Occurrence of Polypodium × mantoniae and new localities for P. interjectum in the Czech Republic confirmed using flow cytometry

Polypodium × mantoniae a nové lokality P. interjectum v České republice, potvrzené pomocí průtokové cytometrie

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Flow cytometry measurements confirmed the occurrence of Polypodium × mantoniae (P. interjectum × P. vulgare) at three localities in the eastern part of the Czech Republic (Blansko and Rudice N of Brno and Javoříčko WNW of Olomouc). Nuclear DNA contents (± Sx) were determined for P. vulgare (2C = 29.00 ± 0.32 pg), P. × mantoniae (2C = 37.18 ± 0.38 pg) and P. interjectum (2C = 45.24 ± 0.31 pg) using a PAS Partec GmbH flow cytometer (PI staining / standard Vicia faba, 2C = 26.9 pg). The relative DNA content ratio was measured in all pairs of taxa (± Sx range), i.e. P. × mantoniae : P. vulgare = 1.340 ± 0.008; P. interjectum : P. vulgare = 1.681 ± 0.003; P. interjectum : P. × mantoniae = 1.255 ± 0.008. Six new localities for Polypodium interjectum were found in the region of Moravský Kras (= Moravian Karst, N of Brno). From the PI/DAPI index it can be inferred that the AT/GC ratio (or heterochromatin occurrence) is 1.05× bigger in P. × mantoniae than in P. vulgare and 1.08× bigger in P. interjectum than in P. vulgare. Anatomical data (number of thick-walled cells in the anulus, spore length and stomata length) of selected specimens and live samples from the Czech Republic were in good agreement with the range of variation of these features published by earlier authors from other European countries. A brief historical survey of the knowledge of P. interjectum in the Czech Republic is included.

Key words: AT frequency, C-value, ferns, flow cytometry, genome size, nuclear DNA content, PI/DAPI index, Polypodiaceae, Pteridophyta

Introduction

The genus Polypodium L. s. str. includes approximately 100 species (Dostál & Reichstein 1984, Haufler et al. 1993); some authors mention as many as 150–170 species (e.g. Hassler & Swale 2001). The center of species diversity is situated in the tropical parts of America; relatively few species occur in the Old World. Taxonomically complicated, the Polypodium vulgare complex is a monophyletic and primarily temperate species group (Haufler et al. 1995a).

Three species of the P. vulgare complex are native in Europe:

= P. australe Fée, Gen. Fil.: 236, 1852.


**Brief historical survey of the taxonomical treatment of Polypodium in Europe**

**Habitual and morphological delimitation**

Linnaeus (Sp. Pl.: 1085–1086, 1753) distinguished between *P. vulgare* and *P. cambricum*, but he based *P. cambricum* on plants belonging to an extreme variety with 2-pinnate leaves. Therefore, from the first half of the 18th century *P. cambricum* was distinguished by European botanists mainly as *P. vulgare var. serratum* Willd. or *P. australe* Fée on the basis of an acute lateral lobe or leaf blade shape. This, the oldest concept of *P. cambricum*, however, includes present-day *P. interjectum*. The geographical range of *P. cambricum* was first sketched by Milde (1865). During the second half of the 19th century and the first half of the 20th century, many infraspecific taxa within the *P. vulgare* complex were described (e.g. Milde 1865, Luerssen 1889, Ascherson & Graebner 1896, Christ 1900, Krieger 1904, Junge 1910, Podpěra 1926, Christensen 1928, Farquet 1933, Slavoňovský 1947). Although most of these taxa have no taxonomic relevance, many local botanists and amateur collectors have tried to distinguish and collect these taxa. Therefore, numerous herbarium specimens of *Polypodium* were collected from the end of the 19th century up to the end of World War II.

**Micromorphological delimitation**

A new qualitative step in the taxonomy of *Polypodium* was the use of microscopic anatomical features. Futó (1904) was the first to publish an anatomical feature for determining this taxon, namely, the lower number of thick walled cells in the anulus of sporangia in *P. vulgare var. serratum*. The existence of three distinct taxonomic entities in Europe within the *P. vulgare* complex was subsequently recognized by Rothmaler (1929). *P. interjectum* was distinguished by Rothmaler (1929) as *P. vulgare subsp. prionodes* (Aschers.) Rothm. on the basis of an older form described by Paul Ascherson (1896) within the framework of Milde’s (1858) variety: *P. vulgare var. attenuatum f. prionodes* Asch., Synops. 1: 94, 1896. Rothmaler (1929), however, based his conception of *P. interjectum* on macroscopic characters; the shape of the lateral lobes and leaf blades, and the numerous secondary veins. Martens (1943, 1949, 1950) described the presence of paraphyses (i.e. trichomes occurring among the sporangia) in *P. vulgare var. serratum*. Such paraphyses are absent in other European species of *Polypodium*.

