



Taxonomic significance of morphological characters of spores in the family Ophioglossaceae (Psilotopsida)

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ARTICLE INFO

Article history:

Received 16 September 2017

Received in revised form 7 February 2018

Accepted 19 February 2018

Available online 21 February 2018

Keywords:

Class Psilotopsida

Key to species identification

Morphology

Spore identification

Spores

Taxonomic features

ABSTRACT

Primary and secondary ornamentation of spores of ferns of the family Ophioglossaceae are important characters, used in the taxonomy of this group. Considering the small number of published data on those characters in the Ophioglossaceae from Central and Eastern Europe, this study aimed (1) to describe morphological characters of spores of *Botrychium* and *Ophioglossum* species and to assess their taxonomic significance; (2) to analyse variation in spore size between and within species of these genera, based on specimens from various habitats and geographic locations; and (3) to create a key to species identification based on the diagnostic characters of the spore ornamentation. We examined spores of 6 species from 16 localities in Central and Eastern Europe. Results of cluster analysis based on morphological characters of spores indicate that the species form well-defined groups, partly reflecting the systematics of the Ophioglossaceae. A comparison of our results with data from North-West Europe and North America shows differences in some species. The broad range of variation in the size of spores from European populations, in comparison with American ones, may be linked with polyploidy of the studied species. Our findings will help in identification of spores of the family Ophioglossaceae in palynological and palaeobotanical studies.

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1. Introduction

Pteridophytes are divided into 4 classes: Psilotopsida, Equisetopsida, Marattiopsida, and Polypodiopsida (Smith et al., 2006, 2008). The Psilotopsida are most often subdivided into 2 families, Ophioglossaceae and Psilotaceae, less often into 5, including additionally the Botrychiaceae, Helminthostachyaceae, and Tmesipteridaceae (Smith et al., 2006; Mabberley, 2009; Christenhusz et al., 2011). Depending on classification, the Ophioglossaceae include 4–9 genera: *Botrychium* s.s., *Sceptridium*, *Botrypus*, *Japanobotrychium*, *Cheiroglossa*, *Ophioderma*, *Ophioglossum*, *Helminthostachys*, and *Mankyua* (Kato, 1987; Hauk et al., 2003; Smith et al., 2006, 2008; Mabberley, 2009; Christenhusz et al., 2011).

In Europe the family Ophioglossaceae is represented by 11 species of 2 genera: *Botrychium lunaria* (L.) Swartz, *B. matricariifolium* (Retz.) A. Braun ex Koch, *B. virginianum* (L.) Swartz, *B. simplex* E. Hitchc., *B. boreale* Milde, *B. lanceolatum* (S. G. Gmelin) Ångström, *Ophioglossum vulgatum* L., *O. lusitanicum* L., *O. azoricum* C. Presl., and *O. polyphyllum* A. Braun (Valentine and Moore, 1993). In Central and Eastern Europe, 8 species of this group are found (Mosyakin and Fedoronchuk, 1999;

Didukh and Protopopova, 2000; Mirek et al., 2002; Danihelka et al., 2012; Novikov, 2014; floraweb.de; ibot.sav.sk/checklist).

On the global scale, the genus *Botrychium* Swartz is rich in species (28), as compared to other genera of the family Ophioglossaceae. They include diploids, tetraploids, and hexaploids. Fertile tetraploids result from hybridization between species of this genus. So far no hybrids of *Botrychium* with other genera of this family have been reported (Hauk, 1995). Plants of this genus are relatively small-sized, as their sporophytes are about 1–40 cm high. Trophophores are pinnately divided into lobes of various shapes, either thin or fleshy. Sporophores are variously divided, and their shape somewhat reflects the shape of the trophophores. Sporangia spherical and thick-walled, opening through a horizontal slit.

The genus *Ophioglossum* L. comprises about 30 species. Trophophores and sporophores are not pinnate. Trophophores are single, oblong-ovate or elliptic, on average up to about 20 cm long, margin entire. The base is narrower, slightly decurrent into a petiole, embracing the sporophore. Sporophores are single, with 2 rows of 10–40 sporangia each (Smith et al., 2006).

Morphological characters (including those of spores) of the members of Ophioglossaceae are used in the systematics of this family (e.g. Clausen, 1938; Tryon and Tryon, 1982). Both studied genera have more than 1000 spores per sporangium and are eusporangiate (Smith et al., 2008; Mehltrere et al., 2010). Many researchers emphasize that

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spore ornamentation is highly valuable in taxonomic studies, especially at the species and genus level (Johns, 2000; Regalado and Sánchez, 2002; Wei and Dong, 2012; Wang et al., 2015). There are some publications on spore morphology in single or many species of this family (e.g. Nakamura and Shibasaki, 1959; Bobrov et al., 1983; Burrows, 1999; Goswami, 2007; Zenktele, 2012; Meza Torres et al., 2015), but no studies investigated the variation in spore size of species from various geographic regions and habitat types. Also keys to species identification of the Ophioglossaceae are rarely based on spore characters and most often lead to spore types and spore groups (see Mc Vaugh, 1935; Sahashi, 1979, 1980; Stafford and Paul, 2009).

Our study was aimed (i) to describe morphological characters of spores of *Botrychium* and *Ophioglossum* species found in Central and Eastern Europe, and to assess their significance; (ii) to analyse the variation in spore size between and within species of the same genus coming from populations from various geographic regions and habitat types; and (iii) to create a key to species identification on the basis of spores ornamentation of the Ophioglossaceae from Central and Eastern Europe.

2. Material and methods

2.1. Plant material

The spores used in this study originate from herbarium specimens from the Herbarium of the Department of Plant Taxonomy of Adam Mickiewicz University in Poznań (POZ), Herbarium of the M. G. Kholodny Institute of Botany of the NAS of Ukraine in Kiev (KW), and from material collected specifically for this study (Table 1). The analyses do not include *Botrychium lanceolatum*, which is extinct in Poland (Michalik, 2014), in herbarium materials no specimens with spores were found, and the species is absent from other countries of Central and Eastern Europe. For comparison, we used specimens of *Psilotum nudum* (L.) P. Beauv., from the Botanical Garden in Poznań, and results of measurements and micrographs of spores of this species are also included in this paper. From each population, a random sample of 30 spores was examined. The analysis of variation in spore size between populations from various geographic regions and habitat types, was conducted for *B. lunaria* and *O. vulgatum*.

