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ES designed the project, performed field research, literature survey; EMG performed morphological analyses; IJ performed flow cytometry analyses; ES, EMG, and IJ wrote the manuscript

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Competing interests

EMG is a deputy editor of the *Acta Societatis Botanicorum Poloniae*; other authors: no competing interests have been declared

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ORIGINAL RESEARCH PAPER

It is worth checking old data – validation of *Asplenium onopteris* L. presence in the most northeastern sites in Europe (Sudetes, SW Poland)

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Abstract

In Poland, isolated serpentine rocks are exclusive habitats of some *Asplenium* species, reaching here their north or northeastern border range. One of them was *Asplenium onopteris*, a diploid European species native to Mediterranean and Atlantic areas. Since the nineteenth century, Polish out-of-range sites of *A. onopteris* have been quoted in literature without critical verification. Thus, to verify occurrence of this species in Poland, we analyzed the nuclear DNA content and micromorphological features as well as critically reviewed the literature data. We proved that all individuals from Polish populations resembling *A. onopteris* were tetraploids and should be classified as *A. adiantum-nigrum*. In addition, we validated a taxon *silesiacum* reported as co-occurring with *A. onopteris*. The proposed diagnostic features are insufficient to indisputably delimit this taxon, and distinguishing it as a separate unit is not justified. Analyses of the DNA content revealed also the presence of a triploid *A. xcentovallense*, a new hybrid for Polish flora.

Keywords

Asplenium onopteris; *Asplenium silesiacum*; *Asplenium xcentovallense*; *Asplenium adiantum-nigrum* complex; flow cytometry; micromorphological features

Introduction

The European *Asplenium adiantum-nigrum* complex includes three related and morphologically variable taxa: diploid *A. cuneifolium* Viv. and *A. onopteris* L. and their stable tetraploid hybrid *A. adiantum-nigrum* L. [1]. *Asplenium cuneifolium* is associated with serpentine rocks in Southern Europe, up to southern Germany and southwestern Poland [2]. The most frost-resistant, circumpolar, and cosmopolitan *A. adiantum-nigrum* can colonize also other types of rock [3]; its European range covers the Atlantic and sub-Mediterranean area up to western Norway, southwestern Poland, Slovakia, Ukraine, and Romania [4]. *Asplenium onopteris* is the most thermophilous species, tolerant to the substratum type, with a continuous range in Macaronesia, northwestern Africa, and the Mediterranean area [4,5]. The species has also been confirmed in a discontinuous location in Ireland [5,6] and reported from Poland [6,7]. In Poland, all species of the complex with the only Central European population of *A. onopteris* and the locus clasticus of *A. silesiacum* Milde are associated exclusively with serpentine rock outcrops in the Sudetes. The latter taxon is currently included in *A. adiantum-nigrum* though

its origin, taxonomic rank, and diagnostic features vary and are sometimes contradictory [8–10]. Both last mentioned taxa were of particular interest for botanists due to suggested endemism and the distance to the continuous range.

Our research primarily focused on biology and ecology of peripheral populations of the complex due to their biological importance (e.g., [11–13]), in order to estimate the habitat conditions limiting the *A. onopteris* occurrence to only four out of several serpentine rock outcrops in Poland. The long-term continuous monitoring of *A. onopteris* populations revealed enormous morphological variability related to the fern age and habitat conditions. Therefore, the main goal of the presented research was to validate the identity of the ferns quoted in the literature as the only Central European populations of *A. onopteris*. Also, we aimed to elucidate the current status of the co-occurring taxon “*silesiacum*”. We applied three approaches: (i) a critical review of the published data; (ii) estimation of the nuclear DNA content by flow cytometry, and (iii) morphological analyses.

Material and methods

To check the changes in the nomenclature of the revised taxa, we have reviewed all available publications since the first paper by Milde [14].

The field research was conducted between 1996 and 2016 on all populations of *A. onopteris* and *A. silesiacum* reported in the Sudetes (SW Poland). As comparative material, we used *A. adiantum-nigrum* and *A. cuneifolium* sampled in the neighboring serpentine rock outcrops and *A. onopteris* collected in its continuous distribution range, in Pitigliano (Tuscany, Italy), Bari Sardo (Sardinia, Italy), and Tenerife (Canary Islands, Spain).

