Genome size and morphology of the *Dryopteris affinis* group in Central Europe

Velikost genomu a morfologie skupiny *Dryopteris affinis* ve střední Evropě

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Ekrt L., Trávníček P., Jarolímová V., Vít P. & Urfus T. (2009): Genome size and morphology of the *Dryopteris affinis* group in Central Europe. – Preslia 81: 261–280.

The agamosporous and taxonomically critical *Dryopteris affinis* group was investigated as part of a cytogeographic and morphometric study of ferns in Central Europe. Material from 27 localities in the Czech Republic, Slovakia, Poland and Austria was sampled and evaluated using both morphometric multivariate and karyological analyses. Chromosome counts and flow cytometric analyses revealed the existence of two distinct triploid taxa (2n = 123) of differing genome size, which correspond to *D. borreri* and *D. cambrensis,* and of a rare pentaploid hybrid (2n = 205) *D.* ×*critica* (*D. borreri* × *D. filix-mas*). Morphometric analyses confirmed a clear separation between both triploid taxa. New quantitative characters were selected based on a discriminant analyses, and a key for the identification of the species is presented.

K e y w o r d s : Central Europe, chromosome number, cytotype, DNA ploidy level, *Dryopteridaceae*, ferns, flow cytometry, multivariate morphometrics, polyploidy

Introduction

Dryopteris is a highly diverse genus containing about 225 species distributed mainly in both cold and warm temperate parts of S, SE and E Asia (Kramer 1990). There are several taxonomically intricate groups with a complicated classification and identification. *Dryopteris* is among the most hybrid-prone genera, and hybridization and polyploidization events play an important part in their evolution, resulting in a particularly vague delimitation of species in closely related taxa (Werth & Windham 1991). Such a trend is evident within agamosporous (apomictic) species that are estimated to make up about 10% of all pteridophyte species (Walker 1979). The majority of agamosporous species (ca 50–70%) are triploid (Lovis 1977). Agamospory in ferns involves the production of unreduced spores followed by agamosporous reproduction in the gametophyte. Agamosporous triploids are thought to have been derived from hybrids between sexual and agamosporous (functionally male) diploids, or triploids formed between diploids and tetraploids (Manton 1950, Lovis 1977, Walker 1979). *Dryopteris affinis* agg. represents a group of agamosporous species of European sub-Atlantic and sub-Mediterranean distribution, ranging from SW Norway to N Africa and from Macaronesia to Caspian Iran (Fraser-Jenkins 1980, 2007). In the widest sense *Dryopteris affinis* has long been known as

a member of the *Dryopteris filix-mas* group (Manton 1950), with the latter in the sect. *Dryopteris*. Recently, however, it has been treated as a member of *Dryopteris* sect. *Fibrillosae* Ching (Fraser-Jenkins 1986). The major morphological differences between the sect. *Fibrillosae* and sect. *Dryopteris* are a more or less coriaceous lamina that is somewhat dark-green and glossy above, segments more rectangular and parallel-sided, and the stipe and rhachis more densely covered with narrowly lanceolate scales. Dense hair-like scales (fibrillae) are also present on the rhachis and are also often found on the lamina. Indusia are usually thicker and turned down or inflected at the edges and are more persistent. The majority of the species are agamosporous (Fraser-Jenkins 1986).

Taxa of the *D. affinis* group are notorious for their morphological variability, which has resulted in the recognition of multiple taxa described at various ranks. In Central Europe, three species differing in ploidy level and evolutionary history are currently recognized (Widén et al. 1996, Fraser-Jenkins 2007; Fig. 1). Diploid (2n = 82) *D. affinis* (Lowe) Fraser-Jenk. s.s. is restricted to the western and southern parts of Central Europe. Two triploid (2n = 123) species, *D. borreri* (Newman) Newman ex Oberh. & Tavel and *D. cambrensis* (Fraser-Jenk.) Beitel & W. R. Buck, are known from Central Europe. The other triploid European taxa recognized at the species level occur in W Europe (*D. pseudodisjuncta* (Tavel ex Fraser-Jenk.) Fraser-Jenk.) or in the surroundings of the Caucasus (*D. schorapanensis* Askerov, *D. pontica* Fraser-Jenk.; Fraser-Jenkins 2007).