2.2. Description of spore morphology

The spores were studied using light microscopy (LM) and scanning electron microscopy (SEM). LM was used to measure spore size: polar and equatorial diameters of spores (Fig. 1). SEM images allowed us to

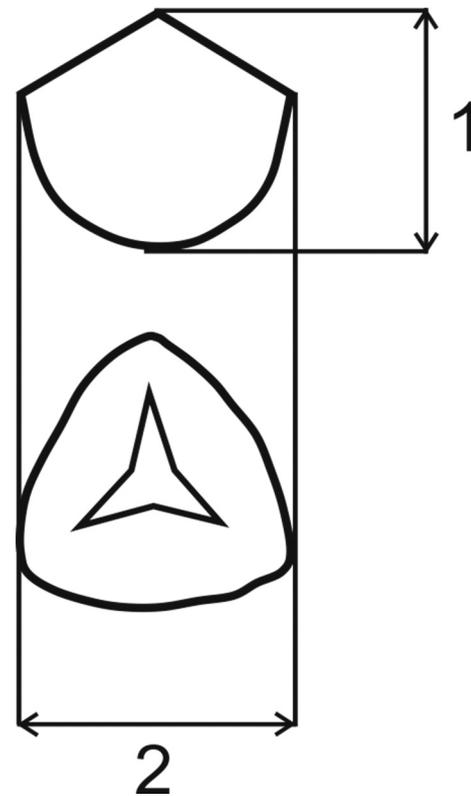


Fig. 1. Schematic diagram of spore measurements (after Nakamura and Shibasaki, 1959): 1 – polar diameter (equatorial view); 2 – equatorial diameter (proximal view).

describe the spore surface (exine). We examined primary and secondary ornamentation. Primary ornamentation was defined as the sculpture of spore surface, while secondary ornamentation consisted of tiny structures, smaller than 1 μm , found on larger elements of spore sculpture. When describing the spores, we used terms from the *Glossary of pollen and spore terminology* (Punt et al., 2007) and the commonly known terms used in palynological publications (Nakamura and Shibasaki, 1959; Lellinger, 2002; Stafford and Paul, 2009).

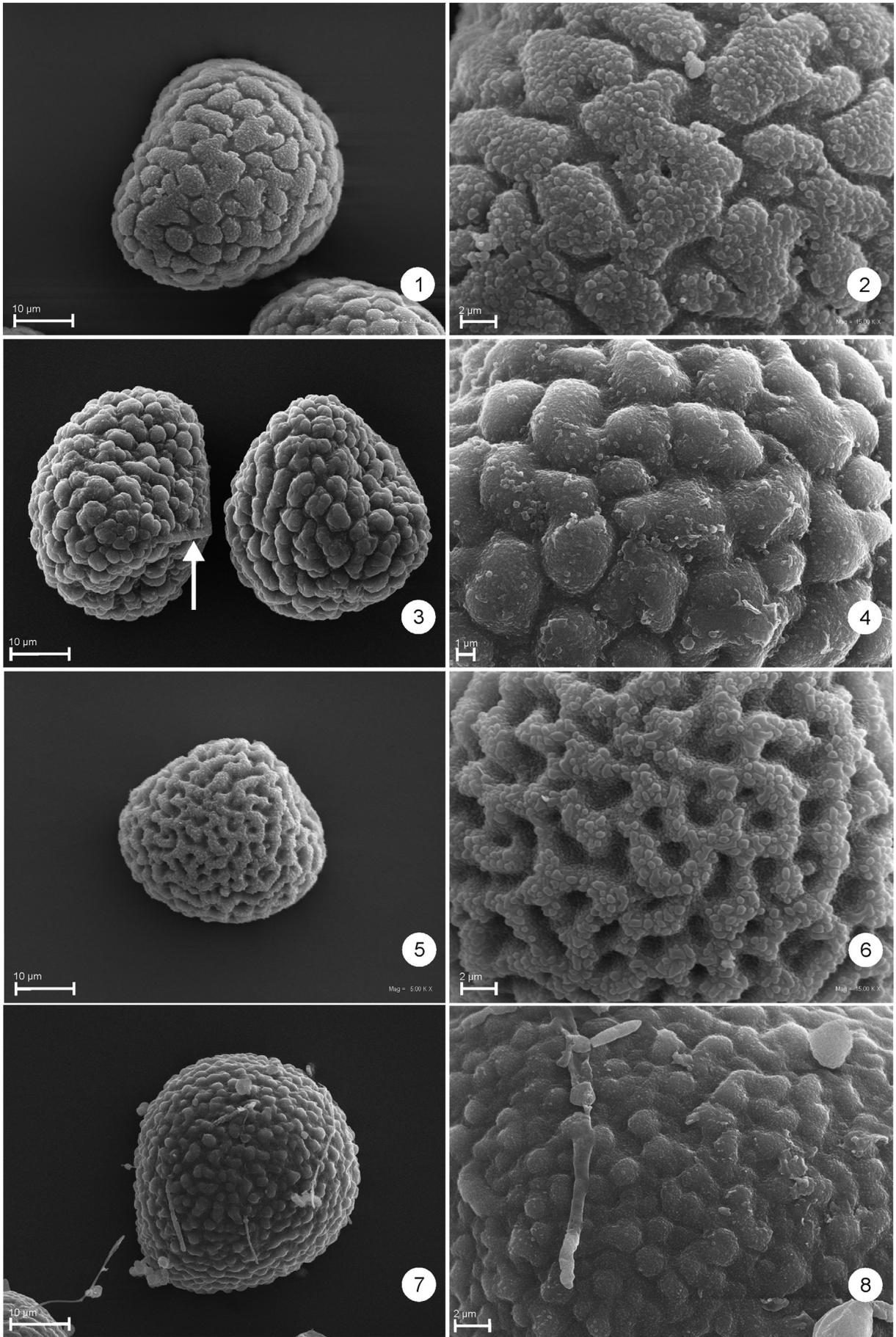
2.3. Taxonomic analysis

On the basis of our study of spore morphology, we selected the characters that can be considered crucial. These are multistate qualitative

Table 1
Origin of spore samples of ferns of the family Ophioglossaceae.

