 2001). In *T. caerulescens*, Cd was proposed to enter the cell via either a high or low affinity uptake system for iron or calcium/zinc, respectively, depending on the population studied (Lombi et al., 2001, 2002; Zhao et al., 2002; Roosens et al., 2003). Furthermore, a high affinity transporter for Cd was also proposed for specific populations due to differences in observed Cd uptake (Lombi et al., 2001; Zha et al., 2004). Despite many studies on Cd hyperaccumulation physiology (e.g. Bernard et al., 2004; Cosio et al., 2004; Papoyan and Kochian, 2004; Zha et al., 2004; Zhao et al., 2002), little work has been done on the population genetics of this trait in *T. caerulescens*, where Cd hyperaccumulation is considered a population-specific rather than a species wide characteristic (Assunçao et al., 2003b). Some studies in controlled conditions suggested marked differences between *T. caerulescens* populations in their ability to hyperaccumulate Cd (Lombi et al., 2000, 2001; Roosens et al., 2003). Investigating natural population systems is of great importance to understand the genetic basis of this trait and the selective forces acting on it (Pollard et al., 2002). Recently, methods to identify genomic parts affected by natural selection have been developed (e.g. Beaumont and Nichols, 1996; Schmid et al., 2005). To detect loci under recent selective effects, patterns of variation observed at one genetic marker need to be compared with the variation expected under a neutral model.

The aim of this study was to examine the genetic differentiation of *T. caerulescens* natural populations in relation to Cd hyperaccumulation using molecular markers at a regional scale. *T. caerulescens* populations were sampled and characterised for their Cd hyperaccumulation capacity. Two groups of populations were defined based on their capacity to hyperaccumulate Cd in their shoots. Amplified fragment length polymorphism (AFLP) markers and plastid DNA (cpDNA) polymorphism were used to reveal the patterns of genetic differentiation between these two groups. Markers diverging from the neutral model expectations were identified using computer simulations. The effects on population structure of geography, environment, soil and metal (Zn, Pb, Fe and Cu) plant concentrations were also investigated.

2. Material and methods

2.1. Sampled sites and population characteristics

Sampling locations were selected based on the distribution map of *T. caerulescens* in Switzerland (Welten and Sutter, 1982) and, for the Alps region, on the mineral distribution maps [Wallis, Bernese Oberland (Cavalli et al., 1988); Tessin (Kündig et al., 1990)]. Among the 22 studied populations (Table 1), 15 were from the Swiss Jura (coded J1, J3 to J15 and J18), two from the Prealps (PA1, PA2) and five from the Western Alps (Saas valley, A1–A3 and Grimsel-pass, A4 and A5). Environmental parameters [the topographic wetness index (wetness index), the monthly potential direct shortwave radiation (solar radiation), the degree–days, the number of precipitation days (precipitation days), the number of frost days during the growing season (frost days)], soil (pH, HNO_3-extractable Cd, Zn, Pb, Cu concentrations as, respectively, S[Cd], S[Zn], S[Pb], S[Cu]) and plant (Zn, Fe, Pb, Cu concentrations as P[Zn], P[Fe], P[Pb], P[Cu], respectively) parameters were evaluated for each sampled population according to Basic et al. (2006).

Cadmium concentration in shoots (P[Cd]) was measured and the capacity to hyperaccumulate Cd for the studied populations was characterised according to Basic et al. (2006). A multiple linear regression model was used to normalize the Cd hyperaccumulation among populations with regards to wetness index, solar radiation, degree–days,

Table 1
The 22 studied Swiss populations of *Thlaspi caerulescens*: latitude, longitude, wetness index (unitless), solar radiations (kJ day^{-1}), degree–days (day × deg), number of precipitation days (nday) and number of frost days during the growing season (nday)

