The genetic diversity of *Asplenium viride* (Aspleniaceae) fern colonizing heavy metal-polluted sites

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**Abstract**

*Asplenium viride* is a diploid species, belonging to the largest genus of the cosmopolitan fern family Aspleniaceae and occurring on various types of alkaline rocks. It is known to colonize sites with high concentrations of heavy metals, exhibiting changes in frond morphology. *A. viride* can sometimes form new substrate-dependent ecotypes that can be morphologically and genetically different from parental populations. This study aimed to evaluate the morphological and genetic diversity of *A. viride*, and test for a potential correlation between variability and heavy metal concentration. Analysis of *A. viride* specimens from one metalliferous and five non-metalliferous sites showed elevated concentrations of heavy metals in roots of metalliferous plants. The concentrations were higher in roots than in aboveground organs, especially in the case of Cd and Pb, suggesting an excluder strategy for these metals. Both metalliferous and non-metalliferous sites were populated by plants with similar ploidy levels. The overall genetic diversity was low (*H*<sub>T</sub>=0.25) and concentrated between populations (*G*<sub>ST</sub>=0.62). The obtained 2C DNA content ranged from 8.67 pg/2C to 8.69 pg/2C. STRUCTURE analysis revealed two groups among the studied populations which did not correlate with heavy metal concentrations and were not significantly supported by AMOVA. This suggests that factors influencing genetic diversity of *A. viride* are a consequence of intragametophytic selfing caused by patchy habitats and subsequent founder effects, resulting from long-distance colonization by single spores. The species has a potential to colonize heavy metal polluted sites; however, it does not seem to form genetically distinct ecotypes at those sites.

**Keywords** *Asplenium viride* · Genetic diversity · Genome size · Heavy metals · Historical mining · Ploidy level

**Introduction**

Heavy metal pollution may influence the metabolism, ecology, and microevolution of plants. An excess of heavy metals can exert harmful effects on plants due to the disturbance of important metabolic pathways by interfering with the functions of various enzymes (DalCorso 2012). This results in significant differences between affected and control plants in morphological and anatomical features such as growth rate, size, the thickness of leaves, and density of root hairs (Meyer et al. 2010; Przedpełska and Wierzbicka 2007).
High concentrations of heavy metals also have significant effects on plant reproductive processes and fertility which are manifested as disorders of male and female gametophyte development (Czapik 2002; Koivunen et al. 2004). Microevolutionary processes induced by the presence of heavy metals often result in changes in the structure and number of chromosomes, the formation of new cytotypes (Coulaud et al. 1999), changes in genome size (Temsch et al. 2010), and the generation of new genotypes (Hildebrandt et al. 2006; Słomka et al. 2011).

*Asplenium* L. is the largest genus belonging to the cosmopolitan family of Aspleniaceae Newm., which comprises about 720 species, and is found in humid tropical and temperate zones across all continents (Kramer and Viane 1990; Smith et al. 2006). Some species have a sub-cosmopolitan range, or occur as endemics on islands and oceanic archipelagos such as Madagascar, New Zealand, Juan Fernandez, and Tristan da Cunha (Brownsey 1977; Kramer and Viane 1990; Roux 1993). The mountainous regions of Asia and the Americas are secondary centers of Aspleniaceae species diversity (Kramer and Viane 1990; Werth et al. 1985). Genome size within the *Asplenium* genus ranges from 6.79 pg/2C (*A. dalhousiae* L.) to 51.45 pg/2C (*A. aethiopicum* subsp. *doecaploideum* L., Clark et al. 2015). This variation can be explained by a high variability of chromosome number and multiple polyploidization events during the speciation of the *Asplenium* genus. The ploidy of *Asplenium* species ranges from diploid to dodecaploid (base chromosome number x = 36; Smith et al. 2006; Clark et al. 2015).