Species	Locality, habitat	Collection date	Collector ^a
<i>Botrychium simplex</i>	Wierzchowo, West Pomerania, Poland; escarpment above the lake	05.07.1905	C. Kolhshoff (POZ)
<i>Botrychium matricariifolium</i>	Taczanów Forest District, Wielkopolska region, Poland; broad-leaved forest	13.06.2013	Z. Celka, N. Olejnik
<i>Botrychium virginianum</i>	Koltiv, Lviv Oblast, Ukraine; beech forest	07.07.1936	J. Mądalski (KW)
<i>Botrychium multifidum</i>	Maśluchy, Lublin region, Poland; grassland dominated by <i>Nardus stricta</i>	22.08.2013	Z. Celka, N. Olejnik
<i>Botrychium multifidum</i>	Sianki, Podkarpacie region, Poland; partly overgrown grassland in a former timber store	20.08.2013	Z. Celka, N. Olejnik
<i>Botrychium lunaria</i>	Bobrowiec in Tatra Mts; Małopolska region, Poland; subalpine grassland	12.07.2013	N. Olejnik
<i>Botrychium lunaria</i>	Stupy, Kujawy region, Poland; meadow dominated by <i>Molinia caerulea</i>	18.06.2013	Z. Celka, N. Olejnik
<i>Botrychium lunaria</i>	Dziewicza Góra, Wielkopolska region, Poland; ash forest	02.07.2013	Z. Celka, N. Olejnik
<i>Botrychium lunaria</i>	Śnieżka (Sněžka) Massif in Karkonosze (Krkonosé) Mts.; Lower Silesia, Poland; subalpine grassland in a place where a chalet was burnt	23.07.2013	Z. Celka, N. Olejnik
<i>Botrychium lunaria</i>	Sitno, West Pomerania, Poland; xerothermic grassland	12.06.2013	Z. Celka, N. Olejnik
<i>Botrychium lunaria</i>	Shepit, Chernivtsi Oblast, Ukraine; alpine meadows	05.07.1968	O. Pidgirnyak (KW)
<i>Ophioglossum vulgatum</i>	Porąbka, Małopolska region, Poland; meadow	18.06.2014	N. Olejnik
<i>Ophioglossum vulgatum</i>	Imielenko, Wielkopolska region, Poland; sedge bed	01.08.2013	Z. Celka, N. Olejnik
<i>Ophioglossum vulgatum</i>	Skorzęcin, Wielkopolska region, Poland; meadow	19.06.2013	Z. Celka, N. Olejnik
<i>Ophioglossum vulgatum</i>	Zabudnik, Biebrza National Park, Podlasie region, Poland; meadow	23.08.2013	Z. Celka, N. Olejnik
<i>Psilotum nudum</i>	Botanical Garden of Adam Mickiewicz University in Poznań, Poland	16.06.2015	N. Olejnik

^a If spores were extracted from herbarium specimens, the collector's name is followed by the herbarium acronym in brackets; KW – Herbarium of the M. G. Kholodny Institute of Botany in Kiev; POZ – Herbarium of the Department of Plant Taxonomy of Adam Mickiewicz University in Poznań.