**Karyological delimitation**

The modern conception of European and some American taxa within the *P. vulgare* complex was based on their karyology delimited by Manton (1947, 1950) and Shivas (1961a,
1961b); these authors found three different ploidy levels in Rothmaler’s (1929) subspecies: diploid, (allo)tetraploid and (allo)hexaploid. It was obvious that these cytotypes must be distinguished at the species, not the subspecies level. At that time, the epithets vulgaris and australis indicated species, but prionodes was also previously used within the genus Polypodium to indicate species level by C. H. Wright (in Kew Bull. 1906: 253). Therefore Shivas (1961b) proposed a new name, P. interjectum, as a nomen novum synonymous to Rothmaler’s subspecies. The results of the karyological and experimental hybridization studies of Manton and Shivas became textbook examples (see Briggs & Walters 1997) and inspired many taxonomical “national” or “regional” revisions of the P. vulgare complex in most European countries, especially in Germany (Mergenthaler & Damboldt 1962, Lenski 1962, 1964, Eschelmüller 1970–1971, 1971, Zenner 1972, Jessen 1982, Seraphim 1985, Leonhards et al. 1992, 1993, Philippi 1993, Jäger et al. 1994, Diekjobst 1997) and Great Britain (Benoit 1966, Hughes 1969, Roberts 1970, Synnott 1970, Stirling 1972, Roberts & Synnott 1972, Rutherford & Stirling 1972). Unfortunately, it was clear that these taxa were only determinable using a microscope; therefore the number of herbarium specimens of Polypodium collected by botanists rapidly decreased after 1960. This is confirmed for the BRNU herbarium, and was also mentioned by Hršak (2002).

The reticulate evolution of the Polypodium vulgare complex is apparent from earlier karyological studies and from chloroplast DNA molecular studies by Haufler et al. (1995a, 1995b) (Fig. 1).

Fig. 1. – Reticulate evolution in Polypodium vulgare agg. in Europe, Asia, and North America. Polypodium glycyrrhiza D. C. Eaton occurs in the W part of North America and also in Asia on the Kamchatka Peninsula (Haufler et al. 1993). P. sibiricum Sipliv. has a similar distribution throughout the northern (boreal) part of North America up to SE Greenland (Haufler et al. 1993); it also occurs in Asia from E Siberia up to the Far East of the former USSR, Sakhalin and the Kurile Islands [Bobrov 1964 (ut P. virginianum), Siplivinski 1974], and probably also occurs in some northern Japanese Islands (cf. Hultén 1986). P. virginianum L. occurs in the central and E parts of North America, P. appalachianum Hauffler et Windham is distributed along the E coast of North America (Haufler et al. 1993). Reticulate evolution among the other American Polypodium species and hybrids was confirmed by Haufler & Zongren (1991) and Haufler & Ranker (1995).
Geographical range of European *Polypodium* species and their hybrids

*Polypodium cambricum* occurs mainly in the southwest and south of Europe along the Atlantic and Mediterranean coasts. Its range stretches from central Scotland through W England, Wales, Ireland (Roberts & Synnott 1972, Rutherford & Stirling 1973, Stace 1991), whole of France (Badré & Prelli 1978, Corillion & Denis 1968, Guinchoet & Vilmorin 1973, Jalas & Suominen 1988, Neuroth 1996), SW Switzerland (Farquet 1933, Villaret 1960), Portugal (Fernandes 1968a), Spain, the Balearic Islands (Muñoz Garmendia 1986, Neuroth 1996), Corsica (Berton 1974), Sardinia, the Apennine Peninsula, Sicily (Nardi & Tommei 1976, Pignatti 1982), SW Slovenia (Mlakar 1987), NW and SE Croatia (Hršak 2002), Montenegro (Hayek 1924, Rohlena 1942, Mayer & Horvatić 1967), Herzegovina (Mayer & Horvatić 1967), Albania, Corfu, S Greece (Hayek 1924, Jalas & Suominen 1988, Voliotis 1985, Neuroth 1996), Crete (Neuroth 1996) and Cyprus (Hultén & Fries 1986). It is also found in NE Africa in the Atlas Mts (Hultén & Fries 1986), E Mediterranean up to the islands of Ikaria and Samos (Runemark apud Henderson 1965), Lebanon, W Syria (Mouterde 1966) and coastal regions of Turkey (Henderson 1965). The easternmost localities are along the Russian (Neuroth 1996) and Georgian (Kutateladze et al. 1971) shores of the Black Sea. This species was reported from SW Bulgaria by Assyov et al. (2001), but older reports of *P. vulgare* var. *serratum* Willd. probably belong to *P. interjectum* (cf. Jordanov 1963). In most other southeastern European botanical literature, the distribution of *P. cambricum* is also not clearly separated from that of *P. interjectum*. This species was also introduced once to North America (Lloyd & Hohn 1969, Lloyd et al. 1992). Some authors regard populations from the Canary Islands, Madeira and Porto Santo as the subspecies *P. c. subsp. macaronesicum* (A. E. Bobrov) Fraser-Jenk. in Greuter, Burdet et G. Long. and populations from the Azores Islands as a closely related subspecies *P. c. subsp. azoricum* (Vasc.) E. Nardi. The subspecies *macaronesicum* also occurs, but is rare, in the south of Spain near Cadiz (Hohenester & Welsh 1993, Muñoz Garmendia 1986, Press & Short 1994). A species designation for these taxa is accepted, e.g. by Akeroyd & Jermyn (1992), Bobrov (1964), Fernandes (1968b) and Jalas & Suominen (1988). However, Neuroth (1996), after a detailed morphometric and molecular genetic study, included the populations from the Azores as in the subsp. *macaronesicum*, as var. *azoricum*.

