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Physiological and genetic differentiation between metallicolous and non-metallicolous diploid populations of alpine *Biscutella laevigata* (Brassicacae) in the Tatra Mountains and the northern Carpathian foreland

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We investigated physiological and genetic differentiation between local, metallicolous (M) and non-metallicolous (NM) populations of the pseudometallophyte *Biscutella laevigata* (Brassicaceae) in the Tatra Mountains of the Carpathian range and in the northern Carpathian foreland. The investigated plants did not hyperaccumulate Pb, Zn or Cd. Presence of EDTA (ethylenediaminetetraacetic acid) in the substrate caused increased metal uptake (Pb, Zn) only in accessions from the M population. The content of mineral nutrients (Fe, Ca, Mg and K) was decreased in accessions from the M population grown in EDTA-enriched substrate. No significant changes in the metal uptake or mineral status were recorded in plants from the NM population. Genetic variability assessed using amplified fragment length polymorphism (AFLP) was equal in the M and NM populations. No signs of a significant decrease in genetic diversity were found in the M population. There was a strong genetic differentiation between the plants from the M and NM populations, which suggests long-term genetic isolation and vicariance between the populations.

Introduction

Toxic effects of heavy metals on plants is a widely studied topic in contemporary biology. Heavy metal toxicity is the main cause of many abnormalities in the functioning of plant cells (e.g. Wierzbicka *et al*. 2007, Przedpełska & Wierzbicka 2011, Przedpelska-Wasowicz *et al*. 2012). Plants adapted to heavy-metal-enriched environments are known as metallophytes (Ernst 1990). Within this group two distinct subgroups can be distinguished: obligate metallophytes, which can survive only on soils with elevated content of heavy metals, and facultative metallophytes (pseudometallophytes), which can survive in metalliferous soils, but are not confined to them (Antonovics *et al*. 1971). Within the latter group two types of populations exist: non-metallicolous populations (NM), developing on non-metalliferous soils, and metallicolous populations (M) developing on metalliferous soils rich in heavy metals (Pauwels *et al*. 2005). This differentiation is particularly interesting, as it can be used as a model for studies on local adaptations in plants (Linhart & Grant 1996) and on microevolution initiated by inorganic pollution (Wierzbicka & Rostański 2002, Przedpelska & Wierzbicka 2007, Wójcik *et al*. 2013, Kuta *et al*. 2014).

Biscutella laevigata (Brassicaceae) is a less studied metallophyte. It seems that it has a potential to hyperaccumulate heavy metals, like many other members of Brassicaceae do. However, only a little is known about hyperaccumulation within the species. Wenzel and Jockwer (1999) showed that the accessions investigated by them were able to hyperaccumulate lead, while Anderson *et al*. (1999) evidenced an ability to hyperaccumulate thallium. Those studies, however, were carried out only on plants growing on heavy-metal-enriched substrate. To our knowledge there is just one comparative study focused on *B. laevigata* and employing plants from metallicolous and non-metallicolous populations (Wierzbicka & Pielichowska 2004).

The geographic distribution of *B. laevigata* in Poland is very peculiar as there are only two localities separated by a distance of 120 km. The localities are in the western part of the Tatra Mountains (> 2000 m a.s.l.) and in the northern Carpathian foreland (~300 m a.s.l) (Wierzbicka & Pielichowska 2004). The lowland locality is situated on a calamine waste heap in Bolesław near Olkusz (S Poland). Silver, zinc and lead ores have been extracted and processed in the region since the 13th century. Soils with elevated content of heavy metals are widespread within the area, as well as calamine waste heaps. The waste heap in Bolesław is probably the oldest, and most investigated calamine heap within the Olkusz ore-bearing region, having been a place of floristic and toxicological research for more than a century (Uechtritz 1877, Wóycicki 1913, Dobrzańska 1955, Grodzińska *et al*. 2001, Załęcka & Wierzbicka 2002, Wierzbicka & Pielichowska 2004, Przedpełska & Wierzbicka 2007, Abratowska *et al*. 2012, Przedpelska-Wasowicz & Wasowicz 2013).

In the present study as well as in our previous studies (Wierzbicka & Pielichowska 2004) we focused on *B. laevigata* and its adaptations to calamine soils. Our previous research showed that the M and NM populations differed in many aspects including physiological and morphological characters, which can be interpreted as adaptations to dry environment (Wierzbicka & Pielichowska 2004). Therefore, it seems that the two populations followed two different evolutionary paths to adapt to temporary water deficit. We also found that plants from a calamine, metallicolous population possessed several adaptations to metalliferous soils: they had a significantly higher tolerance to zinc, cadmium and lead than plants from non-metallicolous populations in the Tatra Mts.