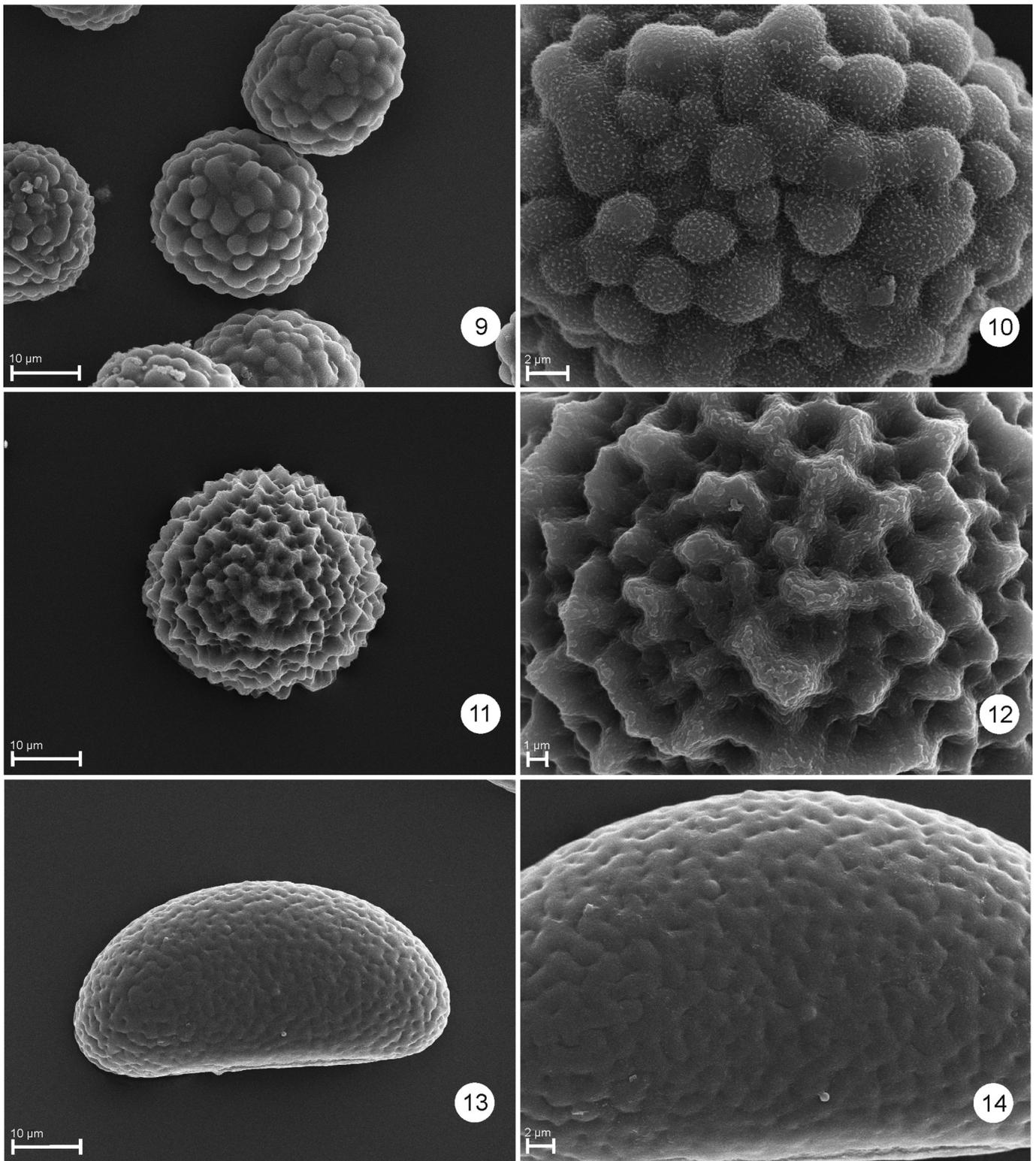


Plate I. SEM micrographs of spores of the studied species of ferns: *Botrychium lunaria* (Stupy) – distal view (1), surface sculpture (2); *B. matricariifolium* (Taczanów) – equatorial view (3 left, arrow – laesura), distal view (3 right), surface sculpture (4); *B. multifidum* (Maśluchy) – distal view (5), surface sculpture (6); *B. simplex* (Wierzchowo) – distal view (7), surface sculpture (8); *B. virginianum* (Koltiv) – distal view (9), surface sculpture (10); *Ophioglossum vulgatum* (Porąbka) – distal view (11), surface sculpture (12); *Psilotum nudum* (Botanical Garden in Poznań) – equatorial view (13), surface sculpture (14).

characters, which were used in the taxonomic analysis. It involved cluster analysis based on Ward's method.

The analysed characters include tetrad type (1 – uniplanar, tetragonal; 2 – multiplanar, tetrahedral); sculpture type (1 – poorly

developed, flat; 2 – prominent, well developed); primary ornamentation (exine) (1 – rugulate; 2 – tuberculate/verrucate; 3 – reticulate); secondary ornamentation (1 – smooth; 2 – microverrucate; 3 – papillate), and spore shape (1 – ellipsoid; 2 – nearly spherical).

2.4. Key to spore types

On the basis of the features distinguished in Section 2.3, we created a key to identification of Central European species of the Ophioglossaceae.

2.5. Statistical analysis

In the analysis of spore size, we used descriptive statistics and the χ^2 test. In the analysis of diagnostic features of spores, we used principal component analysis (PCA).

3. Results

3.1. Morphology

3.1.1. *Botrychium lunaria* (L.) Sw

Spores tetrahedral and trilete, rounded triangular in polar view (Plate I, 1, 2). Laesurae straight, reaching 2/3 of radius. In young spores, proximal surface completely sunken. Ornamentation visible on both distal and proximal surface. Primary ornamentation rugulate to irregular verrucate, muri 3–4 μm thick, striae usually 0.5 to 1 μm , verrucate all over the surface, verrucoid projection irregular. Secondary ornamentation granulate, diameter of granules 0.2–0.5 μm . Equatorial diameter 31–58 μm , polar diameter 29–49 μm .

3.1.2. *Botrychium matricariifolium* (Retz.) A. Braun ex W. D. J. Koch

Spores trilete and tetrahedral (Plate I, 3, 4), proximal face protruding. Proximal and distal faces ornamented. Laesurae straight, reaching 3/4 of radius. Primary ornamentation coliculate to tuberculate, verrucae irregular, diameter 5–6 μm ; secondary ornamentation: scattered microverrucae, diameter 0.3 μm . Polar diameter 29–39 μm , equatorial diameter 33–46 μm .

3.1.3. *Botrychium multifidum* (S. G. Gmel.) Rupr

Spores tetrahedral and trilete (Plate I, 5, 6). Laesurae long, reaching near the equator. Primary ornamentation rugulate to reticulate, muri 1–2 μm thick and irregular, with anastomosing ridges, reticulate; striae of nearly the same width. Secondary ornamentation microverrucate, verrucae larger and more irregular than in *B. matricariifolium*, diameter 0.3 to 0.5 (0.8) μm , densely covering ridges of muri, while sides of muri and striae covered with smaller microverrucae. Equatorial diameter 27–49 μm , polar diameter 24–44 μm .

3.1.4. *Botrychium simplex* E. Hitchc

Spores tetrahedral and trilete. Laesurae straight, reaching 3/4 of radius (Plate I, 7, 8). Primary ornamentation irregular verrucate to

rugulate, verruca diameter 1–4 μm , with spaces between verrucae; Secondary ornamentation granulate, granule diameter 0.1 μm . Equatorial diameter 31–37 μm , polar diameter 26–33 μm .

3.1.5. *Botrychium virginianum* (L.) Sw

Spores tetrahedral and trilete; laesurae long, reaching near the equator (Plate I, 9, 10). Primary ornamentation verrucate, verrucae circular, roundish, diameter 2–3 μm , sometimes 5(7) μm , separated by narrow striae, verrucae developed also on proximal side. Secondary ornamentation: micropapillate, papillae on the whole surface of verrucae and depressions. Well-developed, mature spores convex. Equatorial diameter 24–31 μm , polar diameter 20–27 μm .

3.1.6. *Ophioglossum vulgatum* L.

Spores tetrahedral and trilete, in polar view roundish to triangular roundish, laesurae straight, long, reaching 2/3 of radius (Plate I, 11, 12). Primary ornamentation lopho-reticulate, spore surface covered with projecting, anastomosing ridges forming an open angular, irregular reticulum (limited to proximal face). Muri thick at base, irregular in height, sometimes tapering to narrow, sharp edges, and relatively narrow lumina. Secondary ornamentation: the whole surface of muri covered with microgranules of about 0.1 μm in diameter. Equatorial diameter 31–52 μm , polar diameter 27–51 μm .

3.1.7. *Psilotum nudum* (L.) P. Beauv

Spores uniplanar, tetragonal, and monoete (Plate I, 13, 14). Primary ornamentation rugulate/reticulate (pitted) muricate, muri low, hardly visible. Secondary ornamentation: whole surface covered with microgranules. Equatorial diameter 58–82 μm , polar diameter 24–41 μm .