Hybridization with *D. filix-mas* (L.) Schott is an important determinant of the phenotypic variability of the group (Fig. 1). The resultant hybrids are robust plants with a pentaploid or tetraploid cytotype and fronds with an intermediate morphology close to that of *D. affinis* s.l. Hybrids are found scattered within populations of the parental species (Heckmann et al. 1989, Bär & Eschelmüller 2006, Fraser-Jenkins 2007).

All taxa of the *D. affinis* group are comprehensively described in the publications of Fraser-Jenkins (1980, 1987, 2007) and are mostly based on qualitative characters, which are also used in the majority of European floras (Dostál et al. 1984, Fraser-Jenkins 1993, Frey et al. 1995, Willner 2005). The cytology, chemotaxonomy and local distribution of the *D. affinis* group have also been studied in Europe (Schneller 1974, Murín & Májovský 1980, Piękoś-Mirkowa 1981, Bär & Eschelmüller 1984, Heckmann et al. 1989, Bär & Eschelmüller 1990, Bremer & Koopman 1994, Vinter 1995, Eschelmüller & Eschelmüller 1996, Hilmer 1996, Widén et al. 1996, Ivanova 2004, Bär & Eschelmüller 2006). On the other hand, some of the species are not yet distinguished in some Central European floras, such as those of the Czech Republic (Chrtek 1988, Kubát et al. 2002), Poland (Mirek et al. 1995, Woziwoda 2005) and Slovakia (Marhold & Hindák 1998), and even in the identification key recently prepared for the Slovak flora (P. Mráz, in preparation). There is no evaluation of the use of independent quantitative morphological characters or genome size for identifying species.

Genome size and cytotype are easily assessable and do not require chromosome-squash counts (Bennett & Leitch 2005a, Suda & Trávníček 2006, Suda et al. 2006). Genome size is usually constant within the same taxonomic entity (Greilhuber et al. 2005) but often varies among closely related taxa (Bennett & Leitch 2005a). For this reason, genome size might be a good taxonomic marker at the specific and infraspecific level and has proved to be diagnostically useful for determining species boundaries or identifying cryptic taxa (Dimitrova et al. 1999, Mahelka et al. 2005, Suda et al. 2007). Despite its potential taxonomic value, estimation of cytotype by flow cytometry is rarely used on ferns (but see