Variability in ploidy level within *B. laevigata* is a well-known phenomenon (Manton 1937, Tremtersberger *et al*. 2002). It was shown that diploid, relic populations are scattered across non-alpine areas, whereas tetraploid populations occur in alpine areas of France, Germany, Switzerland, Austria, Italy, and Slovenia (Tremetsberger *et al*. 2002). All the metallicolous populations described hitherto were located in alpine areas, which suggests that they are tetraploids. The diploid (Skalińska 1950) populations of *B. laevigata* studied by us have never been investigated for their physiological adaptations to heavy metal-enriched environment, metal accumulation or plant mineral status. It seems, how-

ever, that studies focused on this problem are of crucial importance for our understanding of the phenomenon of metal tolerance and accumulation within the species.

In the light of these findings we decided to go further in our research on the adaptations of *B. laevigata* to calamine soils. Our research aimed at exploring differences between the M and NM populations on the physiological level (metal accumulation potential and mineral status) and at quantifying the level of genetic differentiation between these populations at neutral loci using AFLP. We also aimed at establishing a plausible scenario that could have caused the emergence of the M population. We tested the following hypotheses:

1. Metal hyperaccumulation (Pb, Zn, Cd) takes place in diploid populations of *B. laevigata*.
2. The presence of EDTA in the substrate increases metal uptake and influences the uptake of selected mineral nutrients (Fe, Ca, Mg and K) in plants from the M and NM populations.
3. Plants from the NM populations are able to grow on polluted substrate.
4. The investigated M population was founded recently from the closest NM populations.

Material and methods

The study species

Biscutella laevigata is an alpine, perennial herb in the Brassicaceae. It is an outcrossing species with a sporophytic self-incompatibility system (Olowokudejo & Heywood 1984). Its pollen is dispersed by insects (generalist *Diptera* and *Lepidoptera*) and seed by gravity and wind (Parisod & Bonvin 2008). It is a mountain plant species occurring from the eastern Pyrenees to the Transylvanian Alps and the Carpathians. The non-metallicolous populations of the species usually grow on shallow, calcareous soils and screes (Tremetsberger *et al*. 2002). Metallicolous populations have been described from tailing basins containing waste material from furnace (Anderson *et al*. 1999), shallow dolomite soils polluted with heavy metals from smelter emission (Vidic *et al*. 2006), from surroundings of a Zn/Pb smelter, screes (calcaric regosols; Wenzel & Jockwer 1999) and from serpentine outcrops (Lombini *et al*. 1998).

Plant material sampling

Rosette leaves were collected in field, dried in silica gel and used for molecular studies. Details on the sampling locations and number of sampled individuals are given in Table 1. Unequal sample sizes in molecular studies resulted from exhaustive sampling and different sizes of the sampled populations. Samples were taken from individual plants separated by at least one metre to avoid sampling clones.

Plant cultivation

Seeds for chemical analyses were sampled in bulk from two populations (Table 1). Seeds germinated in Petri dishes on filter paper moistened with water. Seedlings were then transferred to pots with garden soil and grown for

Table 1. Geographic location of the investigated populations of *Biscutella laevigata*. M = metallicolous population, NM = non-metallicolous populations, *n* = number of individuals sampled for molecular analyses. Seeds for chemical analyses were collected from populations marked with an asterisk (*).

Population acronym	Location	GPS coordinates		Elevation (m a.s.l.)	*n*
		N	E		
M*	Bolesław, calamine waste heap	50°17´31.17´´	19°28´22.70´´	322	40
NM_1*	Jaworzynka Valley, Tatra Mts.	49°15´44.38´´	19°59´07.13´´	1097	26
NM_2	Mała Łąka Valley, Tatra Mts.	49°15´25.81´´	19°54´24.89´´	1178	5
NM_3	Stoły, Tatra Mts.	49°13´37.77´´	19°53´55.67´´	1687	6

two months. After that period the plants were transferred to pots with different substrate conditions as follows: (i) calamine soil, (ii) calamine soil + 1.5 mM kg^{-1} EDTA (ethylenediaminetetraacetic acid), (iii) garden soil (control group), (iv) garden soil + 1.5 mM kg^{-1} EDTA and grown for 4.5 weeks. Calamine soil is a substrate rich in heavy metals (40 000 mg kg^{-1} Zn, 1650 mg kg^{-1} Pb, 170 mg kg^{-1} Cd, pH = 7.3) and was collected from the Bolesław waste heap. EDTA was used to increase metal bioavailability (Liphadzi & Kirkham 2006). Plants were cultivated in a greenhouse at a photoperiod of 16 h/8 h (light/dark), in a temperature of 25 °C/18 °C, relative humidity of 45% ± 5% and light intensity of ~120 μmol m^{-2} s^{-1}. There were five to six plants in every experimental combination. In total, 46 plants were used in the study.