3.2. Variation in spore size

The 6 studied species of the family Ophioglossaceae significantly differed in spore size, in respect of polar diameter ($\chi^2 = 61.65729$, $p = 0.00000$) and equatorial diameter ($\chi^2 = 71.30769$, $p = 0.00000$). Interpopulation variation in spore size was investigated for *O. vulgatum* and *B. lunaria* on the basis of spores originating from 4 and 6 populations, respectively.

The analysis of spore size in *O. vulgatum* revealed that irrespective of geographic location and habitat type, the mean, minimum, and maximum dimensions of spores were similar (Fig. 2). Thus geographic location and local environmental conditions apparently do not affect spore size. A similar pattern of variation in spore size was observed in *B. lunaria* (Fig. 3). In this species, our results show that 4 out of 6 studied populations have spores of similar size, but the remaining 2 populations slightly deviate from the others. This applies to a population from a

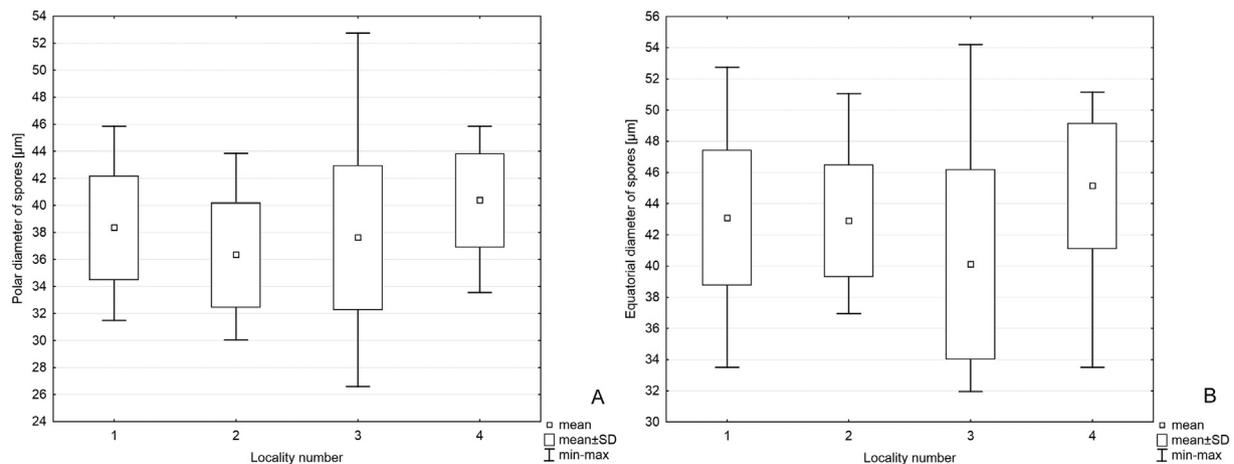


Fig. 2. Diagrams of interpopulation variation in spore diameter of *Ophioglossum vulgatum*: A – polar diameter (equatorial view); B – equatorial diameter (proximal view); 1 – Imielenko; 2 – Porąbka; 3 – Skorzęcin; 4 – Zabudnik.

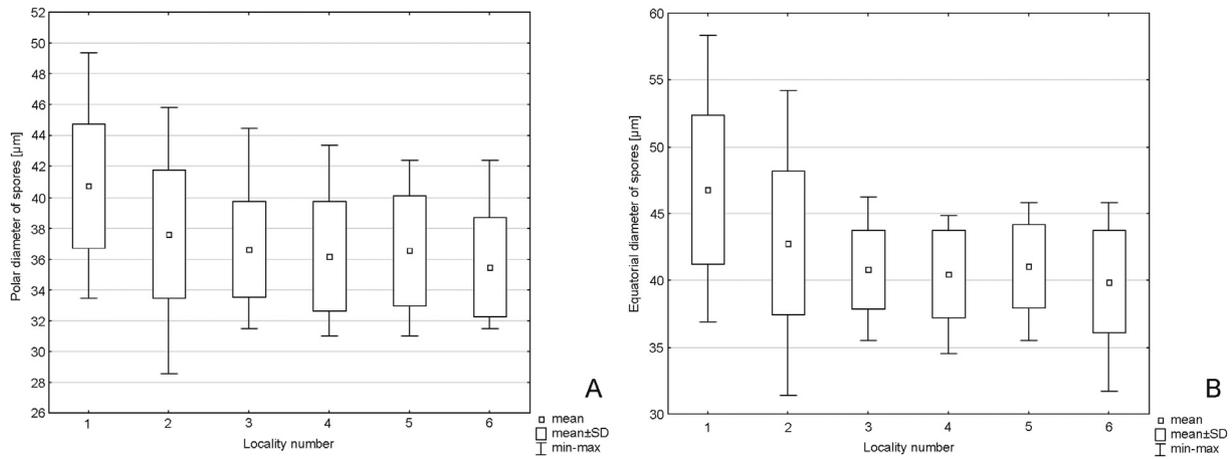


Fig. 3. Diagrams of interpopulation variation in the spore diameter of *Botrychium lunaria*: A – polar diameter (equatorial view); B – equatorial diameter (proximal view); 1 – Słupy; 2 – Bobrowiec in the Tatra Mts.; 3 – Dziewicza Góra Mt.; 4 – Śnieżka (Sněžka) Massif in the Karkonosze (Krkonoše) Mts.; 5 – Sitno; 6 – Shepit.

meadow dominated by *Molinia caerulea* (L.) Moench (Poaceae) in central Poland (Słupy) and a population from a subalpine grassland in the Tatra Mts. Spores from those populations have broader ranges of variation and slightly higher mean values, but the differences are small. The typical values determined by the mean \pm standard deviation overlap, and only maximum values clearly distinguish those populations (Fig. 3). Similar results of analysis of raw data are provided by PCA conducted for both species on the basis of spore dimensions (Figs. 4–5).