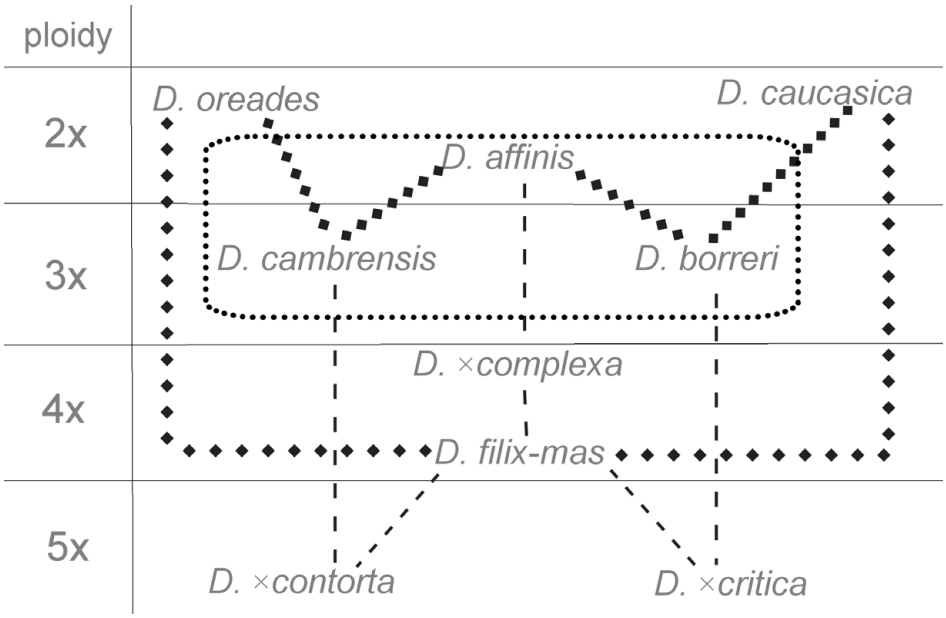

Fig. 1. – Evolutionary and hybrid relationships among taxa of the *Dryopteris affinis* and *D. filix-mas* groups in Central Europe. – – – recent hybrids; ◆ ◆ ◆ putative historical hybrids; ••• *D. affinis* group, other non hybrid species of the *D. filix-mas* group (compiled according to Widén et al. 1996, Fraser-Jenkins 2007).

Bureš et al. 2003, Ekrt & Štech 2008). The plant DNA C-values database (Bennett & Leitch 2005b) does not contain any record for the *D. affinis* group.

In this study, genome size and cytotype data together with multivariate morphometrics were used to identify the boundaries between taxa, find the best diagnostic characters and estimate the frequency of hybridization within the *D. affinis* group in Central Europe (the Czech Republic and adjacent countries).

Nomenclature

The nomenclature of the group *D. affinis* is still not satisfactorily resolved. We accepted names cited in the most recent foreign literature, mainly in the papers of Fraser-Jenkins (1979, 1980, 1987, 2007). However, the name *D. pseudomas* is often used for this group, and not only in the Czech and Slovak literature (Holub 1967, Chrtek 1988, Bremer & Koopman 1994, Marhold & Hindák 1998). The name *Dryopteris pseudomas* (Wollaston) Holub & Pouzar 1967, with basionym *Lastrea pseudomas* Wollaston 1855, was regarded as nomen illegitimum by Fraser-Jenkins (1979), because he believed that the epithet *Nephrodium affine* Lowe 1838 should be adopted for this combination in *Dryopteris*. Fraser-Jenkin's opinion is not justified because neither Holub (1967) nor Wollaston (1855) included the type of the name *Nephrodium affine* in their *Dryopteris/Lastrea pseudomas*. From this reason, the name *Dryopteris pseudomas* (Wollaston) Holub & Pouzar 1967 is not illegitimate from a nomenclatural point of view and from a taxonomic point of view

may only be considered synonymous with *Dryopteris affinis* (Lowe) Fraser-Jenk. 1979, if both taxa are considered to be conspecific.

If a separate taxonomic status at the rank of species is accepted for both taxa, the name is defined by nomenclatural types of both names. The name *Dryopteris affinis* (Lowe) Fraser-Jenk. is definitely linked with diploid plants (Fraser-Jenkins 1979). However, the taxonomic interpretation of the name *Lastrea pseudomas* Wollaston is more complicated and the lectotypification is not yet published. Wollaston (1855) published the name *Lastrea pseudomas* as a taxon identical with *Dryopteris filix-mas* var. *borreri* Newman 1854. Following Holub's analysis (Holub 1967), the epithet *pseudomas* seems to be the oldest specific epithet for var. *borreri*, which is triploid and for which the name *Dryopteris borreri* (Newman) Oberh et Tavel 1937 is currently usually used. Fraser-Jenkins, who studied the original material of Wollaston, believes that this material contains both triploid and diploid plants (C. R. Fraser-Jenkins, in preparation). Accordingly he is preparing lectotypification based on diploid plants. But his selection of the accessible material is not convincingly justified. It appears that it would be nomenclatorially more correct to select the triploid plant as the lectotype of *Lastrea pseudomas*. This selection seems to be more in accordance with Recommendation 9.A2,3 and 5 of International Code of Botanical Nomenclature (McNeill et al. 2006), which requires a special respect to original author's intention and to later interpretations if they are not in conflict with the protologue. In this case, Wollaston (1855) assorted his *Lastrea pseudomas* to *Dryopteris filix-mas* var. *borreri* Newmann distinctly and unequivocally. Although Holub did not know about the cytotype structure within the complex when he made the combination (Holub 1967), he restricted the name *Dryopteris pseudomas* (Wollaston) Holub & Pouzar to triploid plants in his later publication (Holub 1984). Moreover Fraser-Jenkins himself considered the name *Lastrea pseudomas* Wollaston as a synonym of *Dryopteris affinis* subsp. *borreri* (Newman) Fraser-Jenk. in his earlier study (Fraser-Jenkins 1980). So, we cannot but consider the nomenclature as provisional until the lectotypification is published and the selection of a suitable specimen justified.