*Polypodium interjectum* has a distribution similar to that of *P. cambricum*, but the limits of its subatlantic-submediterranean distribution are situated further to the north and east. This species occurs from SW Norway (Nielsen & Johnsen 2000) through Denmark (Pedersen 1969, Nielsen & Johnsen 2000), Belgium, Luxembourg (Beckers 1966, Laminbon et al. 1992), the Netherlands (Meinders-Groeneveld & Segal 1967, Graaf & Heukels 1982), Great Britain, Ireland (Stace 1991), Portugal (Fernandes 1968a, Neuroth 1996), Spain and the Balearic Islands (Muñoz Garmendia 1986, rarely also in Madeira – Press & Short 1994), France (Badré & Prelli 1978, Corillion & Denis 1968, Guinchoet & Vilmorin 1973, Neuroth 1996), Switzerland (in NW, W and S), the Apennine Peninsula, Sicily (Nardi & Tommei 1996, Pignatti 1982), Elba (Neuroth 1996), Slovenia (Mlakar 1987), Croatia (Hršak 2002), S and E Austria (Adler et al. 1994), Germany (many authors, see Rothmaler 2002 for a detailed survey of distribution), Czech Republic (Čvančara 1988, Holub & Kubát 1999), S Slovakia (Murin 1982, Holub & Kubát 1999), W and N Hungary and SW Rumania (Beldie 1977, Popesku & Sanda 1998). It has not been reported from Serbia (cf. Josifović 1970) or Bulgaria, but its occurrence in these countries is probable (see above). The easternmost localities are near
Kaliningrad, SE of Moscow, near Rakhov in the W Ukraine on the Crimean Peninsula (Fedorov 1974), near Bursa in NW Turkey, near Bahçe in S Turkey (near the Iskenderun Gulf of the Mediterranean Sea) and in N Iran (Henderson 1965).

*Polypodium vulgare* is widely distributed throughout Europe from the top of the Scandinavian Peninsula and Iceland to the Azores, the Canary Islands, Madeira and N Africa; in E Europe and W Asia it is distributed mainly in the mountains (Bobrov 1964, Hultén & Fries 1986, Jalas & Suominen 1988, Neuroth 1996).

Besides the three above-mentioned native European species, there is also a North American species, *Polypodium hesperium* Maxon, naturalized in one locality near Bruges in Belgium (Roberts 1982). Many species (either European or extra-European) of the genus *Polypodium* and their hybrids are cultivated as garden plants (Hoshizaki 1982, Page & Bennell 1986).

*Polypodium ×font-queri* Rothm. in Cadevall et Font Quer, Fl. Catalun. 6: 353, 1937 (= *P. cambricum × P. vulgare*) is known from Great Britain (Rutherford & Stirling 1973), France (Shivas 1961b, Neuroth 1996), Switzerland (Shivas 1961b), Italy (Shivas 1961b), and Spain (Redondo et al 1999a, 1999b).

*Polypodium ×shivasiae* Rothm., Kulturpflanze Beih. 3: 245, 1962 (= *P. cambricum × P. interjectum*) occurs in Great Britain (Rutherford & Stirling 1972), France (Shivas & Walker 1970 sec. Badré & Prelli 1978), Germany (Neuroth 1996), probably also in Portugal (Fernandes 1968a), Spain (Redondo et al. 1999a, 1999b), Italy (Shivas 1961b), Slovenia (Mlakar 1987) and the European part of Turkey (Shivas 1961b).


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**Historical survey of the *Polypodium vulgare* complex in the Czech Republic**

*Polypodium vulgare* s.l. was mentioned as a medicinal plant by Křišťan (from Prachatice) in the oldest Czech botanical manuscript, the Latin Herbarius from cca 1400 (Čižek 1994). Czerny (1517) mentioned *Polypodium* and included a schematic picture, but without localities, in chapter CCLIII; Hájek (1562) also mentioned this plant from the Czech Kingdom, without localities, but with a perfect illustration in the Czech translation of Mattioli’s Herbal. Julius Milde, a professor at a secondary school and later at the University in Breslau (now Wróclaw in Poland), was a pteridologist who collected plants particu-

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1. *Polypodium ×font-queri* nothosubsp. encumeadense (= *P. cambricum* subsp. macaronesicum × *P. vulgare*) was described by Neuroth et al. (1998) from Madeira vers. nothosubsp. *font-queri* (= *P. cambricum* subsp. *cambricum* × *P. vulgare*), and occurs in other parts of Europe.