Site	Locality	Latitude	Longitude	Wetness	Solar radiations	Degree–days	Precipitations	Frost days
J1	VD, St-Cergues	46°27′20″	6°9′46″	−19	15,763	1841	34	2
J3	VD, Col du Marchairuz	46°32′43″	6°15′4″	−118	16,700	1619	37	10
J4	VD, Vallée de Joux	46°36′24″	6°12′54″	−176	16,546	1869	35	14
J5	VD, Col du Mollendruz	46°38′44″	6°21′26″	−201	15,672	1699	36	22
J6	VD, Dent de Vaulion	46°40′48″	6°21′18″	−353	17,685	1479	37	28
J7	VD, Le Suchet	46°45′12″	6°26′45″	−110	19,240	1382	36	34
J8	VD, S^{te}-Coix	46°50′25″	6°31′57″	−68	18,168	1044	39	38
J9	VD, S^{te}-Croix	46°50′10″	6°31′25″	−223	18,698	1036	39	38
J10	VD, Chasseron	46°51′18″	6°33′29″	13	17,959	1153	39	36
J11	VD, Chasseron	46°52′8″	6°33′21″	−111	17,543	1150	39	35
J12	NE, Creux-du-Van	46°55′5″	6°43′29″	−127	16,945	1470	40	29
J13	NE, Mont d'Amin	47°5′16″	6°54′6″	−141	13,053	1509	39	5
J14	NE, Mont d'Amin	47°5′8″	6°54′7″	−265	16,622	1483	39	5
J15	NE, Mont d'Amin	47°4′55″	6°54′13″	−221	17,269	1443	39	6
J18	NE, Tête de Ran	47°3′15″	6°51′15″	−289	15,861	1478	40	11
PA1	VD, Leysin	46°20′33″	6°59′54″	57	19,691	1590	39	3
PA2	VD, Leysin	46°21′19″	7°1′12″	11	16,196	1659	39	2
A1	VS, Vallée de Saas	46°9′50″	7°55′27″	−96	16,084	1509	28	8
A2	VS, Vallée de Saas	46°5′20″	7°57′42″	−366	14,065	1122	32	32
A3	VS, Vallée de Saas	46°5′44″	7°56′53″	−241	9278	1009	32	49
A4	BE, Haslital	46°39′22″	8°18′1″	−355	15,017	1700	42	2
A5	VS, Ulrichen	46°30′14″	8°19′12″	−388	14,734	1364	37	50

precipitation days, frost days, pH, S[Cd], S[Zn], S[Pb], S[Cu], P[Zn], P[Fe], P[Pb], P[Cu] (for more details see Basic et al., 2006). The residuals of the best multiple linear regression model for P[Cd] quantified Cd hyperaccumulation differences among populations defined in the present study as Cd hyperaccumulation capacity (Cd HA capacity). Two groups of populations were defined according to their Cd HA capacity. The high Cd HA capacity group was composed of populations presenting positive residuals of the best linear model for P[Cd]. The low Cd HA capacity group was represented by populations with negative residuals.

For the further analyses, environmental (wetness index, solar radiation, degree–days, precipitation days, frost days), soil (pH, S[Cd], S[Zn], S[Pb], S[Cu]) and metal plant (P[Zn], P[Fe], P[Pb], P[Cu]) parameters were summarized in two synthetic environmental, two synthetic soil and two synthetic plant variables by, respectively, the first two components of the Principal Component Analysis (PCA) based on a correlation matrix of the environmental, soil and metal (P[Zn], P[Fe], P[Pb], P[Cu]) plant parameters. Analyses were performed using R 1.7.1 (Ihaka and Gentleman, 1996).

2.2. Amplified fragment length polymorphism (AFLP) and plastid DNA (cpDNA) analyses

Frequent inbreeding events occur in *T. caerulescens* (Dubois et al., 2003; Koch et al., 1998). Since neutral genetic variation in selfing or inbreeding species occurs between rather than within populations (Allard et al., 1968), the genetic characterization of a single individual per population gives a sufficient measure of the diversity between populations (e.g. for *Arabidopsis thaliana*; Jorgensen and Mauricio, 2004). In addition to high inbreeding rates, the genetic differentiation between *T. caerulescens* populations is enhanced by their limited pollen and seed dispersals (Dubois et al., 2003; Riley, 1956), and, for the studied populations, by their discontinuity and isolation by forests or mountain ridges. An initial study, sampling two individuals for each of six populations (J3, J4, J9, PA1, A2, A5), showed higher AFLP-based genetic similarity within than among populations (Supplementary material). The two plants per population were collected at least 1 m apart from one another to avoid genetically related individuals (Dubois et al., 2003). Note that those individuals were included in further analyses. For the remaining 16 populations, one individual was sampled.

Total DNA was extracted from 100 mg of dried leaf using the FastDNA kit (Qbiogene, Inc., Carlsbad, CA). Individuals were characterised by their AFLP profiles, which were considered as phenotypes (or haplotypes). The AFLP™