Material and methods

Plants used for the study

Twenty-seven localities and 55 plants were sampled (both for morphometrics and flow cytometry) in the Czech Republic (24 localities) and adjacent countries – Slovakia (1 locality), Austria (1) and Poland (1) during 2004–2008 (see Appendix 1 for the list of localities). Members of the *Dryopteris affinis* group were neglected in the Czech Republic and other countries of Central Europe for a long time, and therefore only a restricted number of localities are known. In the study area (particularly in Bohemia), the group is usually very sporadically and sparsely distributed. The plants were collected from most of the known localities. In the case of *D. cambrensis* all currently known localities in the Czech Republic were sampled.

Herbarium voucher-specimens are deposited in PR. The nomenclature of the *D. affini*s group follows Fraser-Jenkins (2007).

Chromosome counts

Three plants of *Dryopteris borreri* (locality 13, sample PEC05), *D. filix-mas* (locality 12, sample STO) both in the Šumava Mts (Bohemian Forest) and *D. cambrensis* (locality 1, sample JEZ05) were used for determining the chromosome number. Chromosomes were counted in actively growing spore mother cells (and root-tips of *D. cambrensis*) of cultivated adult plants. Samples were pretreated with a saturated solution of p-dichlorbenzene (3 h, room temperature), fixed in a 3 : 1 mixture of ethanol and acetic acid and stained with lacto-propionic orceine. The number of chromosomes was ascertained using a Carl-Zeiss Jena NU microscope equipped with an Olympus Camedia C-2000 Z camera.

Flow cytometry

Flow cytometry was employed for estimating DNA ploidy level (Suda et al. 2006) and variation in genome size (the list of plants used is in Appendix 1). The measurements of DNA ploidy level were done using a Partec PloidyAnalyser II (Partec GmbH, Münster, Germany) at the Institute of Botany, Academy of Sciences in Průhonice, and genome size was estimated using Partec CyFlow (Partec GmbH, Münster, Germany) equipment at the Department of Botany, Faculty of Science, Charles University in Prague. In both studies we used the two-step procedure of nuclear isolation and staining, originally described by Otto (1990) and slightly modified by Suda & Trávníček (2006).

We mainly used sterile pinnae without sori but occasionally used developing shoot-apex buds for flow cytometric analyses. Approximately 4.5 cm^2 of fresh pinnae tissue were finely cut up together with the appropriate amount of an internal DNA standard plant using a new razor blade, in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). *Vicia faba* cv. Inovec (2C = 26.90 pg, Doležel et al. 1992) was selected as a suitable internal DNA standard with a genome size close to, but not overlapping, the taxa examined. The suspension was filtered through a nylon mesh (42 μm). After incubation (20 min at room temperature with occasional shaking), 1 ml of staining solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), fluorochrome (4 μg/ml DAPI) and β-mercaptoethanol (2 μl/ml) were added. The staining took 1–2 min. Fluorescence intensity of 3000 particles was recorded and the coefficient of variation for each analysed plant was calculated. The same method, but with fluorochrome propidium iodide (PI) together with RNase IIa (both in a final concentration of 50 μg/ml) replacing DAPI fluorochrome in the staining solution, was used for genome size estimation. *Pisum sativum* 'Ctirad' (2C = 8.76 pg, Greilhuber et al. 2007) was used as an alternative internal DNA standard, and the fluorescence intensity of 5000 particles was recorded.