2. Not confirmed by chromosome counting or cytometry.

3. The oldest transcription of Křišťan’s Herbarium, made by Matěj from Zlín under the title “Erbarius Reverendi Mgri Cristiani”, is dated 1416 and kept in the Cerroni collection no. II.87. of the Moravian Land Archive in Brno.
<table>
<thead>
<tr>
<th>Location, collector and date</th>
<th>Original determination</th>
<th>Specimen no. in BRNU</th>
<th>Mean spore length (mm)</th>
<th>Mean stoma length (mm)</th>
<th>Mean no. of annulus cells</th>
<th>No. of basal cells (range)</th>
<th>Our determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Žulosyenit: skála u cesty Adamov – Alexandr. rozhledu; F. Bílý 1929/09/26</td>
<td>P. v. var. prionodes Aschers</td>
<td>218829</td>
<td>77.4</td>
<td>60.6</td>
<td>8.1</td>
<td>2–4</td>
<td>interjectum</td>
</tr>
<tr>
<td>Údolí Punkvy mezi huť a skalským mýlným (mezi 2 až 3. žlíbkem); F. Bílý 1930/09/14</td>
<td>P. v. f. acuminatum J. Schmid</td>
<td>218831</td>
<td>75.1</td>
<td>57.0</td>
<td>8.5</td>
<td>2–4</td>
<td>interjectum</td>
</tr>
<tr>
<td>Blansko, Josefské údolí, proti Býčí skále; F. Bílý 1937/06/11</td>
<td>P. v. prionodes Aschers</td>
<td>374735</td>
<td>64.5</td>
<td>60.8</td>
<td>10.3</td>
<td>1–2 (–3)</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, Josefské údolí, u mostu Černých; F. Bílý 1935/10/04</td>
<td>P. v. acuminatum J. Schmidt</td>
<td>sin no.</td>
<td>71.4</td>
<td>58.7</td>
<td>14.9</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, Josefské údolí, Bílá skála za Černými; F. Bílý 1935/10/29</td>
<td>P. v. f. attenuatum in comb. with m. m.</td>
<td>sin no.</td>
<td>64.8</td>
<td>53.3</td>
<td>11.4</td>
<td>0–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Žleb &quot;Myší díra&quot; u Bilovic n./S. F. Veselý; 1921/07/14</td>
<td>P. vulgare</td>
<td>57427</td>
<td>66.9</td>
<td>50.8</td>
<td>9.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Žleb &quot;Myší díra&quot; u Bilovic n./S. F. Veselý; 1921/07/14</td>
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<td>57427</td>
<td>66.9</td>
<td>50.8</td>
<td>9.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Letovice, ježlinatý les pod severním svahem Děkanové; Mrázklová</td>
<td>P. vulgare</td>
<td>504382</td>
<td>66.6</td>
<td>56.6</td>
<td>9.9</td>
<td>(0)</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Skála u cesty z Adamova k Alexandrově rozhledně; F. Bílý 1930/03/27</td>
<td>P. v. var. grandifrons Lange</td>
<td>218840</td>
<td>61.4</td>
<td>55.8</td>
<td>9.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko nad mlýnem; F. Bílý 1937/09/24</td>
<td>P. v. f. attenuatum Milde in comb. with f. m.</td>
<td>sin no.</td>
<td>66.9</td>
<td>56.7</td>
<td>10.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Litovel: skály na vrcholu Rampachu; S. Lachman 1931/07/25</td>
<td>P. vulgare</td>
<td>224125</td>
<td>62.9</td>
<td>49.1</td>
<td>11.4</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Josefské údolí u Adamova; J. Podpěra 1924/10</td>
<td>P. v. var. attenuatum Aschers</td>
<td>39343</td>
<td>65.6</td>
<td>52.4</td>
<td>10.5</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, Josefské údolí, za Černými; F. Bílý 1931/10/14</td>
<td>P. v. f. acuminatum Milde</td>
<td>sin no.</td>
<td>67.4</td>
<td>55.7</td>
<td>10.5</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Josefské údolí u Adamova; J. Podpěra 1924/10</td>
<td>P. v. f. attenuatum Milde s.</td>
<td>sin no.</td>
<td>69.4</td>
<td>51.6</td>
<td>10.3</td>
<td>(0–)</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko: ve stinné rokle mezi granit. skalami nad 1. tunelem; J. Müller</td>
<td>Indeterminavit</td>
<td>373292</td>
<td>64.8</td>
<td>51.6</td>
<td>11.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, Josefské údolí; F. Bílý 1936/10/01</td>
<td>P. v. f. acuminatum J. Schmid</td>
<td>sin no.</td>
<td>63.8</td>
<td>56.6</td>
<td>10.4</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, Josefské údolí; F. Bílý 1934/03/29</td>
<td>P. v. f. acuminatum J. Schmid</td>
<td>sin no.</td>
<td>67.4</td>
<td>55.2</td>
<td>10.4</td>
<td>0–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>In valle Josefovské údolí prope Adamov ad rupes graniticas muscosas; J. Podpěra &amp; F. Švestka 1924/05/</td>
<td>P. v. f. acuminatum J. Schmid</td>
<td>sin no.</td>
<td>67.6</td>
<td>55.2</td>
<td>11.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Poštů Zleb. U stejného žlíbku proti Košiímu svědu; F. Bílý 1930/06/29</td>
<td>P. v. f. acuminatum Milde</td>
<td>sin no.</td>
<td>63.8</td>
<td>56.6</td>
<td>10.4</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, nad pilou; F. Bílý 1938/09/27</td>
<td>P. v. f. prionodes Aschers</td>
<td>218827</td>
<td>67.6</td>
<td>55.0</td>
<td>10.6</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Blansko, nad pilou u Lažánk; F. Bílý 1931/10/11</td>
<td>P. v. f. serratulatum Moore</td>
<td>sin no.</td>
<td>64.8</td>
<td>53.4</td>
<td>11.0</td>
<td>0–1 (–2)</td>
<td>×mantoniae</td>
</tr>
<tr>
<td>Žulosynit. Josefovské údolí: Stráň mezi mostem a Švýcarnou; F. Bílý 1934/04/24</td>
<td>P. v. f. serratulatum</td>
<td>285087</td>
<td>68.7</td>
<td>52.9</td>
<td>10.9</td>
<td>1–2</td>
<td>×mantoniae</td>
</tr>
</tbody>
</table>