To determine the species identity, we estimated the nuclear DNA content and measured the size of the spores and stomata. Due to the rarity of the *A. adiantum-nigrum* complex in Poland, only one leaf was taken from each tuft. Leaves were collected from all ferns resembling *A. onopteris* and *A. silesiacum* sensu Milde (41 samples). As a reference, 31 leaves of *A. onopteris*, 31 of *A. cuneifolium*, and 34 of *A. adiantum-nigrum* were analyzed.

The nuclear DNA content was analyzed for all taxa studied from the Sudetes populations (18 leaves of potential *A. onopteris* plants, eight of *A. cuneifolium*, and 10 of *A. adiantum-nigrum*), and additionally for *A. onopteris* from Pitigliano (three leaves). Samples were prepared according to Galbraith et al. [15], with some modifications. Prior to the genome size analysis, the presence of propidium iodide (PI) staining inhibitors in plant tissues was tested [16]. Leaves of *Vicia faba* ‘Inovec’ (2C = 26.90 pg) [17] were used as an internal standard. Plant tissues of the target taxa and internal standard were chopped in 1 mL of Tris-MgCl₂ nucleus isolation buffer supplemented with 2% (w/v) polyvinylpyrrolidone (PVP-10), PI (50 µg/mL), and ribonuclease A (50 µg/mL). For each sample, 7000–10 000 nuclei were measured using a Partec CyFlow SL Green flow cytometer (Partec GmbH, Germany). At least five replicates were performed for each taxon. The obtained histograms were analyzed using FloMax software (Partec GmbH, Germany). The genome size was estimated using the linear relationship between the ratio of the target taxon and internal standard 2C peak positions on the histograms. The mean coefficients of variation of the 2C DNA content were estimated for all samples. The 1C genome size was calculated after the conversion of values into base pair numbers using the formula 1 pg = 978 Mbp [18].

The size of stomata and spores was analyzed in voucher materials. Prior to the measurements, basal fragments of dried pinnae were soaked with warm water for ca. 1 h and the strips of abaxial epidermis were peeled. Specimens were analyzed and documented by an Olympus Microscope BX50 – DP71 camera – Cell[^]B software system (Olympus Optical, Poland). The length of the spores (with perispore) and stomata was measured with AxioVision LE 4.2 software (Carl Zeiss MicroImaging GmbH, Germany). For each taxon, six to eight specimens were analyzed. For each specimen, 100 randomly selected stomata and 30 spores, when available, were measured. The results were analyzed in Statistica 12.

Results

Review of published information

The presence of *Asplenium onopteris* in the Sudetes refers mainly to one population from a serpentine rock outcrop in the Gozdnik Hill (previously Weinberg, Winna Góra; Ślęza Massif, Sudetes Foreland) discovered and recorded by Milde as *A. silesiacum* [14,19]. This name was maintained during the subsequent division of the *A. adiantum-nigrum* group into three species [19]: (i) *A. adiantum-nigrum* with five varieties, (ii) *A. serpentini* Tausch (currently *A. cuneifolium* Viv.) with four forms, and (iii) *A. silesiacum* Milde, for which *A. adiantum-nigrum* subsp. *silesiacum* was given as a synonym. Next, the last taxon was categorized following Heufler's [20] classification as a form of *A. adiantum-nigrum* subsp. *onopteris* (L.) Heufl. [21–24]. Luerrsen [25], based on herbarium materials from Central Europe, also divided *A. adiantum-nigrum* into three subspecies: *adiantum-nigrum*, *serpentini*, and *onopteris*, the latter with var. *silesiacum* exclusively from the Gozdnik Hill.