The GLM procedure (LS means) available in SAS 8.1 (SAS Institute 2000) was used to assess the differences in genome sizes (Cx-values).

Morphometry

Each individual was taken as an operational taxonomic unit (OTU), and population aspects were omitted, because of occurrence of mixed populations. Only plants with fully developed sori were collected. A list of localities of the plants used for morphometric analyses is given in Appendix 1.

Table 1. – Morphological characters used in multivariate analyses of the *Dryopteris affinis* group.

Acronym	Character [unit]
StL	stomatal length [μm] / mean of 15 stomatal guard cells
La	lamina length [cm]
Pe	petiole (stipe) length [cm]
Pi	pinna length in central part of lamina [mm]
PuL	length of central pinnule in central part of lamina [mm]
PuW	width of central pinnule in central part of lamina [mm]
PuT	pinna segments with sharp teeth (1) or obtuse or without teeth (0)
PuN	number of pairs of segments on basal half of pinna in central part of lamina
trightScL	basal petiolar scale length [mm] / mean of 5 scales
ScW	basal petiolar scale width [mm] / mean of 5 scales
ScC	length of scales from centre of rhachis [mm] / mean of 5 scales
La/2xPi	ratio of lamina length and 2x pinna length
La/Pe	ratio of lamina length and petiole length

In total, 13 morphological and micromorphological characters of the triploid taxa (*D. borreri*, *D. cambrensis*) were measured (Table 1). Diagnostic quantitative characters presented in previous papers (Fraser-Jenkins 1980, 2007) and other important characters were included in our study.

Spore size was not analysed because of the lack of spores for some samples. Stomatal length was measured on the lower (abaxial) surface of pinnae through a thin nail-varnish layer moistened with a drop of water using a light microscope at the magnification of 1000×.

Prior to running multivariate analyses, the quantitative data were log-transformed [$x' = \ln(x + 1)$] to improve their fit to a normal distribution. Qualitative characters (shape of pinna segments – PuT) were coded as binary (dummy) variables. Principal components analysis (PCA) was applied to the primary data matrix, which includes all the morphological characters recorded. The PCA provided an insight into the overall pattern of variation and revealed morphological discontinuities among the taxa studied. The analysis was conducted using CANOCO for Windows (ter Braak & Šmilauer 2002, Lepš & Šmilauer 2003) and the results visualized using CanoDraw for Windows 4.0 (ter Braak & Šmilauer 2002).

Linear discriminant analysis (LDA; Klecka 1980, Krzanowski 1990) with forward selection was used to find the morphological characters that resulted in the maximum separation of the taxa. The linear discriminant function was then calculated and its predictive ability tested by cross-validation. Computation of the discriminant analyses were carried out using STATISTICA 5.5 software (StatSoft 1998).

Results

Chromosome counts

In order to evaluate the accuracy of the flow cytometric results chromosome squash counts were obtained for samples of *Dryopteris borreri*, *D. cambrensis* and *D. filix-mas.* The chromosome counts confirmed the triploid cytotype for plants identified as *D. borreri* ($n = 123^{I}$, Fig. 2A) and *D. cambrensis* (2n = ca 123) and the tetraploid cytotype ($n = 82^{II}$, Fig. 2B) of *D. filix-mas*. Moreover, these counts represent the first chromosome counts for all three taxa from the Czech Republic.

