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

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A B S T R A C T

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 species 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., Septrium, Botrypus, Japanobotrychium, Cheioglossa, Ophioderma, Ophioglossum, Helminthostachys, and Munkyua (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 sporophores 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; Mehltreter et al., 2010). Many researchers emphasize that
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; Zenkteler, 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 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality, habitat</th>
<th>Collection date</th>
<th>Collector*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botrychium simplex</td>
<td>Wierczowo, West Pomerania, Poland; escarpment above the lake</td>
<td>05.07.1905</td>
<td>C. Kohlstoff (POZ)</td>
</tr>
<tr>
<td>Botrychium matrixicarifolium</td>
<td>Taczadow Forest District, Wielkopolska region, Poland; broad-leaved forest</td>
<td>13.06.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium virginianum</td>
<td>Kołtiv, Lviv Oblast, Ukraine; beech forest</td>
<td>07.07.1936</td>
<td>J. Maďalski (KW)</td>
</tr>
<tr>
<td>Botrychium multifidum</td>
<td>Małuchy, Lublin region, Poland; grassland dominated by Nardus stricta</td>
<td>22.08.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium multifidum</td>
<td>Sianki, Podkarpackie region, Poland; partly overgrown grassland in a former timber store</td>
<td>20.08.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Bobrowiec in Tatra Mts; Małopolska region, Poland; subalpine grassland</td>
<td>12.07.2013</td>
<td>N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Słup, Rujary region, Poland; meadow dominated by Molinia caerulea</td>
<td>18.06.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Dziewicza Gór, Wielkopolska region, Poland; ash forest</td>
<td>02.07.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Śnieżka (Sněžka) Massif in Karkonosze (Krkonoše) Mts.; Lower Silesia, Poland; subalpine grassland in a place where a chalet was burnt</td>
<td>23.07.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Sitno, West Pomerania, Poland; xerothermic grassland</td>
<td>12.06.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Botrychium lunaria</td>
<td>Shipit, Chernivtsi Oblast, Ukraine; alpine meadows</td>
<td>05.07.1968</td>
<td>O. Pidigrnyak (KW)</td>
</tr>
<tr>
<td>Ophioglossum vulgatum</td>
<td>Porąbka, Małopolska region, Poland; meadow</td>
<td>18.06.2014</td>
<td>N. Olejnik</td>
</tr>
<tr>
<td>Ophioglossum vulgatum</td>
<td>Imielenko, Wielkopolska region, Poland; sedge bed</td>
<td>01.08.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Ophioglossum vulgatum</td>
<td>Skorzęcin, Wielkopolska region, Poland; meadow</td>
<td>19.06.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Ophioglossum vulgatum</td>
<td>Zabudnik, Biebrza National Park, Podlasie region, Poland; meadow</td>
<td>23.08.2013</td>
<td>Z. Celka, N. Olejnik</td>
</tr>
<tr>
<td>Psilotum nudum</td>
<td>Botanical Garden of Adam Mickiewicz University in Poznań, Poland</td>
<td>16.06.2015</td>
<td>N. Olejnik</td>
</tr>
</tbody>
</table>

* 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.
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 $\chi^2$ 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). Laesaeae 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: micropapillate, papillae on the whole surface of verrucae and de-}

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

Spores uniplanar, tetragonal, and monolete (Plate I, 13, 14). 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.

3.1.3. Botrychium multiradiatum (S. G. Gmel.) Rupr

Spores tetrahedral and trilete (Plate I, 5, 6). Laesaeae long, reaching near the equator. 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.

3.1.4. Botrychium simplex E. Hitchc


3.1.5. Botrychium virginianum (L.) Sw

Spores tetrahedral and trilete; laesaeae long, reaching near the equator (Plate I, 9, 10). Primary ornamentation verrucate, verruculae 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, laesaeae 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


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). Inter-population 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 – Tarnobrzeg; 2 – Porąbka; 3 – Skorzęcin; 4 – Zabudnik.
Those differences concern spore ornamentation in also observed qualitative differences during analysis of SEM images.