Plant Mapping kit protocol was used (Perkin–Elmer Applied Biosystems, USA). A 100 ng of genomic DNA were digested using the *Eco*RI and *Mse*I restriction enzymes and adaptors were ligated to the restriction fragments using T4 DNA ligase. A preamplification PCR was performed using standard *Eco*RI and *Mse*I adaptors having one additional selective nucleotide. For selective amplification, six primer combinations were chosen (B4: *Eco*RI ACA × *Mse*I CAT; B8: *Eco*RI ACA × *Mse*I CTT; B16: *Eco*RI ACT × *Mse*I CTT; G4: *Eco*RI AAG × *Mse*I CAT; G8: *Eco*RI AAG × *Mse*I CTT; G20: *Eco*RI AGG × *Mse*I CAT). For each primer combination, one primer was labelled in 5′ with a fluorochrome (Applied Biosystems). Reaction mixtures were prepared according to the provider recommendations (Perkin–Elmer Applied Biosystems, USA) and were incubated in a thermocycler (T1, Biometra). Electrophoresis of the PCR products was directly carried out on a denaturing 5% polyacrylamide gel using an automated sequencer (ABI 377; Applied Biosystems). Raw data were collected using ABI's GENESCAN®. Each differently sized fragment identified, in the range of 75–500 bp, was scored either present or absent in each individual analysed. A label was given at each locus according to the code of its set of primers and the fragment's size (e.g. B8.141 refers to combination B8, fragment size of 141 bp). To reduce bias in fragments assignment, only clearly amplified polymorphic fragments (loci), with frequency superior to $3/N$ in addition of those inferior to $1 - (3/N)$ (Lynch and Milligan, 1994), where N is the sample size, were retained for further analyses.

The plastid DNA (cpDNA) polymorphism was investigated to control for the genetic structure of populations based on a maternal marker (Harris and Ingram, 1991). We analysed length polymorphism at one highly variable cpDNA microsatellite locus (located in the intergenic spacer *trn*S–*trn*G) which is present in phylogenetically distant Brassicaceae genera (i.e. *Arabidopsis*, *Biscutella*, *Lepidium* and *Sinapis*; Gaskin et al., 2005; Parisod et al., 2005). For the PCR amplification, mixtures contained 20 ng of total genomic DNA, 1× reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 μmol of each oligonucleotide primer (one 5′ labelled with a fluorochrome; Applied Biosystems; Parisod et al., 2005) and 0.75 U of *Taq* DNA polymerase (Qbiogene) in a total volume of 25 μL. Reaction mixtures were incubated in a thermocycler (T1, Biometra) firstly for 4 min at 94 °C and then for 36 cycles consisting of 1 min at 94 °C, 1 min at the annealing temperature of 53 °C and 1 min at 72 °C. The last cycle was followed by a 10 min extension at 72 °C. Electrophoresis of the PCR products was directly carried out on a denaturing 5% polyacrylamide gel using an automated sequencer (ABI 377; Applied Biosystems).

2.3. Genetic analyses

2.3.1. Identification of outlier loci

Identification of candidate loci that show unusually high levels of genetic differentiation between high and low Cd HA capacity groups of populations was carried out following the method described by Beaumont and Nichols (1996). Expected F_{ST} values between the high versus low Cd HA capacity group of populations for neutral loci can be simulated using a coalescent approach assuming an infinite alleles mutation model (Beaumont and Nichols, 1996). The neutral expectation was based on the overall mean value of F_{ST} calculated on all markers from the observed data. The distribution of calculated F_{ST} as a function of heterozygosity (H_e) was characterised by estimating the 0.05, 0.50 and 0.95 quantiles of the null distribution. Observed F_{ST} value at each locus between the high versus low Cd HA capacity group of populations obtained with AFLP and the cpDNA locus can then be compared to the simulation results to identify loci that show unusually high levels of genetic differentiation between high and low Cd HA groups of populations. The F_{ST} and H_e were calculated with the Beaumont and Nichols (1996) approach based on the number of presence and absence of each haplotype in each group of populations. Using an iterative procedure, markers with F_{ST} values outside the 0.95 limits were then removed and a new analysis was performed with a recalculated mean value of F_{ST}. Markers with F_{ST} values that fell outside the 0.95 limits after the second analysis were considered as outlier loci (Acheré et al., 2005). Calculations were done using the Fdist2 software (Beaumont and Nichols, 1996). In order to test whether the grouping of populations had an effect on the identification of outlier markers, permutations of populations were performed. Thus, 20 replicates of random assignment of populations to high and low Cd HA capacity group were obtained and the number of outlier loci was determined for each replicate as described above.

2.3.2. Genetic differentiation between populations in relation to Cd hyperaccumulation capacity

The characterization of the genetic differentiation between the two groups of populations with different Cd HA capacities, over all AFLP loci and across the outlier loci identified by the Beaumont and Nichols (1996)

approach, was done by an analysis of molecular variance (AMOVA; Excoffier et al., 1992) using Arlequin version 2.000 (Schneider et al., 2000). Significance was obtained using 1000 permutations.