1 Selection was based on morphology, ecology and relationship to the data discussed in the chapter Historical survey of the P. vulgar complex in the Czech Republic.
2 This specimen, which consists of only one leaf, was determined by F. Slavoňovský as Polypondium vulgare L. f. Thompsoni Monk, combined with f. auritum Wall.
3 This specimen was also determined by Čvančara to be P. interjectum, and he considered the locality to be the only one from the Moravian Karst (Čvančara 1987)
larly in the Krkonoše Mts, Jeseníky Mts and Kralický Sněžník Mts and described some infraspecific taxa within *P. vulgare* in the second half of the 18th century (Milde 1858). Among these taxa was *P. vulgare* var. *attenuatum* from the Gesenke (now Jeseníky Mts, i.e. Nízký Jeseník and Hrubý Jeseník in the Czech Republic), Zobten (now Sobótká, SW of Wróclaw in Poland) and Trebnitz (now Trzebnitza, N of Wróclaw in Poland). German botanists in particular collected plants they assigned to Milde’s var. *attenuatum*, which are now recognized as *P. interjectum*. Because *P. interjectum* is not known in Poland (cf. e.g. Mirek et al. 1995, cf. etiam Dostál & Reichstein 1984), Milde’s original conception of this taxon is considered to have been different from the present *P. interjectum* (also on the basis of Ascherson’s conception of f. *prionodes* – see above). On the other hand, *P. interjectum* is documented by a herbarium specimen from the locality Hrubá Voda (Ferdinand Weber 1936, PR – Kubát 1986, Čvančara 1987) in the Nízký Jeseník Mts, i.e. in the area from which Milde collected var. *attenuatum*. Milde’s original herbarium collection is deposited in Wróclaw. Podpěra mentioned var. *attenuatum* from the Josefovské údolí valley in the region of Moravian Karst (Formánek apud Podpěra 1926) and from other localities in Moravia, in his treatment of *Pteridophyta* in the Flora of Moravia (Podpěra 1926). Otruba (1930) recorded the occurrence of *P. vulgare* var. *attenuatum* on the calcareous hill Kotouč near Štramberk. However, the herbarium specimen from this locality determined as var. *attenuatum* by Otruba (1928 BRNU) belongs to *P. vulgare* L. s. str. (Table 1). Moravian botanists František Bílý and František Unzeitig (= Slavoňovský after 1945) studied the variability of *P. vulgare* in the karst region Moravský Kras N of Brno before World War II. Their results were published by Bílý (1929, 1931, 1937) and Slavoňovský (1947). They used and described many infraspecific taxa in the sense of the narrow-species “Aschersonian” approach, as did Podpěra (1926). Among numerous varieties, forms and subforms, they also used a taxon named *P. vulgare* f. *prionodes* Aschers., which they recorded from the following localities: at the forest road from Adamov to the Alexandria rozhledna outlook-tower (Bílý 1931), “nad pilou” and “proti Kolbence” near Blansko, and from Josefovské údolí valley near Adamov (Slavoňovský 1947). They also used the name *P. vulgare* f. *attenuatum* f. *acuminatum* (originally belonging to a taxon described by J. Schmidt from Holstein in Germany where *P. interjectum* is frequently recorded). Some of the specimens from these localities collected by Bílý and determined by Slavoňovský belong to *P. interjectum* or *P. ×xantoniae* (Table 1). Therefore František Slavoňovský, on the basis of plants collected by František Bílý, was probably the first to publish the occurrence of *P. interjectum* in the Czech Republic (Slavoňovský 1947), albeit under the name *P. vulgare* f. *attenuatum* f. *acuminatum*. This accomplishment by Slavoňovský and Bílý is somewhat diminished by the many redundant infraspecific and teratologic taxa without taxonomic value they used. The famous Czech taxonomist Josef Holub reported the occurrence of *P. interjectum* in the Czech Republic at Podmoky, Střekov, Šárka and Moravský Kras (Holub 1960). Perhaps on the basis of contacting M. G. Shivas or W. Rothmaler, he used the name *P. interjectum* as early as 21 March 1960 [cf. Holub 1960 (published in October)], whereas Shivas did not propose the name *P. interjectum* until 1961⁴. Shivas is known to have used this name in the Exhibition Meeting at Chelsea College, 26 November 1960 (Walker et al. 1961). Because the occurrence of *P. interjectum* in the Czech Republic

⁴ However, Holub’s *Polypodium interjectum* is not validly published (ICBN 2000, 32.1.c) therefore Shivas’ later homonym should not be rejected (ICBN 2000, 45.3).
was not accepted by the authors of the Flora Europaea (Valentine 1964), neither on the basis of the announcement by Holub (1960) nor of his later comments (Holub 1964), Holub noted this failure once more in 1966.

K. Kubát found a new locality for *P. interjectum* near the village Brná nad Labem in the České středohoří Mts (Kubát 1972). Skalický (1979) published a new occurrence at a ruin of the castle Skála near the village Vlčí u Přeštic in W Bohemia. J. Holub found *P. interjectum* during a field course of the Czechoslovak Botanical Society at Moravský kras in 1980 at the following localities: 1. slopes along the left shore of the Punkva river at Skalní mlýn near the village Lažánky, 2. forest road on the northern slope of Josefovské údoli valley between the places called “U sedmi dubů” and “Býčí skála” (Vaněčková et al. 1997).