Reticulate evolution also plays an important role in the *Asplenium* diversity as many species are of hybrid and allopolyploid origin and frequently exhibit parallel and recurring polyploid speciation (Dyer et al. 2012; Perrie et al. 2010; Schneider et al. 2013). *Asplenium viride* Huds. is a diploid species (2n = 2x = 72) which occurs mainly in the mountainous regions of Europe (Ivanova and Piękoś-Mirkowa 2003; Valentine and Moore 1993). In Poland, the species’ range includes the areas of the Sudetes and the Carpathians, the Kraków-Częstochowa Upland, the Lublin Upland, and the Świętokrzyskie Mountains (Zając and Zając 2001). It occurs in rock crevices, in damp and shaded places. *A. viride* prefers alkaline rocks, mostly limestone (Valentine and Moore 1993) and serpentine rocks (Johnston and Proctor 1979; Marin and Tatić 2001; Proctor and Woodell 1971), being an indicator of ultrabasic soils in some regions, for example in Scandinavia (Lepp 2001).

The aim of this study was to (1) estimate ploidy level and genome size, and evaluate morphological and genetic variability of selected populations of *Asplenium viride*, as well as to (2) ascertain whether there is a correlation between heavy metal concentration and specimen variability.

### Materials and methods

#### Study areas and sampling

Plant material was collected from both metalliferous (HM) and non-metalliferous (NM) sites in the summer of 2013. The polluted sites (heaps of waste Triassic dolomite rocks left by historical Zn-Pb ore mining) are located in western Olkusz Upland (Southern Poland), in the vicinity of the former mining village of Galman (G), approx. 5 km north-east of the town of Trzebinia. Detailed characteristics of the geology and climate of the region, the history of mining, and the mine waste heaps, as well as the vegetation covering them are described in Woch (2015) and Woch et al. (2017). Plants at the metalliferous site were collected from five heaps, scattered over approx. 2 km². Three fern specimens were taken from each heap and pooled to obtain one sample per heap (n = 5). Additionally, three soil samples (0–15 cm in depth) were collected from the same heaps and pooled into one composite sample per heap. Control ferns were taken from five non-metalliferous sites located in Southern Poland: (1) the Czupel (C) mountain (n = 4), located in the Beskid Male mountains, 6 km south-east of the city of Bielsko-Biała, 480 m ASL, the Carpathian flysch; (2) the Cisowe Skały (CS) mountain (n = 2) located in western Olkusz Upland, 5 km north-east from the town of Trzebinia, 400 m ASL, the Triassic dolomite; (3) the Murońka (Mu) mountain (n = 2) located in the Beskid Śląski mountains, 10 km south-west from the town of Żywiec, 950 m ASL, the Carpathian flysch; (4) the Kletno (K) village (n = 2) located in the Masyw Śnieżnika mountains, 7 km south-west from the town of Stronie Śląskie, 830 m ASL, the Devonian marble; (5) the Marzysz (M) village (n = 5) located in the Świętokrzyskie Mountains, 9 km south-east from the city of Kielce, 240 m ASL, the Devonian limestone.

#### Analysis of element concentrations in the soil and plant tissues

Soil samples were sieved using a 2 mm mesh and dried at 105 °C. Total As, Cd, Pb, Tl, and Zn were extracted by digestion of the ground soil with hot concentrated HClO₄ (Foss Tecator Digestor Auto). *Asplenium viride* specimens from the metalliferous site were washed carefully in running tap water, followed by double-distilled and deionized water. The plants were divided into shoots and roots, and dried at 80 °C for 48 h. To analyze As, Cd, Pb, Tl, and Zn contents, the plant material was ground and digested in a hot concentrated mixture of HNO₃ and HClO₄ (4:1; Foss Tecator Digestor Auto). Element concentrations in the soil and plant extracts were analyzed using flame or graphite furnace atomic absorption spectrometry (Varian AA280FS; Varian...
AA280Z, GTA 120). Certified reference materials were used to estimate the quality of the metal analyses in soil (CRM048-050; RTC) and plants (Oriental Basma Tobacco Leaves, INCT-OBTL-5; The Institute of Nuclear Chemistry and Technology and moss Pleurozium schreberi M2; The Finnish Forest Research Institute).