At that time, Lower Silesia was a province of Prussia but *A. onopteris* was not included in the flora of Germany [26,27] until 1903, when it was mentioned as *A. adiantum-nigrum* subsp. *onopteris* Heufl. with var. *silesiacum* Milde [28]. In the same year, the shortened name written as “*A. onopteris* Heufl.” was used by Schube [29] in reference to the Gozdnik population as a separate name of the species and a synonym of *A. adiantum-nigrum* subsp. *onopteris* (L.) Heufl. The author reported also its new stands in the Kielczyńskie Hills (Ślęza Massif) and near the village of Kamionki (Sowie Mts), both with f. *silesiacum*. Pax [30] quoted all data and presented *A. onopteris* f. *silesiacum* as an endemic taxon to the Ślęza Massif serpentine rock. In the second edition of Hegi's *Flora*, the taxon was named by Bergdolt *A. onopteris* var. *silesiacum* ([31] and re-editions in 1961, 1965). In the latest edition, *A. silesiacum* Milde was synonymized with *A. cuneifolium* and described as a form similar to *A. onopteris* [32]. It was also classified as *A. cuneifolium* var. *silesiacum* Dostál [33].

After 1945, the majority of authors quoted *A. onopteris* sites according to the last and most complete flora of Silesia by Schube [29]. *Asplenium onopteris* L. was included into Polish and European floras, keys to plant identifications [6,7,34] (additionally, [35] and earlier editions), geobotanical and floristic research [36–41], Polish checklists and red lists [42–46]. The species is protected by Polish law [47].

Field research on *A. onopteris* focused mostly on the Gozdnik Hill population. The presence of *A. onopteris* was noted here, with var. *silesiacum* [48] or subsp. *silesiacum* [49] as an endemic taxon. Żołnierz [50] questioned the distinctiveness of “*silesiacum*” as a separate taxon but reported the presence of *A. onopteris* and added its new stand from the Kamienny Grzbiet Hill (Ślęza Massif); the chromosome number was cited after the literature, without revision. Next, *A. onopteris* and f. *silesiacum* were recorded in the Gozdnik Hill but big and glossy *Asplenium* ferns in the Kielczyńskie Hills were classified as *A. cuneifolium* Viv. var. *anthriscifolium* Milde [51]. On the other hand, the presence of the species was also questioned in Kamionki (after [52]), and in general in Lower Silesia [53].

Nuclear DNA content

Three different values of the 2C DNA amount, corresponding to three ploidy levels, were obtained in the studied plants. The determined 1C genome size of all specimens ranged from 4690 Mbp to 8993 Mbp (Tab. S1). The 2C nuclear DNA content for the diploid *A. cuneifolium* was 9.59 pg, and similar for the diploid *A. onopteris* from Italy (9.04 pg), whereas for the tetraploid *A. adiantum-nigrum*, the 2C DNA amount reached 18.45 pg (Fig. S1a–c, Tab. S1). In the ferns from the putative population of *A. onopteris* in the Sudetes, the genome size was 18.38 pg/2C and typical of *A. adiantum-nigrum* tetraploid plants (Fig. S1d, Tab. S1). The presence of the diploid *A. onopteris* was excluded at all stands – almost all examined plants similar to *A. onopteris* were tetraploids. The only exception were some ferns collected in Kamionki. Their nuclear DNA content was of about 13.90 pg/2C, approximately in between diploid and tetraploid species. These specimens represented *A. xcentovallense* D. E. Meyer (Fig. S1e, Tab. S1), a triploid hybrid

between *A. cuneifolium* and *A. adiantum-nigrum*; both parental species co-occurred at the stand. In the Kielczyńskie Hills, only *A. cuneifolium* was found.

The obtained histograms of the 2C DNA content displayed distinct G_0/G_1 peaks (Fig. S1) with a mean coefficient of variation (CV) ranging from 4.82% for the analyzed diploid plants, 4.83% for triploids, to 4.87% for tetraploid plants.

Size of stomata and spores

The stomata size varied mostly in *A. adiantum-nigrum* and in the representatives of the putative *A. onopteris* from the Sudetes. In the remaining ferns, stomata size variability was less pronounced and similar in analyzed populations (Fig. 1, Tab. S1). The smallest stomata, with mean lengths ca. 46 μm , were present in *A. cuneifolium*, whereas for other taxa, mean values were between 52 μm and 60 μm . In addition, the values in diploid *A. onopteris* overlapped with those in *A. adiantum-nigrum*. Stomata in ferns from the Sudetes resembling *A. onopteris*, which according to our DNA analysis were tetraploids, were of a similar size as in *A. adiantum-nigrum* and had ca. 60 μm in length. Ferns identified as a triploid hybrid, *A. xcentovallense*, had stomata ca. 54 μm long.