*P. interjectum* was included in the category C1 (critically threatened taxa) in the first version of the Czech Red List (Holub et al. 1979). In the current version of the Red List of the Czech flora (Holub & Procházka 2000, Procházka 2000), *P. interjectum* was moved to group C2 (strongly threatened taxa). A detailed Czech determination key for *P. vulgare* and *P. interjectum* was first published in the Catalogue of the Moravian Flora by Smejkal (1980). Dostál (1982, 1989) mentioned both *P. vulgare* and *P. interjectum* as well as their hybrid *P. ×mantoniae* from the former Czechoslovakia – the latter being found in Slovakia. Dostál (1982) also mentioned an erroneous former report of *P. cambricum* from Czechoslovakia. This note was made by Dostál (Dostál & Reichstein 1984, Dostál 1989) on the basis of a paper by Domin (1937) who published comments on a specimen collected by Klement Ptačovský at Svätojurský Šúr near Bratislava in Slovakia. While Domin (1937) mentioned that the specimen resembled var. *serratum*, he unambiguously determined it as *P. vulgare* f. *prionodes*. Therefore we consider the notes of Dostál (1982, 1989) and Dostál & Reichstein (1984) about a doubtful report of *P. cambricum* from Czechoslovakia as superfluous (see also the map in Holub & Kubát 1999). Opiz (1852) included the name “*P. vulgare* γ. *serratum*” in the Checklist of the Czech Flora. However, on the basis of “*P. vulgare* γ. laciniiis alterniantibus, serratis”, previously published by Opiz in Kratos 2/1: 2 (1820), Domin (1937) believed that Opiz’s conception was probably not that of Wildenow.

A distribution map of *P. interjectum* in the Czech Republic was first published by Slavík (1986) and subsequently by Kubát (1986) who mentioned new localities from NW Bohemia documented by his own herbarium specimens in LIT – Střekov: along the road to Nová Ves; Velemín: rocks of Dubický vrch Hill under Milešovka Mts; Štáhlavy: ruin of the castle Lopata. Kubát (1986) also mentioned new localities discovered by V. Čejka near Klatovy – Mezihoří u Švihova; Kaliště u Švihova; Tupadly. *P. interjectum* from a locality in the Nature Reserve Tupadelské skály near Tupadly by Klatovy was published by Čejka (1994). Two localities S of Rokycany, i.e. rocky hill E of the village Mešno and rocks 1 km NW of the village Vísky, discovered by K. Kubát in 1986 were published by Nesvadbová & Sofron (1996). *P. interjectum* was collected by the German botanist S. Jessen at the Punkevní jeskyně caves in the Moravian Karst and mentioned in the chapter Material by Neuroth (1996). The second locality, calcareous rocks in the valley above the village Borinka in the Malé Karpaty Mts, is in Slovakia, not in the Czech Republic as reported by Neuroth (1996).

Kubát (1986) also mentioned that the hybrid *P. ×mantoniae* very probably occurs in some localities in the Czech Republic where both parent species occur together. Čvančara (1987) published a detailed determination key and extensive descriptions of *P. vulgare* and
He also noted the probable occurrence of *P. × mantoniae* in the localities at Nezdice near Přeštice, Hamr na Jezeře and Josefovské údolí in the Moravian Karst, and emphasized the need for karyological confirmation of the identity of these populations; the same opinion was expressed by Holub & Kubát (1999). Probably because of the poor translation of Čvančara’s note about *P. × mantoniae* (Čvančara 1987), Neuroth (1996) considered the occurrence of this hybrid in the Czech Republic cytologically confirmed. Kubát (2002), who published a good field key for *Polypodium*, revised his opinion and reported *P. × mantoniae* from the Czech Republic.

Because there is no certainty about the occurrence of the hybrid *P. × mantoniae* in the Czech Republic, we have tried to confirm the presence of this nothotaxon in native populations of *Polypodium*, using flow cytometry.

**Confirmation of the presence of *Polypodium × mantoniae* in the Czech Republic and new localities for *P. interjectum***

New localities of *Polypodium interjectum* in karst areas of S and C Moravia were investigated using flow cytometry. Nineteen samples collected by L. Tichý in the Moravský Kras (Moravian Karst) and Javoříčský Kras were measured. Three DNA content levels (for *Polypodium × mantoniae, P. interjectum* and *P. vulgare*, see Fig. 2) were found in samples from the following localities:
A PA-I ploidy analyzer (Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp was used. Sample preparations were carried out in a two-step procedure (Otto 1990, Doležel & Göhde 1995) in the Laboratory of Flow Cytometry, Department of Botany, Masaryk University Brno. Young leaf samples (0.5 cm²) from a measured individual and a standard were chopped with a new razor blade for about 20 s in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (4.2 g citric acid monohydrate + 1 ml 0.5% Tween 20 adjusted to 200 ml and filtered through 0.22 µm filter), then 0.5 ml more Otto I buffer was added. The solution was filtered through nylon cloth (50 µm mesh size). For DNA staining, 2 ml of Otto II buffer (0.4 M disodium hydrogenphosphate dodekahydrate) including DAPI (4',6-diamidino-2-phenylindole; 4 µg/ml final concentration) was used.