Atomic absorption spectroscopy

After cultivation, plants were thoroughly washed with deionized water and divided into root and shoot portions, dried in owen at 100 °C and weighed. Plant material was then ground in agate mortar. Samples of 0.5 g were incubated for 24 h in the mixture of HNO_3 and $HClO_4$ (4:1), and put in oven for 48 h at 270 °C. Subsequently, samples were diluted and the content of selected elements was determined by atomic absorption spectroscopy (Varian FS220 Flame Atomic Absorption Spectrometer) using GTA-110 graphite furnace. NIST Standard Reference Material 1575 (pine needles) and NIST Standard Reference Material 1570a (trace elements in spinach) were used as control plant material.

Statistical analysis

Non-parametric tests were employed due to the relatively small sample sizes. Data on element content collected by atomic absorption spectroscopy were analyzed using a Mann-Whitney *U*-test or a Kruskal-Wallis test ($\alpha = 0.05$ in both cases) to test for significant differences between the investigated groups. All statistical analyses were carried out using the Statistica 9.0 software (Statsoft Inc.). The value of translocation index was calculated by dividing the metal concentration in the shoot by the metal concentration in the root.

Molecular analyses

DNA isolation and AFLP fingerprinting

Genomic DNA was extracted from 77 plants using the Plant DNeasy Mini Kit (Quiagen), and was quantified spectrophotometrically. Integrity and purity of the extracted DNA was verified by 1% agarose gel electrophoresis (stained with EtBr 0.1 μg ml^{-1}) in TBE buffer.

Protocol of the AFLP fingerprinting followed Vos *et al*. (1995) with some minor modifications according to Bednarek *et al*. (2002). Samples of genomic DNA (0.5 μg) were digested with *Eco*RI/*Mse*I, followed by ligation of adaptors. For the selective amplification we used six ^{32}P-labeled primer combinations: E–AAA/M–CAA, E–AAC/M–CAC, E–AAG/M–CAC, E–ACA/M–CAA, E–ACG/M–CAA and E–AGT/M–CTT. The products of selective amplification were separated on 7% polyacrylamide gels and visualized by autoradiography.

Data analysis

AFLP patterns were compiled into a binary (presence/absence) matrix. The resulting data matrix was analyzed to estimate the parameters of genetic variation and patterns of genetic structure. Proportion of polymorphic loci (%PLP), Nei's gene diversity (Hj) and Shannon's information index (*I*) were calculated for each population as a measure of within-population diversity. Shannon's information index (Lewontin 1972) was calculated across all the loci as $I = -[p_i \log_2(p_i)]$, where p_i is the relative frequency of the *i*th AFLP fragment in a population, using POGENE 1.32 (Yeh & Boyle 1997). Genetic distance between populations was estimated using Nei's genetic distance (Lynch & Milligan 1994). Number of polymorphic loci (#Ploc), %PLP, Hj and Nei's genetic distance between populations were estimated by a Bayesian method with nonuniform prior distribution, and Wright's coefficient of inbreeding, F_{IS}, set to zero, using the AFLP-SURV 1.0 (Vekemans

et al. 2002). We also calculated an additional measure of population divergence, frequency-down-weighted marker values (DW), following Schönswetter and Tribsch (2005).

To even out the unequal sample sizes all the parameters of diversity, DW index and Nei's genetic distances (Nei 1972) were calculated on the basis of the data from five randomly chosen individuals from each population.

NJ tree was constructed on the basis of the data from all sampled individuals with Treecon 1.3b (Van de Peer & de Wachter, 1994) using the Nei and Li (1979) genetic distances. The robustness of the branches was estimated using 1000 bootstrap replicates. A NeighbourNet was constructed based on a matrix of *P*-distances, the proportion of sites at which two samples being compared is different, using the program SplitsTree 4.6 (Huson & Bryant 2006).

Bayesian analysis of genetic structure was performed with STRUCTURE 2.2.3 (Pritchard *et al*. 2000) using no admixture model and uncorrelated allele frequencies. Each simulation was run using the length of burn-in and MCMC (Markov Chain Monte Carlo) of 50 000 and 500 000 respectively. The range of possible *K*s was tested from 1 to 10. Each simulation was run 10 times in order to quantify the amount of variation of the likelihood for each *K*. Estimation of the uppermost hierarchical level of the genetic structure was made using *ad hoc* statistics *K* and followed the procedure described by Evanno *et al*. (2005). Computations were made using BioPortal (Kumar *et al*. 2009).

Analysis of molecular variance was performed with Arlequin 3.5 (Excoffier & Lischer 2010). The statistical significance of the respective fixation indices (F_{ST}, F_{SC} and F_{CT}) was tested with 10 100 permutations (Excoffier *et al*. 1992).

Results

After the end of cultivation, plant growth and the common symptoms of phytotoxicity were assessed in every experimental combination as well as in the control group. All investigated plants were in good physiological condition (leaves showed no signs of necrosis, chlorosis or of decreased turgidity), most of them were in flowering stage and were reproducing vegetatively by formation of "daughter" rosettes.