To test the relation between genetic and Cd HA capacity distances while controlling the effects of geographic distances between populations, environmental, soil and metal plant synthetic variables on genetic diversity, partial Mantel tests (Smouse et al., 1986) were done using FSTAT 2.9.3 (Goudet, 1995). Pairwise comparisons between populations were performed independently of the Cd HA capacity grouping of populations. Partial Mantel test investigates the correlation between two variables while controlling the effect of a third one (or more) and thus tries to remove spurious correlations. Estimates of genetic resemblances or similarity (GS_{ij}) between pairs of AFLP haplotypes were expressed as Dice (asymmetrical) coefficients of similarity (Dice, 1945), over all loci and across outlier loci identified by the Beaumont and Nichols (1996) approach, using R Package version 4.0 (Casgrain and Legendre, 2001). Genetic distances were expressed as the complement to one of the coefficient of similarity. Other pairwise population matrices were obtained by calculating Euclidean distances for environmental, soil, metal plant (P[Zn], P[Fe], P[Pb], P[Cu]) synthetic variables and Cd HA capacity, and the log-Euclidean distances for the geography (Rousset, 1997).

2.3.3. Outlier loci characterizing the Cd hyperaccumulation capacity

As a complementary approach to the Beaumont and Nichols (1996) methodology, a Canonical Correspondence Analysis (CCA) was used to test which of the outlier loci are effectively related to the variance of Cd HA capacity. This analysis did not consider any grouping of populations in regards to Cd HA capacity but environmental and plant factors were taken in account. Considering AFLP products as phenotypes, this method allowed relating variation of genetic components to ecological factors (Angers et al., 1999) over all populations. The ecological factors used for CCA were the environmental, soil, plant (P[Zn], P[Fe], P[Pb], P[Cu]) synthetic variables and Cd HA capacity. A permutation test was performed to assess the significance of the CCA (1000 permutations). The analysis was carried out using R 1.7.1 (Ihaka and Gentleman, 1996).

3. Results

3.1. Population characteristics

The Cd concentration in plant (P[Cd]) was correlated to the degree–days (slope $= -0.17$, $t = -1.31$, $p < 0.05$), precipitation days (slope $= 17.65$, $t = 3.11$, $p < 0.01$), S[Zn] (slope $= -1.16$, $t = -2.12$, $p < 0.05$) and S[Cd] (slope $= 63.35$, $t = 6.74$, $p < 0.001$). This model explains 84.5% of the variance with S[Cd] alone explaining 72.1%. The residuals of this linear model were called Cd HA capacity of populations. It must be mentioned here that pseudo-total (HNO_3-extractable) soil Cd concentration (OIS, 1998) could be used because it was also correlated to plant available Cd as defined by a 0.1 M $NaNO_3$ extraction (FAC, 1989) ($r = 0.68$, $p < 0.001$). $NaNO_3$-extractable Zn concentrations were hardly detectable probably due to the generally low pseudo-total Zn concentrations and high pH in the majority of soils (Basic et al., 2006).

Environmental parameters of *T. caerulescens* populations are given in Table 1. The two first axes of the PCA on environmental parameters explained 69% of the total variance, the first one explaining almost 43%. The first component was positively correlated with the number of days of frost during the growing season ($r = 0.56$) whereas the second component of the PCA was negatively correlated with the solar radiations ($r = -0.60$) and positively with the degree–days during the growing season ($r = 0.52$).

S[Cd], S[Pb], S[Zn] and S[Cu] as well as soil pH are given in Table 2. The first two components of the PCA on soil parameters explained 75% of the total variance, the first one explaining almost 50%. The first component was positively correlated with S[Pb] ($r = 0.55$), S[Zn] ($r = 0.49$) and pH ($r = 0.49$) whereas the second component of the PCA was positively correlated with S[Cd] ($r = 0.78$) and negatively with S[Cu] ($r = -0.60$).

P[Zn], P[Fe], P[Pb] and P[Cu] are given in Table 2. The first two components of the PCA on plant parameters explained 71% of the total variance, the first one explaining almost 42%. The first component was positively correlated to P[Cu] ($r = 0.71$) and P[Pb] ($r = 0.71$) whereas the second component of the PCA was positively correlated to P[Fe] ($r = 0.74$) and S[Zn] ($r = 0.65$).