For some samples identified using flow cytometry stoma length (30 stomata from the underside of the middle part of the leaf from each sample), spore length (30 well-developed spores from mid-leaf from each sample) and number of anulus cells (250 sporangia from each sample) were measured and counted. The most useful character for identification of taxa seems to be the number of anulus cells (Fig. 3). On the basis of morphological characters (leaf blade and lateral lobe shapes), herbarium specimens from BRNU were chosen which seemed to be *P. interjectum*. These specimens were revisited by P. Bureš and Yi-Feng Wang based on the number of anulus cells (30 sporangia from mid-leaf), spore length (well-developed 30 spores from mid-leaf), stoma length (30 stomata from the underside of the middle part of the leaf) and basal cell number (10 sporangia from mid-leaf); the average values for these characters are presented in Table 1. In this material, three specimens belonged to *P. interjectum*, three to *P. ×mantoniae*, and 26 to *P. vulgare* (Table 1).

There is also the question of whether or not to include *P. ×mantoniae* (hitherto unknown in the Czech Republic) in the Red List of Czech Flora, since some authors have included it (e.g. Wolff-Straub 2000). Werth & Lellinger (1992) recommended protection of hybrids whose parents are endangered or absent as “genomically preserved plants”. On the other hand, there is a risk that *P. ×mantoniae* can reduce or displace autochthonous populations of the less competitive *P. interjectum* (Dostál & Reichstein 1984, Holub & Kubát 1999). Although there are some studies on the ecology and population genetics of
there is no good comparative eco-physiological study of \( P. \times mantoniae \) in relation to other sympatric taxa of \textit{Polypodium}. We recommend that \( P. \times mantoniae \) be included in the C2 category (strongly threatened taxa), as are \textit{Equisetum \times moorei} and other hybrids in the present version of the Czech Flora Red List.

\textbf{Variability in DNA content and AT frequency}

Variability in the absolute and relative DNA content of samples is summarized in Tables 2 and 3. For estimates of the relative DNA content using DAPI staining (described above), a sample from the locality Býčí skála (\textit{P. interjectum}) was used as a standard for \textit{P. vulgare} and \textit{P. \times mantoniae} and one sample from the locality Sloup (\textit{P. vulgare}) as a standard for \textit{P. interjectum} and \textit{P. \times mantoniae}. A similar protocol was used for measuring absolute DNA content, using a ploidy analyzer PAS (Partec) equipped with a 488-nm laser (25 mW output) in the Laboratory of Molecular Cytogenetics and Cytometry at the Institute of Ex-
Experimental Botany, Czech Academy of Sciences in Olomouc. Young leaves (0.5 cm²) of the sample and standard were macerated using a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer. The solution was filtered through nylon mesh (50 µm pore size). For DNA staining, 1 ml of Otto II buffer supplemented with propidium iodide (50 µg/ml final concentration) and RNase I (50 µg/ml final concentration) was used.

The range of detected infraspecific variability in relative DNA content is very low. Absolute DNA contents published by Redondo et al. (1999a) are lower than reported here (Table 2), but the ratio between taxa is very similar (Table 3). The difference is probably caused by using different internal standards: Redondo et al. (1999a) used *Pisum sativum* ‘Express Long’ (2C = 8.37 pg) and *Petunia hybrida* ‘PxPC6’ (2C = 2.85 pg); we used *Vicia faba* ‘Inovec’ (2C = 26.90 pg) cultivated in Olomouc (see also http://www.ueb.cas.cz/Olomouc1/lcgcm/index.htm). The DNA content of *Vicia faba* ‘Inovec’ is closer to that of *Polypodium* taxa than is that of *Pisum sativum* ‘Express Long’ and *Petunia hybrida* ‘PxPC6’ and therefore yielded more exact results.

The results of our measurements of *P. vulgare* and *P. × mantoniae* are similar to those of Murray (1985) but differ for *P. interjectum*. Murray (1985) used Feulgen microdensitometry, which is considered to give the most reliable estimate of DNA content of a single nucleus (Bennett & Leitch 1995). Several authors have indeed found that flow cytometric DAPI measurements correlate badly with Feulgen microdensitometry data in a wide cross-section of angiosperms (Doležel et al. 1998).

### Table 2. – Absolute DNA content (in pg) of *Polypodium* taxa. See text for details of the methods.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>This study</th>
<th>Redondo et al. (1999a)</th>
<th>Murray (1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vulgare</em></td>
<td>29.00 ± 0.32</td>
<td>25.0</td>
<td>30.76</td>
</tr>
<tr>
<td><em>P. × mantoniae</em></td>
<td>37.18 ± 0.38</td>
<td>31.5</td>
<td>35.66</td>
</tr>
<tr>
<td><em>P. interjectum</em></td>
<td>45.24 ± 0.31</td>
<td>37.5</td>
<td>39.32</td>
</tr>
</tbody>
</table>

Method: PI flow cytometry (± Sx)

1. *Polypodium vulgare* from loc. Sloup + standard *P. interjectum*, loc. Býčí skála (2C = 45.24 pg), 3 measurements

### Table 3. – Relative DNA content of *Polypodium* taxa.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>This study</th>
<th>Redondo et al. (1999a)</th>
<th>Murray (1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. × mantoniae/vulgare</em></td>
<td>1.340 ± 0.008</td>
<td>1.282</td>
<td>1.26</td>
</tr>
<tr>
<td><em>P. interjectum/vulgare</em></td>
<td>1.681 ± 0.003</td>
<td>1.560</td>
<td>1.5</td>
</tr>
<tr>
<td><em>P. interjectum/× mantoniae</em></td>
<td>1.255 ± 0.008</td>
<td>1.216</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Method: DAPI flow cytometry (± Sx)