Apart from variation in spore size between some populations, we also observed qualitative differences during analysis of SEM images. Those differences concern spore ornamentation in *B. lunaria*. Among the studied populations of *B. lunaria*, spore ornamentation in the Tatra Mts is distinct, in comparison with the other 5 populations. The muri forming primary ornamentation are more crowded there, anastomosing, resulting in reticulate ornamentation, in contrast to spores from the other populations, where ornamentation is rugulate to irregular verrucate (Plate II).

3.3. Key to spore types

Using the analysed characters of spores (see Section 2.3), we constructed a key to species identification.

- 1 – Spore sculpture rugulate, rugulate-reticulate or reticulate 2
- 1* – Spore sculpture verrucate or tuberculate 4
- 2 – Muri with rounded edges 3

- 2* – Muri with narrow, sharp, irregular edges *O. vulgatum*
- 3 – Striae up to 1 μ m wide, several-fold narrower than muri *B. lunaria*
- 3* – Striae more than 1 μ m wide, nearly as wide as muri *B. multifidum*
- 4 – Verrucae regular, nearly hemispherical, densely covered with micropapillae *B. virginianum*
- 4* – Verrucae irregular, not covered with micropapillae 5
- 5 – Verrucae of uneven size, often elongated, usually 2–3 μ m across *B. simplex*
- 5* – Verrucae irregular, knob-like, large, often more than 5 μ m across *B. matricariifolium*

3.4. Cluster analysis

The cluster analysis based on morphological characters of spores allowed us to group the studied species (Fig. 6). One group is composed of *B. lunaria* and *B. multifidum*, which are clearly distinct from the other studied Ophioglossaceae. Another distinguished group consists of *B. simplex* and *B. matricariifolium*. A separate position is occupied by *B. virginianum*, as well as by *O. vulgatum*. *Psilotum nudum*, which has a completely different type of spores, is also clearly separated from the other analysed species.

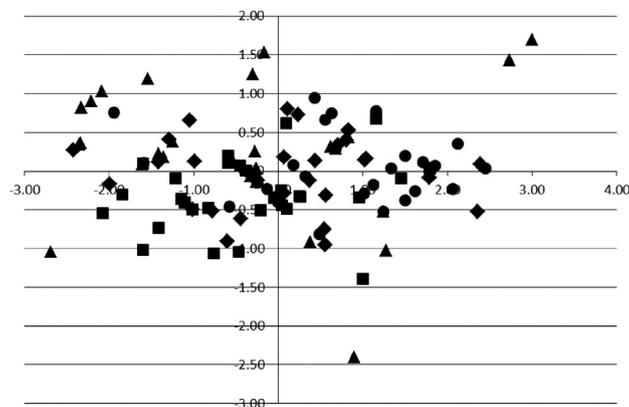


Fig. 4. Results of principal component analysis (PCA) of interpopulation variation in spore diameter of *Ophioglossum vulgatum*: 1 – Imielenko; 2 – Porąbka; 3 – Skorzęcin; 4 – Zabudnik.

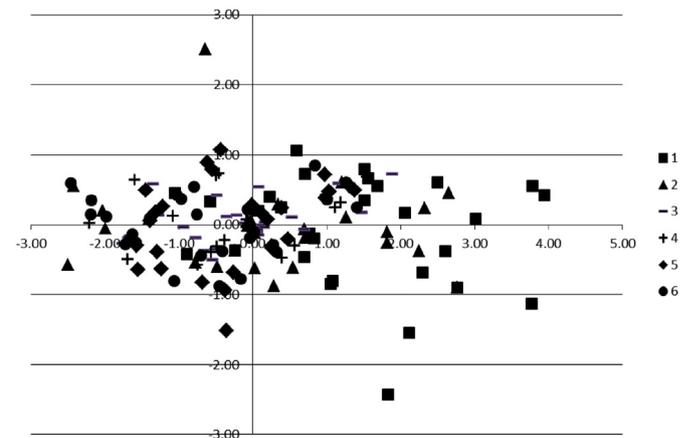


Fig. 5. Results of principal component analysis (PCA) for interpopulation variation in spore diameter of *Botrychium lunaria*: 1 – Słupy; 2 – Bobrowiec in the Tatra Mts.; 3 – Dziewicza Góra Mt.; 4 – Śnieżka (Sněžka) Massif in the Karkonosze (Krkonoše) Mts.; 5 – Sitno; 6 – Shepit.

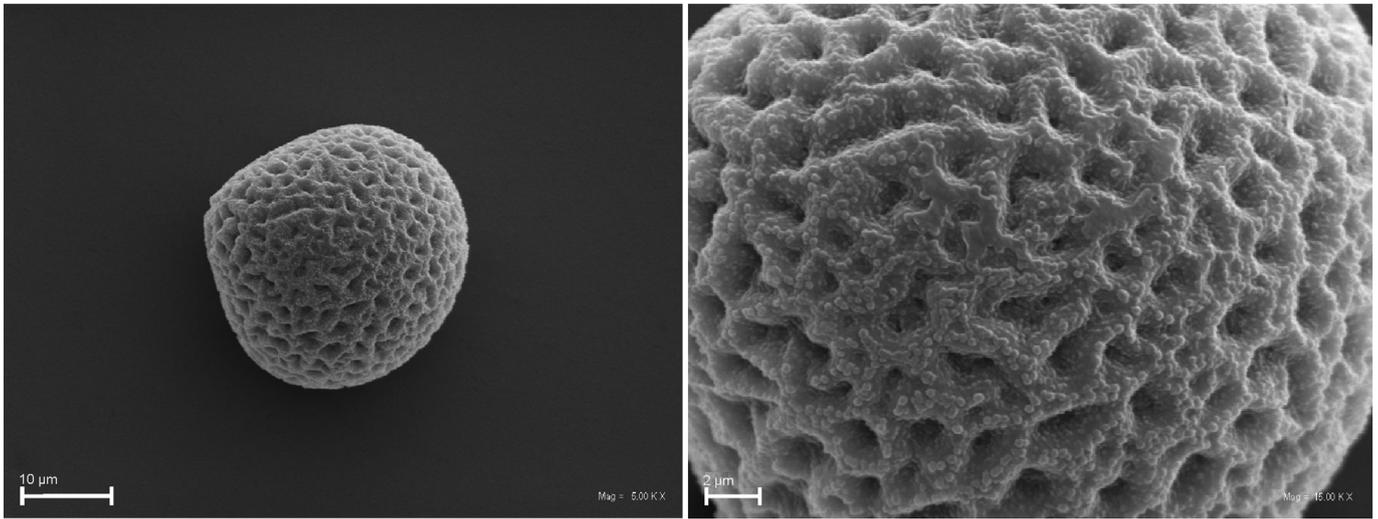


Plate II. Morphology of spores of *Botrychium lunaria* from the population in the Tatra Mts., distal view (left), surface sculpture (right).