Chemical analyses

Heavy metal contents in plants

The total content of heavy metals in plants grown in the garden soil was nearly equal and low in both investigated populations: ~2.0 mg kg^{-1} d.w. for Pb, ~190 mg kg^{-1} d.w. for Zn and ~1.0 mg kg^{-1} d.w. for Cd. The addition of EDTA to the garden soil did not cause changes in the heavy metal content (Mann-Whitney *U*-test (n = 12): Pb: $U = 15$, $p = 0.6889$; Zn: $U = 16$, $p = 0.8101$, Cd: $U = 18$, $p = 0.9361$).

Plants grown in the calamine soil accumulated more heavy metals than those grown in garden soil (Mann-Whitney *U*-test (n = 11): Pb: $U = 0$, $p = 0.0081$; Zn: $U = 0$, $p = 0.0081$, Cd: $U = 0$, $p = 0.0081$). We observed differences in metal accumulation among plants grown in calamine soil. Our results indicate that plants from the NM population accumulated more Pb and Zn than plants from the M population did (Table 2) (Mann-Whitney *U*-test (n = 10): roots: Pb: $U = 0$, $p = 0.0121$; Zn: $U = 0$, $p = 0.0121$, Cd: $U = 4.5$, $p = 0.1172$; shoots: Pb: $U = 0$, $p = 0.0122$; Zn: $U = 0$, $p = 0.0122$, Cd: $U = 12.5$, $p = 0.9168$).

As for the EDTA-enriched calamine soil, plants from the NM populations had a decreased content of Zn (Table 2) when compared with plants from the M population grown in the same type of substrate (Mann-Whitney *U*-test (n = 12): roots: $U = 0$, $p = 0.0051$; shoots: $U = 0$, $p = 0.0051$). There was no significant difference in accumulation of cadmium and lead between plants from the M and NM populations grown in the calamine soil (Table 2) (Mann-Whitney *U*-test (n = 12): roots: Pb: $U = 13.5$, $p = 0.6405$; Cd: $U = 18$, $p = 0.9362$; shoots: Pb: $U = 13.5$, $p = 0.6405$; Cd: $U = 14.5$, $p = 0.6309$).

The addition of EDTA to the calamine soil caused a significant increase in accumulation of Pb and Zn only in the M population (Table 2) (Mann-Whitney *U*-test (n = 11): total content: Pb: $U = 0$, $p = 0.0081$; Zn: $U = 0$, $p = 0.0081$).

The investigated metals were accumulated mainly in the roots, and the translocation index

calculated for both populations (all treatments) and for all heavy metals studied was always less than 1 (Table 2).

The content of mineral nutrients

Plants grown in the garden soil and in the EDTA-enriched garden soil did not differ in the content of mineral nutrients, regardless of the population studied (Table 3) (Kruskal-Wallis test (n = 12): Fe: H = 0.1029, p = 0.7483; Ca: H = 0.2308, p = 0.6310; Mg: H = 0.2308, p = 0.6310; K: H = 2.0769, p = 0.1495).

Plants cultivated in the calamine soil and in the EDTA-enriched calamine soil had a significantly more elevated content of Fe and Mg than those grown in garden soil (Table 3) (Kruskal-Wallis test (n = 17): metallicolous population: Fe: H = 14.2527, p = 0.0008; Mg: H = 14.2527, p = 0.008; non-metallicolous population: Fe: H = 12.7399, p = 0.0017; Mg: H = 14.2353, p = 0.008). The content of K was significantly lower in plants grown in the calamine and EDTA-enriched calamine soils than in plants grown in the garden soil (Kruskal-Wallis test (n = 17): metallicolous population: H = 14.2353, p = 0.0008; non-metallicolous population: H = 11.1568, p = 0.0038). This sharp decrease in the K content was observed in both investigated populations (Table 3).

Our results show that the response to different growing substrates differed between the M and NM population regarding Ca. The content of this element was significantly increased in plants from the M population grown in the calamine soil, and decreased in plants grown in the EDTA-enriched calamine soil (Table 3) (Kruskal-Wallis test (n = 17): H = 14.2353, p = 0.0008). We found that plants from the NM population grown in the calamine and EDTA-enriched calamine soils had a decreased content of Ca in comparison with plants from the same population grown in the garden soil (Table 3) (Kruskal-Wallis test (n = 17): H = 11.2300, p = 0.0036).

As a rule there were no differences in the content of mineral nutrients between plants from the M and NM populations cultivated in the garden soil and EDTA-enriched garden soils (Table 3). Significant differences were found only in plants cultivated in the calamine soil. The content of Ca and K was significantly higher in plants from the M population grown in the calamine soil than in plants from the NM population grown in the same substrate (Table 3) (Mann-Whitney U-test (n = 10): U = 0, p = 0.0122 in both tests).