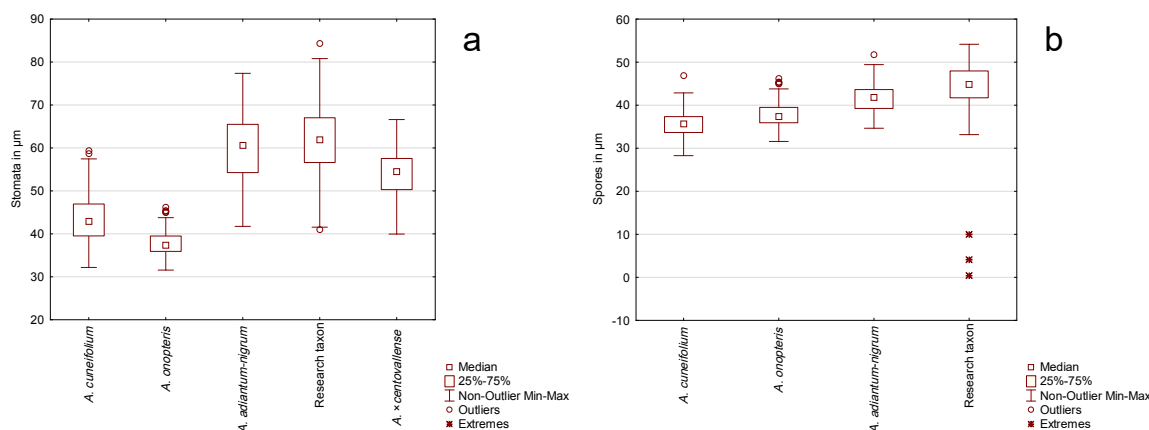


Fig. 1 Comparison of the stomata (a) and spore size (b) in studied taxa of the *Asplenium adiantum-nigrum* complex.

Spore sizes were more related to the ploidy level than stomata sizes and differed between diploid species (*A. onopteris* and *A. cuneifolium*), reaching 36–38 μm , and tetraploid *A. adiantum-nigrum*, for which spores had ca. 42 μm . Spores in ferns resembling *A. onopteris* were of similar length to the latter species, having ca. 45 μm (Fig. 1, Tab. S1). Sporangia in the hybrid *A. xcentovallense* were not properly developed and did not produce spores.

Discussion

The history of out-of-range *Asplenium onopteris* and endemic *A. silesiacum* taxa in the Sudetes started in the nineteenth century, when the peculiar fern population in the Gozdnik Hill was described by Milde [14,19]. At that time, identifications of *Asplenium* taxa were based only on macromorphology. The enormous phenotypic variability of the polyploid complex *A. cuneifolium* – *A. onopteris* – *A. adiantum-nigrum* [1] often resulted in erroneous diagnoses. Isolated populations of the allotetraploid hybrid *A. adiantum-nigrum* can be indistinguishable in gross morphology from the diploid *A. onopteris* and were sometimes deposited in herbaria under this name [5,54]. Therefore, to indisputably identify the *Asplenium* taxa, we analyzed the amount of nuclear DNA.

These analyses revealed three ploidy levels: diploid *A. cuneifolium* and *A. onopteris* from Italy, tetraploid *A. adiantum-nigrum* and revised populations from the Sudetes, and a triploid hybrid *A. xcentovallense*. The genome size of *A. onopteris* and *A.*

adiantum-nigrum established in this study was slightly larger [55] and of *A. cuneifolium* slightly lower [56] than previously reported, however, these differences could result from the different buffers and standards used.

The identification of a triploid hybrid is the first record of this particular taxon for the Polish flora. It is classified as a European nothotaxon *A. ×centovallense* D. E. Meyer nothosubsp. *centovallense* = *A. cuneifolium* subsp. *cuneifolium* × *A. adiantum-nigrum* subsp. *adiantum-nigrum* [54,57] (Fig. S2). We report a 2C DNA C value for this triploid hybrid, to the best of our knowledge, for the first time. It is similar to that given for the triploid *A. ruta-muraria* L. [55] and displayed in the Plant DNA C-value Database [58]. Regarding the DNA content, all studied *Asplenium* species would be defined as a group with intermediate genomes ($1C > 3.5 \text{ pg} < 14.0 \text{ pg}$) [59].