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 *Botrychium 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 tuberculcate ………………………………. 4
2 – Muri with rounded edges ……………………………………………………… 3
2* – Muri with narrow, sharp, irregular edges ……………… O. vulgatum
3 – Striae up to 1 μm wide, several-fold narrower than muri ………………………………………………………………………………………………… 3’ – Striae more than 1 μm wide, nearly as wide as muri …………………………………………………………………………………………… 3’’ – Striae more than 1 μm wide, nearly as wide as muri …………………………………………………………………………………………… 3’’’ – Striae more than 1 μm wide, nearly as wide as muri …………………………………………………………………………………………… 3’’’’ – Striae more than 1 μm wide, nearly as wide as muri …………………………………………………………………………………………… 3
4 – Verrucae regular, nearly hemispherical, densely covered with micropapillae …………………………………… 5
4’ – Verrucae irregular, not covered with micropapillae …………………… 5
5 – Verrucae of uneven size, often elongated, usually 2–3 μm across ……………………………………………………………………………….. 5
5* – Verrucae irregular, knob-like, large, often more than 5 μm across ……………………………………………………………………………….. 6

### 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. 3. Diagrams of interpopulation variation in the spore diameter of *Botrychium lunaria*: A – polar diameter (equatorial view); B – equatorial diameter (proximal view); 1 – Slup; 2 – Bobrowiec in the Tatra Mts; 3 – Dziewicz Góra Mt; 4 – Śnieżka (Śniętka) Massif in the Karkonosze (Krkonoše) Mts; 5 – Sito; 6 – Shepit.](image)

![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.](image)

![Fig. 5. Results of principal component analysis (PCA) for interpopulation variation in spore diameter of *Botrychium lunaria*: 1 – Slup; 2 – Bobrowiec in the Tatra Mts; 3 – Dziewicz Góra Mt; 4 – Śnieżka (Śniętka) Massif in the Karkonosze (Krkonoše) Mts; 5 – Sito; 6 – Shepit.](image)
4. Discussion

The performed spore measurements, especially the analysis of interpopulation 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 mm 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* in 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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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 form 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*.

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

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><em>Botrychium lunaria</em></td>
<td>33.5–40.5 × 49.8–56.2</td>
<td>24.5–33.0 × 35.0–40.5 (53.3)</td>
<td>24.0–40.0 × 42.0–62.0</td>
<td>24.0 × 32.0</td>
<td>29.0–49.0 × 31.0–58.0</td>
</tr>
<tr>
<td><em>Botrychium matricariifolium</em></td>
<td>–</td>
<td>26.0 × 39.6–43.2 (46.8)</td>
<td>22.0–38.0 × 40.0–65.0</td>
<td>–</td>
<td>28.6–38.9 × 33.5–45.9</td>
</tr>
<tr>
<td><em>Botrychium multifidum</em></td>
<td>–</td>
<td>28.8–32.4 × 34.2–36.0 (39.6)</td>
<td>23.0–34.0 × 36.0–47.0</td>
<td>–</td>
<td>24.0–44.0 × 27.0–49.0</td>
</tr>
<tr>
<td><em>Botrychium simplex</em></td>
<td>–</td>
<td>(32.0) 35.0–42.0 (48.0) × 54.0–57.6 (62.0)</td>
<td>32.0–55.00 × 60.0–87.0</td>
<td>40.0 × 50.0</td>
<td>26.0–33.0 × 31.0–37.0</td>
</tr>
<tr>
<td><em>Botrychium virginianum</em></td>
<td>20.3–23.7 × 25.2–30.8</td>
<td>18.0–21.6 × (30.0) 32.4–36.0</td>
<td>–</td>
<td>–</td>
<td>20.0–27.0 × 24.0–31.0</td>
</tr>
<tr>
<td><em>Ophioglossum vulgatum</em></td>
<td>25.8–30.2 × 35.7–40.3</td>
<td>18.0–23.4 × 38.0–50.4</td>
<td>30.0–40.0 × 42.0–50.0</td>
<td>–</td>
<td>27.0–51.0 × 31.0–52.0</td>
</tr>
</tbody>
</table>

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).

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**References**