AFLP fingerprinting

In total, 367 unambiguously scorable fragments

Table 2. Heavy metal content (mean ± SD, mg kg^{-1} d.w.) determined by AAS and translocation indices in the *Biscutella laevigata* plants from M and NM populations grown in calamine soil and EDTA-enriched calamine soil. a and b denote significant differences in metal content between the M and NM population (Mann-Whitney U-test: α = 0.05); A and B denote significant differences in metal content between the groups of plants grown in calamine soil and EDTA enriched calamine soil (Mann-Whitney U-test: α = 0.05). Statistical significance was tested between corresponding pairs (e.g. total content of Pb, pop. M *vs.* total content of Pb, pop. NM etc.), *see* Results.

		Calamine soil (n = 5)			Calamine soil + EDTA (n = 6)		
		Pb	Zn	Cd	Pb	Zn	Cd
M	Total	567 ± 4^{a,A}	3236 ± 51^{a,A}	111 ± 27^{a,A}	892 ± 39^{a,B}	7931 ± 1029^{a,B}	257 ± 147^{a,A}
	Root	454 ± 3^{a,A}	1805 ± 28^{a,A}	101 ± 26^{a,A}	525 ± 23^{a,B}	5334 ± 898^{a,B}	246 ± 141^{a,A}
	Shoot	113 ± 36^{a,A}	1431 ± 185^{a,A}	10 ± 1^{a,A}	367 ± 122^{a,B}	2597 ± 103^{a,B}	11 ± 3^{a,A}
	Translocation index	0.25	0.8	0.1	0.7	0.5	0.05
NM_1	Total	1198 ± 430^{b,A}	5476 ± 765^{b,A}	138 ± 70^{a,A}	710 ± 149^{a,A}	4526 ± 1661^{b,A}	132 ± 26^{a,A}
	Root	1134 ± 407^{b,A}	3772 ± 540^{b,A}	128 ± 65^{a,A}	548 ± 115^{a,A}	3102 ± 1138^{b,A}	126 ± 25^{a,A}
	Shoot	64 ± 35^{b,A}	1704 ± 217^{b,A}	10 ± 4^{a,A}	162 ± 126^{a,A}	1424 ± 802^{b,A}	6 ± 2^{a,A}
	Translocation index	0.06	0.5	0.08	0.3	0.5	0.05

were found in the 77 analyzed individuals of *B. laevigata*. The total number of segregating fragments was 267, accounting for almost 73% of the total number of scorable fragments recorded. The mean number of segregating fragments per individual was 160.4. All the analyzed individuals represented different multilocus genotypes.

The number of polymorphic loci varied between populations, being the highest in the NM1 population (228) and the lowest in the M population (210) (mean ± SD = 218 ± 7.8; Table 4). The proportion of polymorphic loci varied between populations, being the highest in the NM1 population (85.4) and the lowest in the M population (78.7) (mean ± SD = 81.7 ± 2.0; Table 4). Nei's gene diversity index ranged from 0.35024 in the NM1 population to 0.30134 in the M population (mean ± SD = 0.325438 ± 0.021069; Table 4). Values of the Shannon's information index varied narrowly between 0.2825 (population NM1) and 0.2353 (population M) (mean ± SD = 0.2613 ± 0.0207; Table 4).

DW ranged from 77.98 in the M population to 58.43 in the NM3 population (mean ± SD = 65.3 ± 8.8; Table 4). The highest number of 16 private fragments per population was found in the M population. Lower number of private fragments was recorded in the NM populations, ranging from 4 (NM1) to 0 in the two remaining populations. The number of private fragments for the whole area of the Tatra Mountains was, however, much higher and amounted to 33.

The highest values of Nei's genetic distance were obtained between the M population and each of the NM populations (mean distance of 0.2368, SD = 0.0219). The lowest values of the Nei's genetic distance were obtained between the NM populations (mean distance of 0.0076, SD = 0.0086).

The Neighbour Net (Fig. 1) revealed a clear genetic structure within the analyzed data set. Individuals from the M population formed a highly supported group in the neighbour-joining analysis (bootstrap of 99). No significant genetic differentiation was found between the NM populations.

A similar structure was revealed by Bayesian clustering. The modal value of the distribution of *K* showed that the uppermost level of genetic structure in analyzed data set comprised

Table 3. Mineral status of *Biscutella laevigata* plants from the M and NM populations grown in different types of substrate. The total content of mineral nutrients determined by AAS is given in mg kg^{-1} d.w. and expressed as mean ± SD. A, B and C denote significant differences in element content in plants grown in different substrate type (Kruskal-Wallis test, $\alpha = 0.05$); a and b denote significant differences in element content in plants from M and NM population grown in the same substrate type (Mann-Whitney *U*-test, $\alpha = 0.05$).