**Plant material**

Ploidy level and genome size were estimated in fresh and young leaves of *A. viride*, collected from metalliferous (HM) and non-metalliferous (NM) sites. Ploidy level was determined using the diploid plants of *A. viride* as a reference standard. Leaves of *Vicia faba* ‘Inovec’ (2C = 26.90 pg; Doležel et al. 1992) were used as an internal standard for genome size estimation. Molecular analyses were performed on 24 specimens originating from one metalliferous and five non-metalliferous sites.

**Flow cytometry measurements**

Samples for cytometric analysis were prepared according to Galbraith et al. (1983), with some modifications. Plant tissues were chopped simultaneously with a sharp razor blade in a plastic Petri dish in 1 ml of TrisMgCl$_2$ (200 mM Tris, 4mM MgCl$_2$, 0.5% v/v Triton X-100, pH 7.5) nucleus-isolation buffer, supplemented with fluorochrome 4',6'-diamidino-2-phenylindole (DAPI, 2 µg/mL) and 2% (v/v) polyvinylpyrrolidone (PVP-10) for ploidy level, or propidium iodide (PI, 50 µg/mL), ribonuclease A (50 µg/mL) and 2% (v/v) propidium iodide (PI, 50 µg/mL), ribonuclease A (50 µg/mL), and 2% PVP-10 for genome size estimation. Ploidy level was estimated by comparison of the position of the G$_0$/G$_1$ peak of the target sample on a histogram with that of the diploid reference standard. Analyses were performed for 5000–7000 nuclei using a Partec CCA flow cytometer (Partec GmbH, Münster, Germany), equipped with a mercury UV lamp. The obtained histograms were analyzed using DPAC v.2.2 software (Partec, GmbH). For nuclear DNA content, 7000–10 000 nuclei were measured using a Partec CyFlow SL Green flow cytometer (Partec GmbH, Münster, Germany), equipped with a high-grade solid-state laser with green light emission at 532 nm and side (SSC) and forward (FSC) scatters. Analyses were performed on 15 individual samples from the HM and NM sites. The obtained histograms were evaluated manually using FlowMax software (Partec, GmbH). Genome size was estimated using the linear relationship between the ratios of target species and the internal standard 2C peak positions on the histograms. The coefficient of variation (CV) of the G$_0$/G$_1$ peak of Asplenium samples ranged between 4.45 and 4.49%. The 2C DNA contents (pg) were transformed to megabase pairs of nucleotides using the following conversion: 1 pg = 978 Mb (Doležel and Bartoš 2005).

**DNA extraction**

Genomic DNA was extracted from silica gel-dried leaf tissue of HM and NM samples using a CTAB extraction protocol (Rogers and Bendich 1994) modified by Trewick et al. (2002) and Kwiatkowska et al. (2019). Leaves with the sori removed were homogenized in 500 µl 2% CTAB isolation buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA), following the addition of 50 µl 10% sodium dodecyl sulphate buffer (10% SDS, 100 mM Tris-HCl pH 8.0, 20 mM EDTA) and 5 µl 98% β-mercaptoethanol, and the whole mixture was incubated at 60°C for 1 h. An equal volume of chloroform:isoamyl alcohol mixture 24:1 (v/v) was added, mixed for 10 min, and centrifuged for 3 min at 13,000 rpm at room temperature. The collected aqueous phase was mixed with 12.5 µl sodium acetate (pH 5.2) per 100 µl of the mixture. Precipitation was performed by adding a 2/3 volume of isopropanol and incubating at -20°C overnight. DNA pellets were centrifuged at 13,000 rpm for 3 min at 4°C and rinsed in 500 µl 70% ethanol, centrifuging at 13,000 rpm for 1 min. Vacuum-dried pellets were dissolved in 50 µl nuclease-free water (EURx, Gdańsk, Poland). The quality and quantity of extracted DNA were assessed using a UV/Vis Q5000 spectrophotometer (Quawell Technology, Inc, CA, USA) and gel electrophoresis on 1% agarose gel. Only high-quality samples were used for ISSR-PCR.