4. Discussion

The performed spore measurements, especially the analysis of inter-population variation in spore size of ferns of the family Ophioglossaceae, allowed us to determine the ranges of variation in their spore dimensions in Central and Eastern Europe. In comparison with the available data from North-West Europe (Stafford and Paul, 2009) as well as East and North-East Europe (Bobrov et al., 1983), remarkable differences in some species are noticeable (Table 2). In *O. vulgatum* and *B. multifidum*, spore size is rather similar, but in *B. matricariifolium* spores from Central Europe as well as East and North-East Europe (Bobrov et al., 1983) are markedly smaller, up to 45 µm in diameter, whereas Stafford and Paul (2009) report that they are up to 65 µm across. Even greater differences concern spore size in *B. simplex* (Table 2). In our study, its spores were up to 37 µm in diameter, compared to up to 62 µm or even 87 µm in other parts of Europe. In comparison to *B. lunaria*, the analysis of intraspecific variation shows that spores of *B. simplex* from the population in central Poland are similar in size to those described by Stafford and Paul (2009), and spores from the Tatra Mts are also similar to them, but the other populations have much smaller spores (see Fig. 3), similar in size to that reported by Bobrov et al. (1983). Spores of *B. lunaria* from

Central and Eastern Europe clearly differ from those of North American specimens (Farrar, 2006), very much like spores of *B. simplex* (Table 2). Farrar (2006) suggested that spore size may be linked with ploidy level. Thus we can presume that the broad range of variation in the size of spores from European populations, in comparison with American ones, may be linked with polyploidy of the studied species. However, this hypothesis needs to be confirmed by genetic research.

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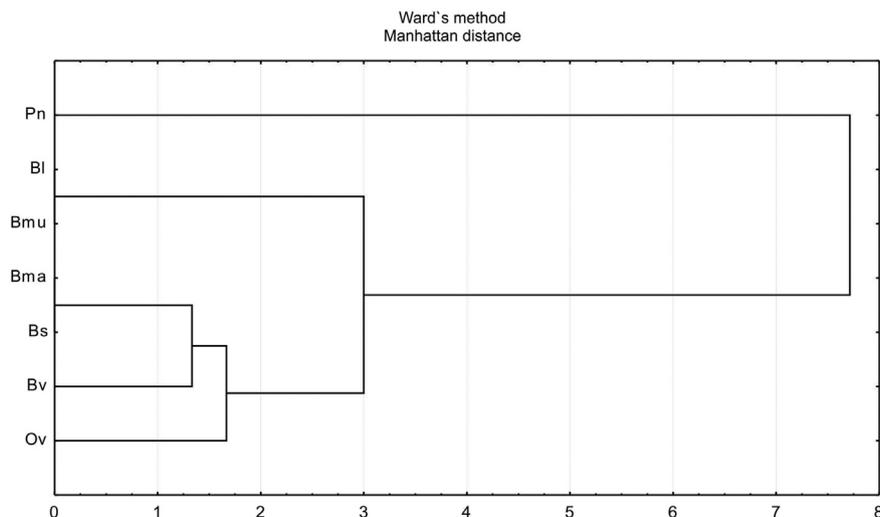


Fig. 6. Dendrogram of spore similarity based on cluster analysis: Ps – *Psilotum nudum*; Bl – *Botrychium lunaria*; Bma – *B. matricariifolium*; Bmu – *B. multifidum*; Bs – *B. simplex*; Bv – *B. virginianum*; Ov – *Ophioglossum vulgatum*.

Table 2
Comparison of spore size (polar diameter × equatorial diameter in μm) of the studied species of Ophioglossaceae based on selected sources.

Species	Nakamura and Shibasaki, 1959	Bobrov et al., 1983	Stafford and Paul, 2009	Farrar, 2006	Original data
<i>Botrychium lunaria</i>	33.5–40.5 × 49.8–56.2	24.5–33.0 × 35.0–40.5 (53.3)	24.0–40.0 × 42.0–62.0	24.0 × 32.0	29.0–49.0 × 31.0–58.0
<i>Botrychium matricariifolium</i>	–	26.0 × 39.6–43.2 (46.8)	22.0–38.0 × 40.0–65.0	–	28.6–38.9 × 33.5–45.9
<i>Botrychium multifidum</i>	–	28.8–32.4 × 32.4–36.0 (39.6)	23.0–34.0 × 36.0–47.0	–	24.0–44.0 × 27.0–49.0
<i>Botrychium simplex</i>	–	(32.0) 35.0–42.0 (48.0) × 54.0–57.6 (62.0)	32.0–55.0 × 60.0–87.0	40.0 × 50.0	26.0–33.0 × 31.0–37.0
<i>Botrychium virginianum</i>	20.3–23.7 × 25.2–30.8	18.0–21.6 × (30.0) 32.4–36.0	–	–	20.0–27.0 × 24.0–31.0
<i>Ophioglossum vulgatum</i>	25.8–30.2 × 35.7–40.3	18.0–23.4 × 38.0–50.4	30.0–40.0 × 42.0–50.0	–	27.0–51.0 × 31.0–52.0

the other populations have much smaller spores (see Fig. 3). Spores of *B. lunaria* from Central and Eastern Europe clearly differ from those of North American specimens (Farrar, 2006), very much like spores of *B. simplex* (Table 2). Farrar (2006) suggested that spore size may be linked with ploidy level. Thus we can presume that the broad range of variation in the size of spores from European populations, in comparison with American ones, may be linked with polyploidy of the studied species. However, this hypothesis needs to be confirmed by genetic research.