	M				NM_1			
	Fe	Ca	Mg	K	Fe	Ca	Mg	K
Garden soil ($n = 6$)	235 ± 139^{A,a}	19636 ± 5039^{A,a}	3561 ± 1177^{A,a}	43759 ± 17164^{A,a}	199 ± 97^{A,a}	23802 ± 3896^{A,a}	4715 ± 1827^{A,a}	36299 ± 15825^{A,a}
Garden soil + EDTA ($n = 6$)	133 ± 39^{A,a}	20588 ± 4935^{A,a}	3352 ± 776^{A,a}	49976 ± 14407^{A,a}	116 ± 120^{A,a}	21188 ± 3774^{A,a}	3799 ± 1691^{A,a}	41179 ± 4458^{A,a}
Calamine soil ($n = 5$)	3728 ± 433^{B,a}	27049 ± 2957^{B,a}	11770 ± 2394^{B,a}	6469 ± 1379^{B,a}	2659 ± 1655^{B,a}	15972 ± 2333^{B,b}	15933 ± 3461^{B,a}	3009 ± 266^{B,b}
Calamine soil + EDTA ($n = 6$)	1615 ± 777^{C,a}	13288 ± 2748^{C,a}	6475 ± 1544^{C,a}	2308 ± 357^{C,a}	3189 ± 2475^{B,a}	16278 ± 3741^{B,a}	5969 ± 1350^{A,C,a}	3182 ± 285^{B,b}

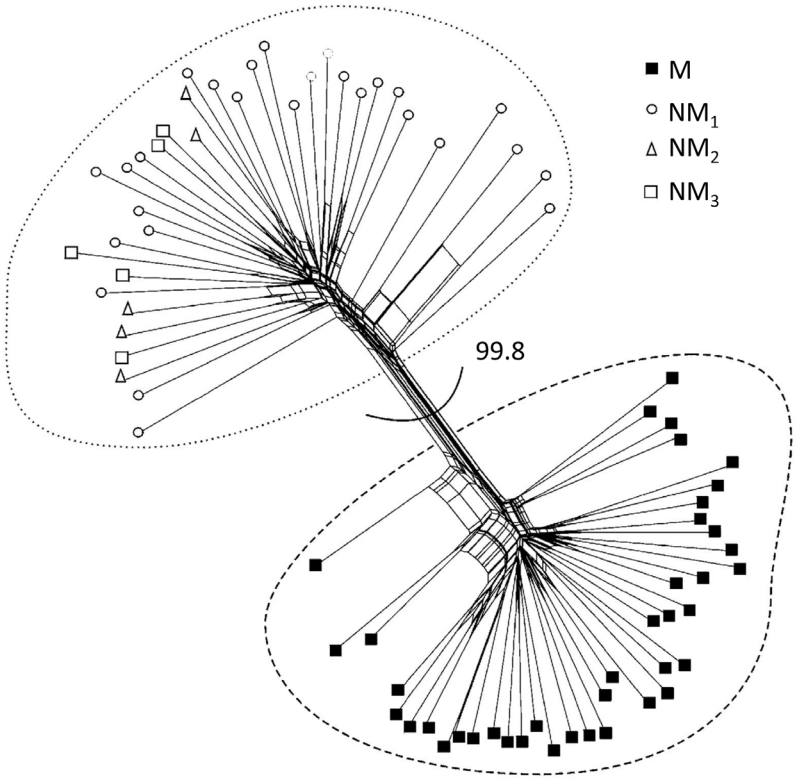

Fig. 1. NeighbourNet derived from AFLP data of *Biscutella laevigata*. The number in the middle is a bootstrap value from neighbour-joining analysis (1000 replicates). Clusters of individuals as detected by Bayesian clustering are marked with dotted and dashed lines. Population names are marked using acronyms (for explanation *see* Table 1).

two groups. Figure 1 presents the results of the Bayesian clustering projected onto the results of the NeighbourNet analysis. Both analyses revealed the same pattern of genetic structure.

A non-hierarchical AMOVA assigned 38.07% of the total genetic variation to variation among the analyzed populations (Table 5). A hierarchical AMOVA attributed 40.65% of the total observed genetic variation to variation among groups revealed by NJ and Bayesian clustering, and 57.50% of the overall genetic variation to variation within the analyzed populations. Only 1.84% of the total genetic variation was attributed to variation among populations within the analyzed groups (Table 5).

Discussion

Metal accumulation in M and NM populations

Depending on the mechanism of metal tolerance metallophytes can be divided into two groups: "excluders" and "accumulators" (Ramirez-Rodriguez *et al*. 2005). Our results combined with

Table 4. Genetic variation in the investigated populations of *Biscutella laevigata*: *n* = number of scored individuals, #loc = number of loci scored, #Ploc = number of polymorphic loci at the 5% level,%PLP = proportion of polymorphic loci at the 5% level, Hj = expected heterozygosity (Nei's gene diversity), SE = standard error of Hj, *I* = Shannon's information index, DW = divergence measure.