In polyploid series, micromorphological features are often used as indirect markers of the taxon, related to its ploidy level [60–65]. However, in the *A. adiantum-nigrum* complex, we found the stomata size less useful in taxon delimitation, varying in populations of a given taxon with values overlapping and similar to each other, regardless of the taxon ploidy. A comparable variability was earlier reported in *A. onopteris* and *A. balearicum* Shivas [66]. Likely, it is a genus-dependent feature, in some ferns not related to the ploidy level [60,62,63], and invalid in the *A. adiantum-nigrum* complex. More stable feature was the spore size, slightly differing between diploid (*A. onopteris* and *A. cuneifolium*) and tetraploid species (*A. adiantum-nigrum*); importantly, in the latter taxon, probably due to its hybrid origin, values overlap with those of both parental species. Numerous abortive or undeveloped sporangia in *Asplenium* specimens indirectly indicated the presence of a triploid hybrid [57], similarly to other fern complexes.

Three vouchers of the typus of *A. silesiacum* collected by Milde survived in the Herbarium in Berlin-Dahlem (B; Germany). They were revised and classified as *A. adiantum-nigrum* subsp. *adiantum-nigrum* var. *silesiacum* [54,67] (Fig. 2). This name was proposed exclusively for the serpentine ecotype similar to *A. cuneifolium* in gross morphology, in contrast to var. *adiantum-nigrum*, which avoids this kind of substratum [8,9,68–70]. This proposition has not been maintained, nor is valid for Polish populations of *Asplenium adiantum-nigrum* as all of them occur on serpentine rocks and are clearly distinguishable from *A. cuneifolium*. Moreover, all examined ferns of the *A. cuneifolium*-shape were diploids. The other proposition was to distinguish the *A. adiantum-nigrum* subspecies based on the origin of maternal genes and highlighting their morphological differences [10]: *silesiacum* attributed to the maternal genes of *A. cuneifolium*, and *adiantum-nigrum* to those of *A. onopteris*. Following this classification, the population in the Gozdnik Hill (locus classicus of *A. silesiacum*) currently presents only features of subsp. *adiantum-nigrum*. Young ferns or specimens growing in open sun have transiently features of subsp. *silesiacum*, which disappear in older or growing in shade tufts. These findings indicate that the taxonomic position and distinguishing features of *silesiacum* are not fully recognized yet and suggest that it is only a synonym of *A. adiantum-nigrum*.

A disjunctive distribution is quite a common phenomenon in ferns [71–73]. Homosporous ferns disperse via wind-blown haploid spores, potentially transported over thousands of kilometers. Due to the ability to reproduce through intragametophytic selfing, new fern populations can develop from a single gametophyte, i.e., from a single spore [74,75]. In this light, the presence of the disjunctive population of *A. onopteris* distant to ca. 500–600 km to the species continuous distribution area cannot be ruled out. However, more suitable habitats can be found between its continuous range and the population analyzed but, remarkably, there is no data on its presence in well-recognized floras of Germany and the Czech Republic [76,77]. The only German population of *A. onopteris*, documented in 1914, was disproved as containing exclusively tetraploid ferns and finally classified as *A. adiantum-nigrum* [78].

To conclude, we verified the identity of supposed *A. onopteris* and proved that the examined populations from the Sudetes should be classified as *A. adiantum-nigrum*; thus we disprove the presence of the species in Poland.