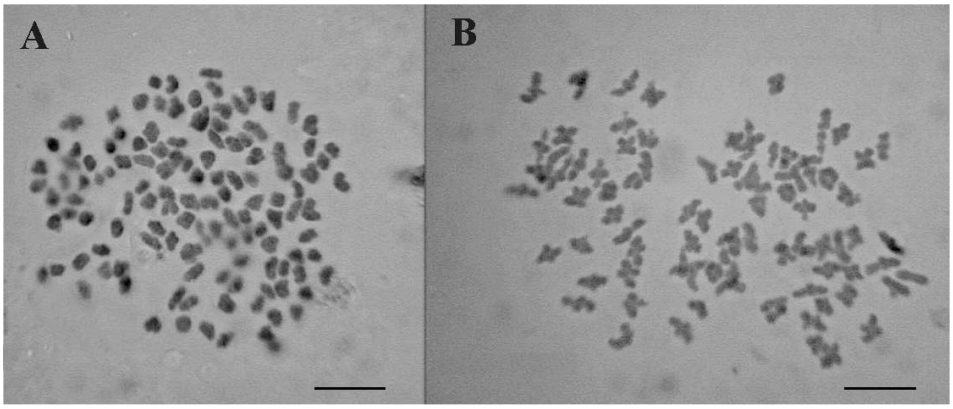

Fig. 2. – Microphotograph of chromosomes at meiosis: A – *Dryopteris borreri* (n = 123^{I}); B – *Dryopteris filix-mas* (n = 82^{II}). Scale bar 1 μm.

Flow cytometry

The cytotypes of all 55 samples of the *Dryopteris affinis* group collected from throughout the study area were examined. Flow cytometric analysis detected triploid and pentaploid plants. Its success in distinguishing taxa based on relative fluorescence is illustrated by the histograms presented in Fig. 3. The triploid cytotype was the dominant ploidy level within the *D. affinis* group (52 out of 55 individuals examined). In concordance with morphological variability, two groups of triploids were detected that were separated significantly by relative fluorescence intensity (Table 2). The first, identifiable by their morphology as *D. borreri* (39 samples), is characterized by a mean relative genome size of 0.883±0.010 (hereafter figures correspond to the mean±S.D., mean fluorescence intensity of the standard given as the unit). The second type, identifiable as *D. cambrensis* (13 samples), is distinguished by a mean relative genome size of 0.844±0.015. One further, uncommon cytotype we found within the *D. affinis* group was pentaploid (3 samples) with a markedly different mean relative genome size of 1.448±0.021 (Table 2). Pentaploids were found either in mixed populations together with *D. borreri* (locality 12 and 26) or isolated as single plants (locality 6). Although the exact taxonomic identity of the pentaploid plants is unclear, flow cytometric results (Table 2, Fig. 3D) indicate hybrid origin, and they most probably are primary hybrids between *D. borreri* and *D. filix-mas*, known as *D.* ×*critica* (Fraser-Jenk.) Fraser-Jenk. Repeated runs of simultaneous analyses of all three taxa (Fig. 3D) revealed the proportions of mean relative fluorescences (given as units) to be 1.000 : 1.276 : 1.642 for *D. borreri*, *D. filix-mas* and *D.* ×*critica*, respectively, which is in good agreement with the suggested genome composition of pentaploid hybrids involving the whole (3x) genome of *D. borreri* and half the genome (2x) of *D. filix-mas*.

Study of the absolute genome size of four selected plants of each triploid taxon and all three available pentaploid plants revealed differences in genome size (Table 3). The mean value (2C-value) was 25.18 pg for plants identified as *D. cambrensis*, 26.01 pg for *D. borreri* and 40.76 pg for *D.* ×*critica.* The genome size of triploids of *D. cambrensis* and *D. borreri* shows a 3.0% difference ($F = 89.4$; $P < 0.001$) in total genome size, while