Population	*n*	#loc	#Ploc	%PLP	Hj	SE	*I*	DW
M	5	267	210	78.7	0.30134	0.01041	0.2353	77.98
NM1	5	267	228	85.4	0.35024	0.00909	0.2825	64.38
NM1	5	267	220	82.4	0.33332	0.00979	0.2724	60.21
NM3	5	267	214	80.1	0.31685	0.01019	0.2548	58.43

data published hitherto allow us to classify *B. laevigata* as an "accumulator". However, the question of metal hyperaccumulation in the species seems to be more complex. Escarré *et al*. (2011) found that plants growing in tailing basins were able to accumulate huge amounts (> 1.5% d.w.) of thallium in shoots. Wenzel and Jockwer (1999), investigating populations from Austrian Alps, showed that the amount of lead in the above-ground parts reached up to 1090 mg kg^{-1} d.w. On the basis of those results, *B. laevigata* could be regarded as a hyperaccumulator. Our results, however, do not agree with those findings. The plants studied by us and grown in different substrates did not have the ability to hyperaccumulate any of the investigated metals (Pb, Zn, Cd), and that lack of hyperaccumulation was observed by us in both populations (M and NM).

Studies on hyperaccumulation in plants have shown that this trait can be either constitutive (i.e. present in all the populations within the species) or not (i.e. present only in some populations). Our results indicate that the trait of metal hyperaccumulation is not present in diploid populations of *B. laevigata*, which suggests that hyperaccumulation is not a constitutive trait in this case.

Metal accumulation and mineral nutrients

Accessions from both populations (M and NM) had the potential to grow in the soil polluted with heavy metals. At the same time, significant differences were found between the M and NM populations regarding their potential of metal accumulation and content of mineral nutrients, especially under increased metal bioavailability caused by adding EDTA. Plants from the two studied populations of *B. laevigata* responded a differently to increased metal bioavailability induced by EDTA. We found that plants from the M population responded with increased metal accumulation (Pb, Zn), while the accumulation pattern observed in the NM population did not change significantly. This suggests major physiological differences between the two investigated populations.

We noticed that in plants from the M population grown in calamine soil an increased accumulation of heavy metals in the presence of EDTA was accompanied by a decreased content of mineral nutrients (Fe, Ca, Mg and K). This effect may have been caused by EDTA. Xu *et al*. (2007) showed that the uptake of many metals in EDTA-chelated form was less efficient than of those in ionic form. It was also shown that the ability to uptake EDTA-chelated metal ions may depend on the species or the metal in question (Xu *et al*. 2007). In our study, the EDTA present in the calamine substrate did not cause any changes in the content of mineral nutrients in the NM population. This difference we observed is another finding suggesting that plants from the local M and NM populations differ significantly in terms of physiology and/or cell biochemistry especially at the root level. These observations require, however, further research and evaluation.

Genetic differentiation between M and NM populations

According to our results the genetic differen-

Table 5. Analysis of molecular variance (AMOVA) for the investigated populations of *Biscutella laevigata*. Statistics include degrees of freedom (d.f.), sum of squares (SS), variance components (VC) and percentage of the total variation explained by each component (%TV). Significance levels are based on 10 100 permutations.

	Source of variation	d.f.	SS	VC	%TV	*p*
All populations	among populations	3	1010.068	19.60144	38.07	< 0.01
	within populations	73	2328.062	31.89125	61.93	< 0.01
Two groups:						
M *vs.* NM	among groups	1	928.83	22.54666	40.65	n.s.
	among populations within groups	2	81.236	1.02179	1.84	< 0.05
	within populations	73	2328.062	31.89125	57.50	< 0.01

tiation between the M and NM populations was high, despite the fact that the research was carried out only at a local scale. The mean genetic distance between the populations from the two different edaphic types was 30 times higher than the mean genetic distance between the NM populations. To our knowledge this has never been observed before and raises a question of the origin of M population.

Genetic diversity was measured in all investigated populations using different estimators. The Shannon index values obtained by us were similar to those found for alpine populations of *B. laevigata* (Parisod & Bonvin 2008). Although the level of genetic divergence was usually lowest in the M population we did not found a sharp decrease in the genetic diversity and all the calculated indices varied only narrowly. It should also be noted that the M population produced the highest number of private bands and the highest value of DW index (which is a measure of genetic divergence).