**ISSR analysis**

After an initial screening of 15 ISSR primers, five were chosen for further analysis. ISSR-PCR amplifications were performed in 25 µl reaction volumes containing 2.5 µl 10X DreamTaq™ Green Buffer (Thermo Scientific), 2 µl 10 mM dNTPs (Thermo Scientific), 0.5 µl bovine serum albumin (BSA), 0.25 µl of each primer, 1 µl template DNA, and 0.25 µl DreamTaq™ DNA polymerase. PCR reactions were performed as follows: initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 44°C for 45 s and elongation at 72°C for 90 s. Final extension was performed at 72°C for 20 min. Obtained ISSR products were separated through gel electrophoresis on 1% (v/v) agarose gel. The bands were visualized using a GelDoc®-it$^2$ imager (UVP, Jena, Germany), sized using GeneRuler 1 kb Plus DNA ladder (Thermo Scientific), and scored by the presence/absence of each feature using PyElph v. 1.4 (Pavel and Vasile 2012).
### Table 1. Element concentrations (mg kg⁻¹) in the tissues of *Asplenium viride* from metalliferous site (N = 5)

|       | Shoot                      | Root                      |       |
|-------|----------------------------|---------------------------|-------|
|       | Minimum (Mean (SD)) | Maximum (Mean (SD)) | Maximum | t-test p |
| As    | 0.004 (0.023) (0.018) | 0.045 | 0.098 (0.244) (0.171) | 0.504 | 0.005 |
| Cd    | 1.1 (2.1) (0.9) | 3.5 | 10.2 (34.7) (20.8) | 56.5 | <0.001 |
| Pb    | 7 | 122 | 297 | 1474 (1341) | 3241 | <0.001 |
| Tl    | 0.04 (0.09) (0.05) | 0.18 | 0.97 | 1.50 (0.49) | 2.27 | <0.001 |
| Zn    | 117 (200) (110) | 376 | 1063 | 2992 (1641) | 5124 | <0.001 |

Student’s t-test for paired samples was used to compare element concentrations between shoots and roots.

### Statistical analysis

Student’s t-test for paired samples was used to compare element concentrations between *A. viride* shoots and roots. The nuclear DNA content results were analyzed using a one-way analysis of variance (ANOVA) and Student’s t-test (STATISTICA v. 10, StatSoft, Poland) to determine possible differences in nuclear DNA content among *A. viride* plants collected from HM and NM sites. Genetic diversity was estimated by calculating parameters both at the population and the whole study area levels. These included: number (P) and proportion (%pop) of polymorphic markers, number of private and discriminating markers (Npr, Ndis; Schlüter 2013), Nei’s gene diversity (Hj; Nei 1973), total gene diversity (Ht), mean gene diversity within populations (Hs; Nei 1973), gene diversity among populations (GST), and estimated gene flow (Nm; Slatkin and Barton 1989). All calculations were performed in FAMD v. 1.31 (Schlüter and Harris 2006) and POPGENE v. 1.31 (Yeh et al. 1999).

A dendrogram, based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and NeighborNet, was constructed using Treecon v. 1.3b (Van de Peer and De Wachter 1994) and SplitsTree v. 4.6 (Huson and Bryant 2006), based on a matrix of Nei-Li coefficients (Nei and Li 1979). Bootstrap analysis was performed using the Neighbor-Joining method, with 2000 replicates. Population genetic structure analysis was completed using Bayesian clustering inference in STRUCTURE v. 2.3 (Falush et al. 2007; Pritchard et al. 2000), following the protocol described in Migdałek et al. (2017) and Kwiatkowska et al. (2019). For these calculations, a recessive allele model for dominant markers, admixture and independent allele frequencies between clusters were assumed. Ten independent runs were performed for each K value ranging from 2 to 5, with a burn-in of 200,000, followed by 1,000,000 Markov Chain Monte Carlo replicates. The estimated mean logarithmic likelihoods of K values and ΔK values were calculated to determine the optimal K value (Evanno et al. 2005) using Structure Harvester v. 0.6 (Earl and VonHoldt 2012). STRUCTURE results were summarized in CLUMPAK (Kopelman et al. 2015), using the LargeKGreedy search method and 2000 random input repeats. A three-level hierarchical analysis of molecular variance (AMOVA) was performed in ARLEQUIN v. 3.5 (Excoffier et al. 2005), aiming at testing the statistical significance of inferred groups. Calculations were run on all individuals and population groups suggested by STRUCTURE using a pairwise difference distance matrix at P = 0.05.