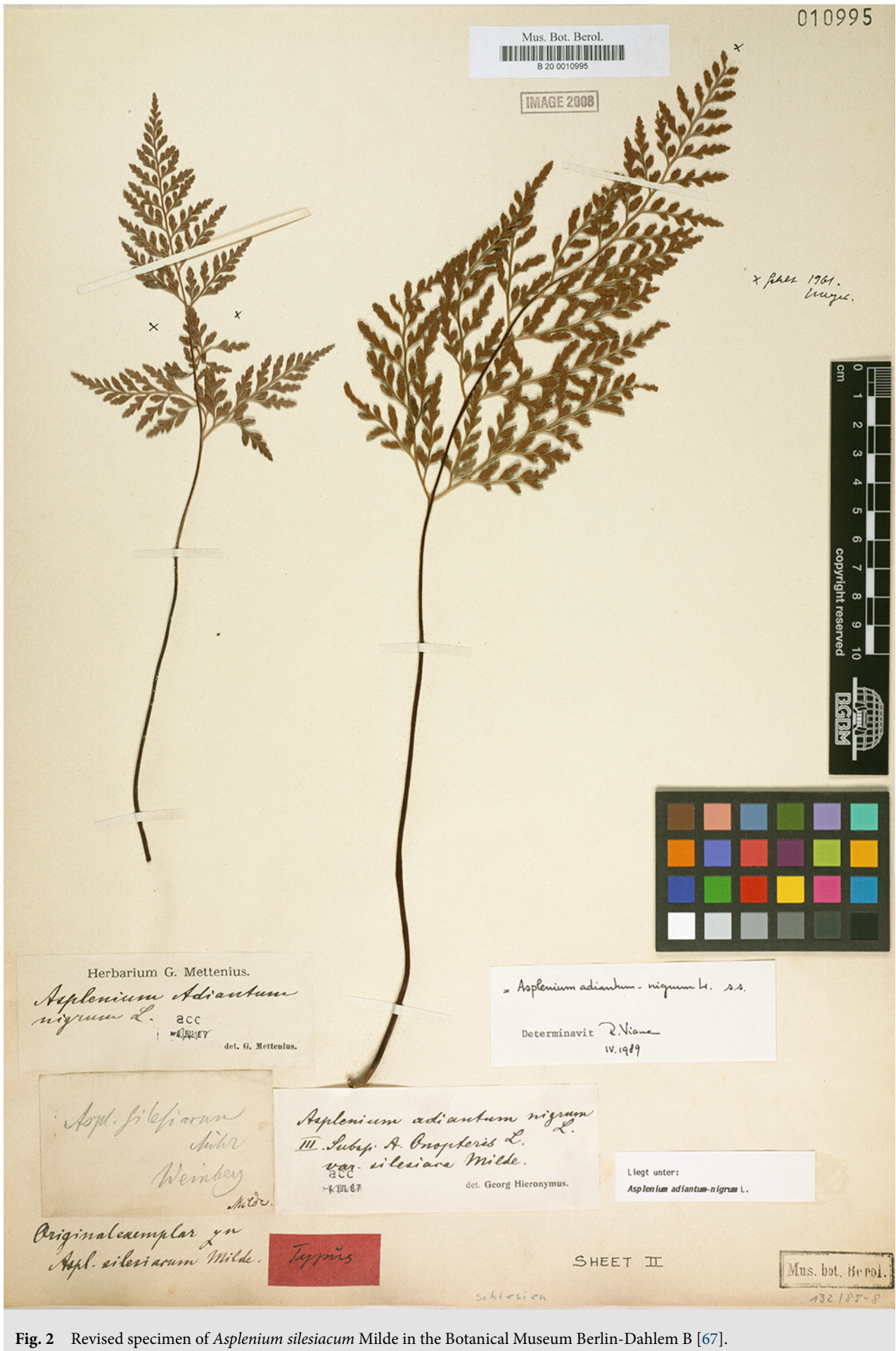


Fig. 2 Revised specimen of *Asplenium silesiacum* Milde in the Botanical Museum Berlin-Dahlem B [67].

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Supplementary material

The following supplementary material for this article is available at <http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3564/0>:

Tab. S1 Specification of chromosome number, ploidy level, nuclear DNA amount, and the size of spores and stomata in ferns representing the *Asplenium adiantum-nigrum* complex, including investigated taxon.

Fig. S1 The nuclear DNA content measured by flow cytometry of the PI-stained nuclei of *Vicia faba* (internal standard) and studied taxa of the *Asplenium adiantum-nigrum* complex.

Fig. S2 Leaves shape of *A. cuneifolium*, *A. xcentovallense*, and *A. adiantum-nigrum*; Kamionki village in the Sowie Mts.

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