It was hypothesized that a significant decrease in genetic diversity should be expected in the M populations. Such a decrease is usually associated with the notion that in natural populations metal-tolerant genotypes are present only at low frequencies; hence, a strong genetic bottleneck effect is expected during the colonization of metalliferous soils (Bradshaw 1984). However, many empirical studies did not confirm that hypothesis and most of the works on the genetic structure of pseudometallophyte populations failed to show clear evidence of genetic bottlenecks (e.g. Vekemans & Lefebvre 1997, Mengoni *et al*. 2001). In our study, the plants from the NM populations were able to germinate (data not shown) and grow (*see* Results) efficiently on the calamine soil sampled from the M population. These findings indicate that the frequency of tolerant genotypes in the NM population was high enough to allow the colonization of calamine soils without a significant bottleneck suggested by the molecular data. We should, however, keep in mind that our experiments were carried out in greenhouse conditions and did not perfectly mimic other environmental factors present in the habitat of the M population (e.g. water deficits, interactions with other species etc.) which could be of major importance for plant fitness. Nevertheless, the considerably high metal tolerance found in the NM populations, although lower than the tolerance level found in the M population (Wierzbicka & Pielichowska 2004), could have been a cause of efficient colonization of metalliferous soils in southern Poland. This scenario, however, does not provide a plausible explanation for the strong genetic difference between the M and NM populations.

In general, two hypotheses can be proposed to explain the origin of the M population of *B. laevigata*: (1) recent long-distance dispersal from the nearest diploid populations in the Tatra Mountains, and (2) a scenario assuming that the disjunct metallicolous population is an old relic population (genetic vicariance).

The first scenario seems to be less probable, assuming the absence of human-related factors, and given the fact that *B. laevigata* possesses no adaptations to long-distance seed dispersal, having the seeds dispersed mainly by gravity (Parisod & Bonvin 2008). However, it should be remembered that long-distance dispersal is much more frequent than previously believed and that recent studies have shown some molecular evidence for presumably postglacial long-distance dispersal in species that are generally treated as "short distance dispersers" (Abbott & Brochmann 2003).

The scenario assuming a relatively old origin of the disjunct M population (vicariance) should imply a clear genetic difference between the M and NM populations that should be confirmed by clustering methods and a considerable amount of genetic differentiation attributable to edaphic subdivision (Kropf *et al*. 2006). Under this scenario, assuming long-term vicariance, a significant decrease in genetic diversity and/or lack of private and rare AFLP fragments is not expected (Kropf *et al*. 2006). It should be stressed that these expectations largely agree with our results and that a clear genetic difference was confirmed by clustering methods and AMOVA. It is usually expected that the genetic diversity in disjunct distribution areas resulting from vicariance should be fairly equal, whereas a significant decrease in genetic diversity should be observed if the disjunct population results from long-distance dispersal (Friar *et al*. 2000). The pattern of genetic variation present

in the investigated populations favors, therefore, the hypothesis assuming long-term persistence rather than recent long-distance dispersal.

Our results clearly show that the contemporary gene flow between the M and NM populations of *B. laevigata* is very limited. The patterns of genetic variation and genetic difference found between the M and NM populations suggest that isolation between these two geographically separated and genetically divergent groups is not of recent origin. The degree of genetic differentiation between the M and NM populations, measured as D_{N72} (Nei 1972, Kropf *et al*. 2009) (data not shown), may suggest that the lineage that has colonized the metalliferous site is not of recent (postglacial) origin but predates the last glacial maximum. This notion is also supported by the value of DW index obtained for investigated populations. The DW index was the highest in the M population, which indicates long-term isolation, as newly established populations are expected to exhibit low values of this index (Schönswetter & Tribsch 2005).

There are also other findings that seem to support the point of view that the M population investigated by us evolved as a product of long-term vicariance. Manton (1937) proposed a well-known and nowadays widely accepted hypothesis (e.g. Parisod *et al*. 2010) in which the diploid populations of *B. laevigata* scattered across non-alpine areas of central Europe are interglacial, if not preglacial, relics. In fact, Tremetsberger *et al*. (2002) have recently confirmed a relic status of the diploid populations. On the basis of fossil data Szafer (1930) also put forward the hypothesis of the relic character of the M population. The fact that the morphological, anatomical and physiological differentiation between the local M and NM populations, observed in our present and previous (Wierzbicka & Pielichowska 2004) research, is unlikely to evolve rapidly (i.e. within several hundred years) is further evidence supporting the vicariance scenario. This scenario has been shown to be extremely rare in metallophytes from the northern hemisphere, especially in areas deprived of vegetation during the Pleistocene glaciations (Ernst 2006). Detailed studies employing gene phylogenies and molecular dating are needed to elucidate this problem further.

Conclusions

Our research shows that: (1) Metal (Pb, Zn, Cd) hyperaccumulation is not taking place in the diploid accessions of *B. laevigata* investigated by us. (2) Presence of EDTA in the substrate changed the heavy metal uptake in the investigated plants, and accessions from the M populations responded to EDTA by an increased uptake of the investigated heavy metals (Pb, Zn) and a decreased content of mineral nutrients (Fe, Ca, Mg, K). No significant change in the heavy metal uptake was recorded in the accessions from the NM populations. (3) Plants from the NM populations have a potential to grow in heavy-metal-polluted environment. (4) We observed that the M population was not recently founded from the geographically closest NM populations. We conclude that the investigated M population could have evolved independently from a local relic population.

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