### Results

#### Heavy metal contamination measurements

The presented study showed that concentrations of As, Cd, Pb, and Zn at the metalliferous site were very high. Concentrations in soils collected from heaps ranged from 11 to 237 mg As kg⁻¹, from 25 to 213 mg Cd kg⁻¹, from 776 to 5437 mg Pb kg⁻¹, from 0.40 to 2.36 mg Tl kg⁻¹, and from 3548 to 44,659 mg Zn kg⁻¹. The maximum concentrations of these elements in soil were approximately 5 (As), 20 (Cd), 10 (Pb) and 45 (Zn) times higher than allowable limits established by Polish law for forest soils (Minister of the Environment, 2016). In contrast, the Tl concentrations were closer to the values measured typically in unpolluted soils (Kabata-Pendias and Pendias 2011).

The concentrations of most elements in *A. viride* samples from the metalliferous site were elevated in both shoots and roots, with quite extreme values in the latter, when compared to the “reference plant” introduced by Markert (1992) (Table 1). The maximum concentrations in the roots reached 0.5 mg kg⁻¹ As, 56.5 mg kg⁻¹ Cd, 3241 mg kg⁻¹ Pb, 2.3 mg kg⁻¹ Tl and 5124 mg kg⁻¹ Zn (Table 1). Maximum element concentrations measured in *A. viride* roots were 5 (As), 1130 (Cd), 3241 (Pb), 45 (Tl) and 102 (Zn) times higher than in the “reference plant” (Markert 1992).

#### Ploidy level study

All samples of *A. viride* collected from HM and NM sites had the same ploidy level as the diploid reference standard (Fig. 1). The genome size of the studied plants of *A. viride* was very similar across all (HM and NM) sites and ranged from 8.67 pg/2C to 8.69 pg/2C, which correspond to 8479 and 8499 Mbp, respectively. Statistical analysis showed no
significant differences in 2C-values between the two studied habitats. The obtained histograms of genome size, along with the internal standard (V. faba ‘Inovec’), showed two distinct G₀/G₁ peaks (Fig. 2).

**Genetic diversity studies**

Samples from the HM and NM groups (24 individuals) were screened using five ISSR primers, which resulted in the amplification of 48 clearly resolved bands (6–14 per primer), with 37 polymorphic loci (Table 2). The percentage of polymorphic loci was in excess of 77%. There was a low overall total genetic diversity (HT = 0.25). The within-population diversity (HS = 0.09) was also much lower than diversity among populations (GST = 0.62). The highest within population diversity was present in the M population (H = 0.17), while the lowest was found in the K population (H = 0.02). The metalliferous group showed similar or lower diversity (H = 0.15) than some of the natural (NM) populations.

The UPGMA clustering and NeighborNet analysis showed a lack of diverging population groups (using bootstrap > 50) (Figs. 3 and 4). The Bayesian STRUCTURE analysis revealed K = 2 as the optimal number of groups (Fig. 5). One main, larger group comprised most of the populations (CS, C, G and Mu), while the remaining two populations (K and M) clustered in a second smaller group (Fig. 3). Within the M population, some of the individuals showed admixture of both genetic groups (Fig. 5). Analysis of molecular variance (AMOVA) showed the highest percentage of variation within populations (55.22%) and the lowest percentage of variation between groups (14.76%) (Table 3). The genetic variation between populations relative to the total variance was also the highest among the studied populations (FST = 0.45), while the variation between groups was the lowest (FCT = 0.15) and was not statistically significant (P = 0.072) (Table 3).
Our results indicate that *A. viride* represents the “excluder” strategy for survival on soils contaminated with heavy metals, which has also been observed by other authors (Punz et al. 1994; Punz and Sieghardt 1993). If the concentration of heavy metals is increased in the above-ground organs, this is typically due to air pollution (Mróz and Rudecki 2009). Some *A. viride* variants can exhibit high bioaccumulation coefficients in in vitro cultures with Pb (Soare et al. 2015). Sub-tropical species (e.g., *A. achilleifolium* and *A. nidus* L.) with high tolerance level and bioaccumulation of As, Pb,
and Ni can be used as hyperaccumulators for the removal of Pb and Ni from the polluted sites (Chang et al. 2009; Liu et al. 2006; Dissanayake et al. 2016).