Table 2
The 22 studied Swiss populations of *Thlaspi caerulescens*: mean and standard deviation of cadmium, copper, lead and zinc HNO_3-extractable concentrations in soils (as S[Cd], S[Cu], S[Pb] and S[Zn] in mg kg^{-1}), soil pH, mean and standard deviation of cadmium, zinc, iron, lead, copper concentrations in plants (as P[Cd], P[Zn], P[Fe], P[Pb], P[Cu] in mg kg^{-1}) and Cd hyperaccumulation capacity (as Cd HA capacity) of populations

Site	ns	S[Cd]	S[Cu]	S[Pb]	S[Zn]	pH	np	P[Cd]	P[Zn]	P[Fe]	P[Pb]	P[Cu]	Cd HA capacity
J1	3	0.7 ± 0.1	5.6 ± 0.6	62.3 ± 7.8	51.0 ± 5.4	5.4 ± 0.0	4	59 ± 36	2534 ± 1141	1064 ± 250	2.7 ± 0.3	3.8 ± 0.7	42.3
J3	2	1.3 ± 0.1	13.8 ± 2.4	50.3 ± 6.7	90.4 ± 9.1	6.4 ± 0.4	4	27 ± 11	3118 ± 130	211 ± 68	2.0 ± 1.4	3.0 ± 0.8	−72.0
J4	7	1.8 ± 0.4	8.0 ± 1.6	48.9 ± 6.9	71.6 ± 8.3	5.9 ± 0.3	5	69 ± 15	2663 ± 1053	814 ± 633	0 ± 2.2	1.8 ± 1.1	−6.5
J5	3	1.0 ± 0.2	9.3 ± 2.0	44.6 ± 6.0	54.1 ± 7.7	5.7 ± 0.2	6	31 ± 19	4953 ± 1307	1206 ± 758	4.1 ± 1.3	4.0 ± 1.1	−59.5
J6	3	1.5 ± 0.2	12.9 ± 1.9	64.5 ± 5.7	95.9 ± 4.2	6.3 ± 0.5	4	66 ± 34	5359 ± 3560	541 ± 167	2.6 ± 3.0	3.3 ± 1.2	−62.5
J7	3	1.8 ± 0.2	9.4 ± 0.6	30.7 ± 1.8	79.7 ± 5.2	5.8 ± 0.1	4	142 ± 44	4857 ± 1745	385 ± 181	0.9 ± 1.9	3.1 ± 1.3	−22.1
J8	3	4.8 ± 0.9	9.8 ± 1.2	48.7 ± 9.1	102.1 ± 12.2	5.6 ± 0.1	1	366	491	635	5.0	3.6	−71.8
J9	9	3.7 ± 0.6	10.4 ± 1.4	45.4 ± 8.6	119.5 ± 33.3	5.9 ± 0.2	6	432 ± 204	5654 ± 2555	468 ± 256	19.5 ± 42.4	7.0 ± 6.1	82.3
J10	3	6.3 ± 1.1	8.3 ± 1.8	43.0 ± 5.0	86.5 ± 11.5	6.1 ± 0.4	4	522 ± 252	2378 ± 1159	545 ± 338	2.1 ± 0.7	3.6 ± 1.1	−11.1
J11	2	2.1 ± 1.2	7.5 ± 2.1	32.6 ± 6.0	66.2 ± 11.5	5.4 ± 0.7	3	352 ± 161	11,071 ± 5042	550 ± 505	1.9 ± 1.0	4.1 ± 1.1	61.4
J12	3	2.0 ± 0.5	8.9 ± 1.8	48.4 ± 11.3	93.0 ± 4.7	5.7 ± 0.4	3	226 ± 68	14,563 ± 6964	341 ± 205	1.2 ± 0.3	4.1 ± 0.6	8.8
J13	8	7.2 ± 3.8	10.9 ± 2.6	49.2 ± 9.3	181.9 ± 59.0	6.0 ± 0.4	4	405 ± 208	8557 ± 648	969 ± 868	4.0 ± 7.0	3.2 ± 1.3	−15.0
J14	3	2.1 ± 0.8	8.4 ± 2.1	83.0 ± 47.6	103.6 ± 49.7	5.3 ± 0.3	4	232 ± 55	8490 ± 2402	267 ± 37	1.6 ± 1.4	2.3 ± 1.1	40.0
J15	3	2.3 ± 0.3	9.4 ± 1.3	36.8 ± 1.6	92.3 ± 4.1	5.6 ± 0.2	1	339	6790	1594	2.7	4.8	115.0
J18	3	2.6 ± 0.6	10.8 ± 1.0	42.0 ± 1.5	108.6 ± 19.8	5.8 ± 0.5	3	260 ± 128	5123 ± 1480	1677 ± 157	2.4 ± 2.1	3.7 ± 1.0	23.8
PA1	7	1.1 ± 0.1	66.8 ± 30.6	99.4 ± 64.7	158.2 ± 77.6	6.6 ± 0.4	6	21 ± 13	8632 ± 8059	581 ± 323	18.1 ± 25.5	3.5 ± 2.0	−26.6
PA2	2	0.9 ± 0.3	12.5 ± 1.5	34.1 ± 0.4	85.9 ± 12.2	4.5 ± 0.3	4	53 ± 39	5201 ± 2133	1376 ± 1161	3.1 ± 0.8	3.8 ± 0.8	−54.4
A1	2	0.1 ± 0.0	22.6 ± 2.4	17.7 ± 2.8	67.4 ± 13.6	5.4 ± 0.2	3	7 ± 1	9299 ± 3634	584 ± 335	1.8 ± 0.3	2.3 ± 1.0	97.9
A2	5	0.2 ± 0.0	20.9 ± 1.5	17.0 ± 1.5	67.3 ± 5.1	5.1 ± 0.2	3	9 ± 5	8090 ± 1980	227 ± 31	2.6 ± 2.5	2.5 ± 0.6	−41.4
A3	8	0.1 ± 0.0	27.8 ± 3.7	10.9 ± 3.5	39.7 ± 10.1	5.4 ± 0.3	2	14 ± 3	6735 ± 2499	355 ± 125	2.6 ± 1.8	2.8 ± 1.2	−80.7
A4	5	0.4 ± 0.1	17.8 ± 8.3	38.5 ± 5.6	155.5 ± 113.1	5.1 ± 0.9	5	34 ± 19	9642 ± 2046	600 ± 312	2.9 ± 2.9	2.7 ± 0.7	−6.9
A5	4	0.3 ± 0.2	13.1 ± 4.3	19.1 ± 6.8	69.4 ± 26.8	5.4 ± 0.6	2	161 ± 197	20,850 ± 9485	1227 ± 820	2.5 ± 0.1	3.4 ± 0	59.1