1. Three samples of *P. × mantoniae*, each measured 3× with standard *P. vulgare*, locality Sloup, 9 measurements.
2. Six samples of *P. interjectum*, each measured 1× with standard *P. vulgare*, locality Sloup + 10 samples of *P. vulgare*, each measured 1× with standard *P. interjectum*, locality Býčí skála, 16 measurements.
3. Three samples of *P. × mantoniae*, each measured 3× with standard *P. interjectum*, locality Býčí skála, 9 measurements.
4. See text for a detailed description of the method used.
5. Ratios are based on average results from Table 2.
DAPI or PI staining indicates that P. xmantoniae is exactly halfway between P. vulgare and P. interjectum. However, there are some differences in the PI/DAPI index or dye factor DAPI (DF_DAPI sensu Barow & Meister 2002) between P. xmantoniae and P. interjectum. This dye factor DAPI is a good parameter for characterizing the AT frequency as it depends only on the AT-specific fluorescence and is independent of the genome size, and does not require any assumption as to the fluorescence dependence on AT (Barow & Meister 2002). If we consider P. vulgare as a reference standard for these taxa, we obtain the following DF_DAPI values from Table 3:

\[
\begin{align*}
\text{DF}_\text{DAPI} \ P. xmantoniae &= 1.34/1.282 = 1.05 \\
\text{DF}_\text{DAPI} \ P. interjectum &= 1.681/1.56 = 1.08
\end{align*}
\]

These results indicate that the AT frequency (or AT/GC ratio) is probably 1.05× higher in P. xmantoniae than in P. vulgare, and is probably 1.08× higher in P. interjectum than in P. vulgare. If we accept the reticulate evolution diagram in Fig. 1 (i.e. 2C of P. interjectum equals approximately 2C of P. vulgare + 2C of P. cambricum: 45.24 pg ≈ 29 pg + ? pg => 2C of P. cambricum = 45.24 pg – 29 pg = 16.24 pg), then P. cambricum is presumed to be the “donor” of this higher AT frequency and the AT frequency (or AT/GC ratio) in P. cambricum may be approximately 1.22× higher than in P. vulgare (from the equation 1 × 29.00 pg + x × 16.24 pg = 1.08 × 45.24 pg).

Acknowledgements

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Souhrn

Cytometricky byl potvrzen výskyt křížence Polypodium xmantoniae (P. interjectum × P. vulgare) na třech lokalitách v České republice: Javoříčko, nad vchodem do Javoříských jeskyní (L. Tichý 15. 2. 2002 BRNU); Blansko, v Suchém zlebu asi 3 km SSV Skalního Mlýna (L. Tichý 8. 7. 2002 BRNU); Rudice, Rudické propadání (L. Tichý 25. 6. 2002 BRNU). Revizí herbariového materiálu byl dále tento kříženec prokázán z lokalit: Blansko, Bílá skála za Černými v Josefovském údolí (F. Bílý 1935 BRNU) a Blansko, u mostu Černých v Josefovském údolí (F. Bílý 1935 BRNU). Cytometricky byl dále doložen z více lokalit také druh P. interjectum: Adamov, u jeskyně Kostelík v Josefovském údolí (L. Tichý 21. 5. 2002 BRNU); Adamov, u jeskyně Jáchymka v Josefovském údolí (L. Tichý 21. 5. 2002 BRNU); Adamov, u jeskyně Býčí skála v Josefovském údolí (L. Tichý 21. 5. 2002 BRNU); Blansko, skály na levé straně silnice u Čertova mostu v Suchém zlebu ca 1 km SSV od Kateřinských jeskyní (L. Tichý 8. 7. 2002 BRNU); Babice nad Svitavou, skalnatý svah 0,5 km JV od obce v chráněném území Čihadlo (L. Tichý 12. 6. 2002 BRNU); Ochoz u Brna, údolí Říčky ca 0,3 km Z od vodní nádrže Hádek (L. Tichý 25. 6. 2002 BRNU).

Absolutní obsah jaderné DNA (± Sx) byl detekován u P. vulgare (2C = 28,00 ± 0,32 pg), P. xmantoniae (2C = 37,18 ± 0,38 pg) a P. interjectum (2C = 45,24 ± 0,31 pg) na průtokovém cytometru PAS Partec GmbH (barvení PI / standard Vicia faba, 2C = 26,9 pg). Relativní obsah jaderné DNA byl zjišťován ve směsi všech tří taxonů (± Sx): P. vulgare : P. xmantoniae = 0,746 ± 0,005; P. vulgare : P. interjectum = 0,595 ± 0,001; P. xmantoniae : P. vulgare = 1,340 ± 0,008; P. xmantoniae : P. interjectum = 0,797 ± 0,005; P. interjectum : P. vulgare = 1,681 ± 0,003; P. interjectum : P. xmantoniae = 1,255 ± 0,008. Podle faktoru barvivnosti DF_DAPI lze předpokládat, že poměr AT/GC (resp. zastoupení heterochromatinu) je u P. xmantoniae 1,05× větší než u P. vulgare a u P. interjectum 1,08x větší než u P. vulgare.
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