During the analysis of intraspecific variation of *B. lunaria*, we examined many samples of spores, collecting also spores from closed sporangia. We then made observations, rarely reported in the literature (see Stafford and Paul, 2009), concerning changes in spore morphology depending on their stage of development (Plate III). Immature spores

are covered with a dense layer, which masks their ornamentation (Stafford and Paul, 2009). This is particularly important when examining spores from herbarium specimens, where it is sometimes difficult to determine if they are mature or not, but the differences can be noticed in some studies (see Tryon and Lugardon, 1991; Goswami, 2007; Stafford and Paul, 2009).

The cluster analysis based on morphological characters of spores revealed interesting relationships. One group is composed of *B. lunaria* and *B. multifidum*, whereas these species are included in different subgenera or even different genera *Botrychium* and *Sceptridium* (Hauk et al., 2003). They are clearly distinct from the other studied Ophioglossaceae. Another group consists of *B. simplex* and *B. matricariifolium*. Separate positions are occupied by *B. virginianum* – classified often as a different subgenus or genus, as *Botrypus virginianus*

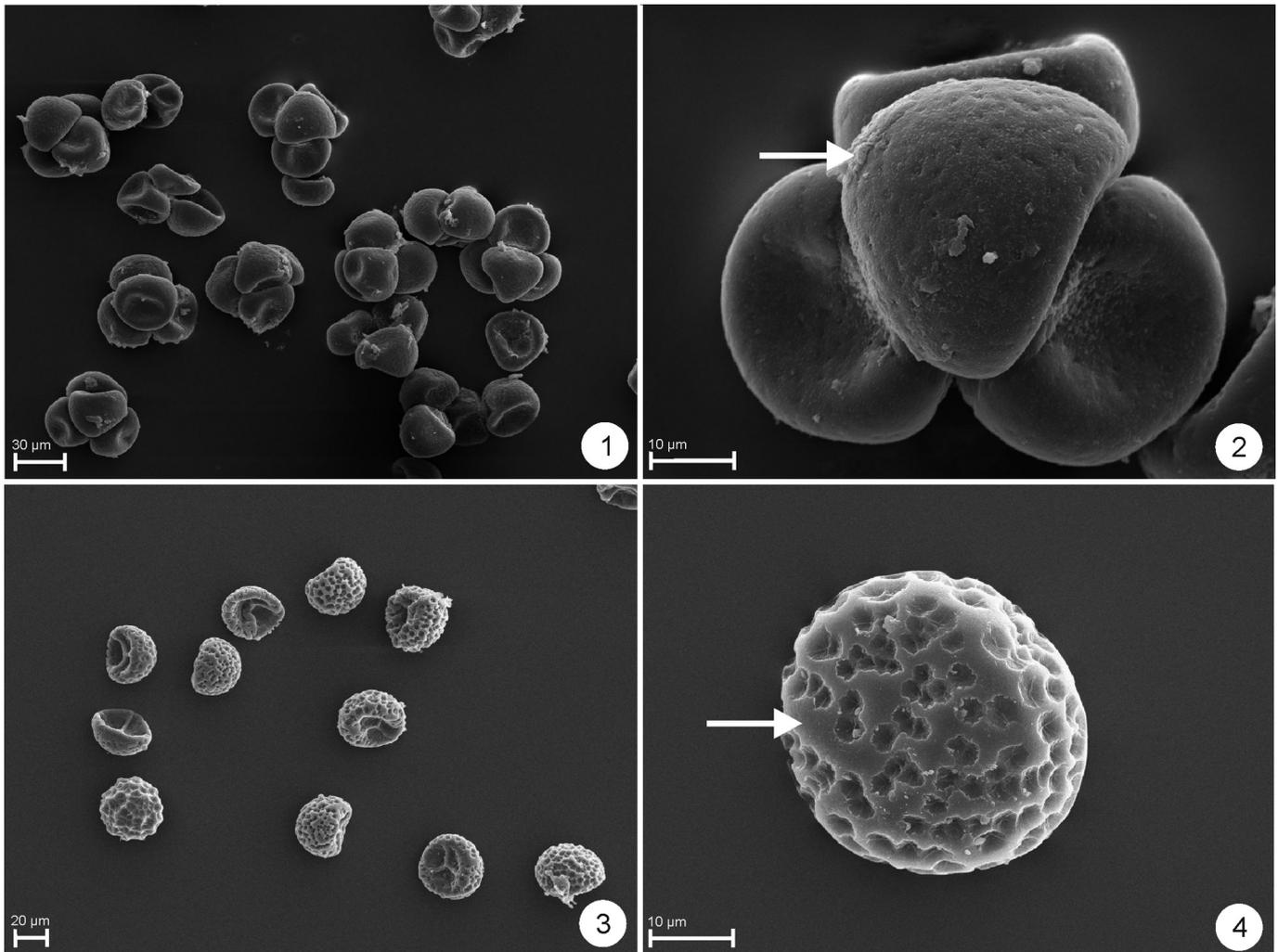


Plate III. Immature spores of *Botrychium lunaria* (1, 2) and *Ophioglossum vulgatum* (3, 4). Arrows – the covering layer of immature spore.

(L.) Michx. (Hauk et al., 2003) – as well as by *O. vulgatum*. As expected, *Psilotum nudum*, as a member of the family Psilotaceae (Christenhusz et al., 2011), is clearly separated from the other analysed species.

Our findings provide new, additional features that can be used in taxonomic analyses of the family Ophioglossaceae (Tryon and Lugardon, 1991; Stafford and Paul, 2009) and certainly can help in identification of spores of this family in palynological and palaeobotanical studies (Demske et al., 2013; Meza Torres et al., 2015; Shatilova et al., 2016).

Acknowledgements

We are grateful to the Regional Directorates of Environmental Protection in Bydgoszcz, Katowice, Lublin, Poznań, and Szczecin, directors of the Biebrza, Bieszczady, Karkonosze, and Tatra National Parks, and curators of herbaria in the Department of Plant Taxonomy in Poznań (POZ), and the M. G. Kholodny Institute of Botany in Kiev (KW), who permitted us to collect spores of the studied species. Special thanks are due to the Head of the Botanical Garden of Adam Mickiewicz University in Poznań, who gave us access to specimens of *Psilotum nudum*, as well as to the Laboratory of Electron and Confocal Microscopy, for professional SEM micrographs of the spores.

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