ns is the number of soil samples whereas np is the number of plant samples analysed by site.

Cadmium concentration in plants varied between 7 mg kg^{-1} and 522 mg kg^{-1} (Table 2). Populations J3, J4, J5, J6, J7, J8, J10, J13, PA1, PA2, A2, A3, A4 were considered as a group with low Cd HA capacity whereas populations J1, J9, J11, J12, J14, J15, J18, A1, A5 were considered as a group with high Cd HA capacity (Table 2).

3.2. Genetic differentiation of populations related to the Cd HA capacity

Seventy-eight AFLP markers were obtained. The number of loci scored was 14, 11, 12, 22, 13 and 6 for AFLP combinations B4, B8, B16, G4, G8 and G20, respectively. Based on the all 78 AFLP markers, the AMOVA showed no significant genetic differentiation between the low and high Cd HA capacity groups ($F_{ST} = 0.02$; $p = 0.07$). Over all loci, the partial Mantel test indicated that the AFLP genetic distances were only correlated to geographic ($r = 0.58$, $p < 0.001$) and the second plant synthetic variable ($r = -0.17$, $p < 0.05$) distances between populations ($R^2 = 36.8\%$). Three cpDNA haplotypes were identified using the *trn*S−*trn*G fragment. These variants were characterised by a band of 267, 297 or 308 bp. No significant genetic differentiation between the low and high Cd HA capacity groups was revealed based on this marker.

The coalescent simulations identified seven outlier AFLP fragments (9% of loci) that exceeded the 95% limits of the simulation distribution of differentiation under neutrality (Fig. 1). The outlier loci exhibited significant F_{ST} with high values. Simulations to test the robustness of the identification of these markers against reclassification of populations gave a mean value of 4.05 ± 1.43 outlier loci. Thus, this analysis showed that the number of loci identified as outliers is significantly greater than expected at random. The AMOVA, based on the seven identified outlier AFLP markers, indicated a significant genetic differentiation between low and high Cd HA capacity groups ($F_{ST} = 0.31$; $p < 0.001$). On these seven outlier loci, the population pairwised AFLP genetic distances were correlated with the geographic ($r = 0.36$, $p < 0.001$) and the Cd HA capacity ($r = 0.21$, $p < 0.01$) distances ($R^2 = 17.5\%$). Among the seven outlier loci, G8-130, B4.281 and B16.151 were found to be particularly related to the Cd HA capacity variance based on the CCA (Fig. 2).