Flow cytometry measurements revealed that all studied plants possessed intermediate genome sizes according to Soltis et al.’s (2003) classification. The mean 2C DNA content estimated for this species was 8.68 pg/2C and was within the range reported by Clark et al. (2015).

The properties of the growing substrate are known to cause morphological variability within Asplenium species which is maintained in later generations. Populations of A. adiantum-nigrum growing on calciferous substrate are morphologically distinct from serpentine populations, and often fail to germinate on serpentine soil (Sleep 1985). A. viride serpentine populations have been proved to be genetically distinct and are probably more primitive than those colonizing limestone soils (James et al. 2008). However, despite some apparent morphological traits of plant populations in heavy metal contaminated areas, notably dichotomic fronds, no evident genetic differences were found between metaliferous and non-metaliferous plants in this study. Heavy metal populations did not form a separate, highly supported clade. The studied populations did not exhibit significant differences in genetic structure when studied with Bayesian analysis. Populations from ore-bearing sites clustered together with non-metaliferous populations, which does not support the presence of a heavy metal ecotype.

All populations exhibited rather high between-population gene diversity, which could suggest some influence of intragametophytic selfing on genetic variability. Selfing as a cause of low within-population genetic variability is particularly apparent in polyploid fern species which are less vulnerable to the effects of inbreeding depression (Schneller and Holderegger 1996; Vogel et al. 1999b). Diploid species generally favor an outcrossing mating system; however, selfing can occur due to the patchy character of their habitats (de Groot et al. 2012a; Vogel et al. 1999a). Long-distance colonization from single spores can also cause founder effects that can increase variation between populations (de Groot et al. 2012a; Groot et al. 2012b). The consequences of a prior founder effect might be responsible for the lower variability observed within the heavy metal population group.

Conclusions

Our studies have shown that A. viride displays high overall tolerance to heavy metals, exhibiting an ‘excluder’ strategy. It has generally low genetic diversity, which is probably more influenced by selfing and long distance colonization from single spores rather than heavy metal concentration leading to formation of new ecotypes.

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Authors’ contributions MWW and GM conceived the idea and wrote the manuscript with input from IJ, AMS, and MP. AMS, GM, and MWW secured funding to support the work. GM, IJ, and AMS performed all the analyses and produced figures and tables. MWW and MP performed the field sampling, taxonomic revision, specimens collection, and conserved material to analyses.

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Data Availability All data and material will be made available upon request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing of interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.
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**Competing interests** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Fig. 4** NeighborNet split network performed on 24 specimens of *A. viride* based on Nei-Li distance matrix with Dice character transformation. Bootstrap was performed on 2000 replicates.
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Table 3 Results of the AMOVA performed on 6 populations of *Asplenium viride* grouped according to STRUCTURE results

| Source of variation | df | Sum of squares | Variance components | Percentage of variation | Fixation Indices |
|---------------------|----|----------------|---------------------|------------------------|-----------------|
| Among groups        | 1  | 23.24          | 1.08                | 14.76                  |                |
| Among populations within groups | 4  | 43.62          | 2.19                | 30.02                  |                |
| Within populations  | 15 | 60.38          | 4.03                | 55.22                  |                |
| Total               | 20 | 127.24         | 7.29                |                        |                |

d.f. – degrees of freedom, $F_{CT}$ – variance among groups relative to total variance, $F_{SC}$ – variance among populations within groups, $F_{ST}$ – variance among populations relative to total variance, $P$ – significance level

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