4. Discussion

4.1. Genetic differentiation in relation to Cd hyperaccumulation capacity

On allozymes, which are assumed to be neutral markers, Dubois et al. (2003) have found low genetic differentiation between metalliferous and non-metalliferous *T. caerulescens* populations from southern France although they were

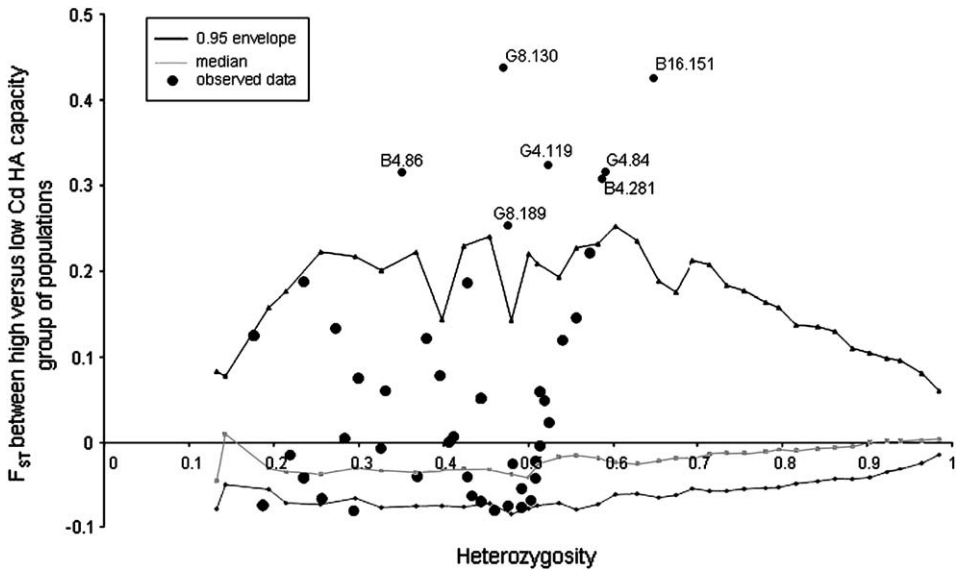

Fig. 1. F_{ST} values estimated from 78 AFLP loci and a cpDNA marker *trn*S−*trn*G, between populations with high versus low Cd hyperaccumulation capacities, plotted against heterozygosity. The 0.95 quantiles have been estimated following Beaumont and Nichols (1996).

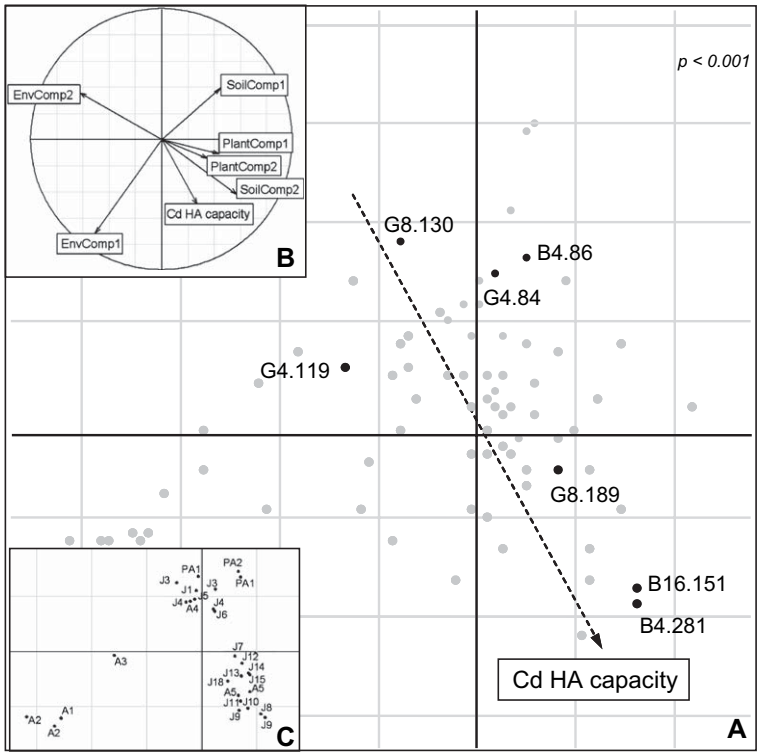

Fig. 2. (A) Representation of the 78 AFLP loci (grey and black points) contributing to the genetic differentiation between the 22 studied populations of *Thlaspi caerulescens* according to the first two components of the Canonical Correspondence Analysis (CCA). The black points represent the outlier loci obtained following the Beaumont and Nichols (1996) approach. (B) Contribution of the population characteristics to the first two components of the CCA. SoilComp1, SoilComp2, PlantComp1, PlantComp2 and EnvComp.1, EnvComp.2 are the first two components of the Principal Component Analysis on the soil, plant and environmental parameters, respectively, whereas Cd HA capacity is the Cd hyperaccumulation capacity. (C) Representation of the CCA among the 22 *Thlaspi caerulescens* populations.

differentiated for Cd tolerance and accumulation (Escarré et al., 2000). In the present study, on the overall genome there was no significant AFLP genetic structure of *T. caerulescens* populations in relation to Cd hyperaccumulation. Although the AFLP fingerprints are also presumably neutral markers, they are *a priori* distributed throughout the complete genome and could therefore characterize genetic fragments located close to or in genomic regions under selection. Indeed, our study revealed a significant genetic structure of *T. caerulescens* populations related to their Cd HA capacity on seven AFLP fragments (9% of the loci). These fragments are probably from the nuclear genome since they were not related to the plastid DNA polymorphism (cpDNA). The structure of genetic polymorphism in a genome can be influenced by different evolutionary processes affecting the whole genome (e.g. geographic population structure) or acting locally across the genome (e.g. selection) (Schmid et al., 2005). Different environments can generate significant barriers to gene flow (e.g. via effects on phenology) or different selective pressures and lead to genetic heterogeneity of plant populations (Linhart and Grant, 1996). Geographic distances between plant populations can as well influence their genetic structuring (e.g. via isolation by distance, distinct origins). However, the correlation between genetic and Cd HA capacity distances remains still significant after taking into account the effects of environmental, soil, metal plant (P[Zn], P[Fe], P[Pb], P[Cu]) parameters and geographical distances between populations. Thus, some genetic differences between *T. caerulescens* populations correspond to their Cd HA capacities independently of their environments and origins. Seven loci can be considered putatively under selective pressures or closely linked to loci subject to natural selection (Beaumont and Balding, 2004) since the genetic differentiation on them was higher than the one expected under a neutral model. Heavy metals are known to represent strong ecological constraints for plants (Macnair, 1987). Therefore, the study of metal tolerant species, including populations with various metal tolerances, provides an excellent system to understand recent adaptive evolutionary processes (Van Rossum et al., 2004). Since Cd tolerance and hyperaccumulation seem to be linked in *T. caerulescens* (Basic et al., 2006; Lombi et al., 2000), one could expect

strong selective pressures related to the Cd HA capacity. Our observations support the hypothesis that selective pressures induce a genetic differentiation between high and low Cd hyperaccumulating *T. caerulescens* populations on specific genomic regions.

4.2. Loci related to the Cd hyperaccumulation capacity variance

According to the CCA, three of the seven outlier AFLP fragments were particularly related to the Cd HA capacity variance. Those fragments can represent genomic regions with genetic factors involved in the Cd hyperaccumulation in *T. caerulescens*. Genetic mapping of these markers would allow to test if they are physically linked on a same genomic block and associated to genes putatively involved in Cd hyperaccumulation. Thus, this approach may help to locate and identify polymorphic genes involved in Cd hyperaccumulation. However, in selfing species where individuals are more likely to be homozygous at a given locus, the recombination is less effective, and the linkage disequilibrium (LD) can be important (Flint-Garcia et al., 2003). LD is also known as a non-random association of alleles at different loci. It will also increase in small and isolated populations where the effects of genetic drift result in the consistent loss of rare allelic combinations (Flint-Garcia et al., 2003; Gupta et al., 2005). Consequently, in *T. caerulescens*, LD may allow identification of large genomic blocks with genetic factors involved in Cd hyperaccumulation but the precise identification of these factors under selection will require additional investigations.

In summary, we report a significant genetic structure of *T. caerulescens* populations regarding their Cd HA capacity. A genomic scan (with mapped genetic markers) coupled with candidate genes may allow identification of the genome blocks putatively under selection in a heterogeneous habitat (i.e. Acheré et al., 2005; Schmid et al., 2005). *T. caerulescens* ecotypes, with regard to Cd hyperaccumulation, were proposed to have different Cd hyperaccumulation pathways (Lombi et al., 2001; Zhao et al., 2002). Several candidate genes involved in heavy metal responses have been identified in *T. caerulescens* and *Arabidopsis halleri* (e.g. ZNT1, IRT1, TcHMA4, Nramp3; Bernard et al., 2004; Lombi et al., 2002; Papoyan and Kochian, 2004; Pence et al., 2000; Weber et al., 2004). As a next step, we propose to analyse the polymorphism of such genes in natural populations (Basic and Besnard, 2006) using a similar approach to this developed in the present study selecting more extreme Cd HA capacity of populations and analyzing more individuals per population. This will allow testing the supposed importance of these genes in the variation observed for the Cd hyperaccumulation mechanism and therefore in the adaptability of *T. caerulescens* populations.

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Appendix A. Supplementary data

Supplementary information for this manuscript can be downloaded at doi:10.1016/j.bse.2006.04.001.

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