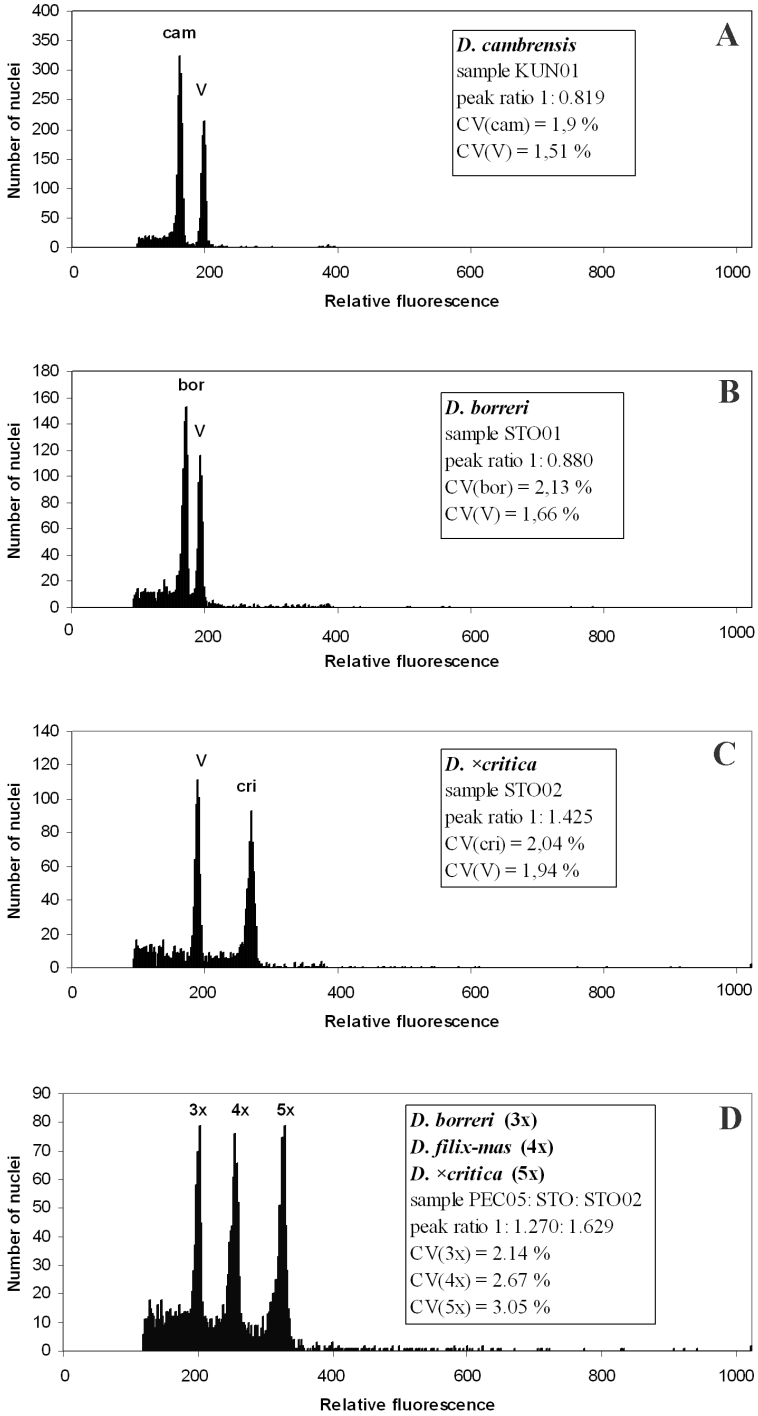

Fig. 3. – Histograms of the relative DNA content obtained by analyzing the DAPI-stained nuclei isolated from the species studied and the reference standard (*Vicia faba* = V). – A: *Dryopteris cambrensis*; B: *D. borreri*; C: *D.* ×*critica*; D: simultaneous FCM analysis of triploid *D. borreri*, tetraploid *D. filix-mas* (both of known chromosome number) and their pentaploid hybrid.

Table 2. – Summary of relative genome sizes of taxa of the *Dryopteris affinis* group compared to the standard *Vicia faba* (given as unit relative genome size). N = number of samples analysed; 2C = somatic relative nuclear DNA content; SD = standard deviation; CV = range of values of coefficient of variance of sample peaks.

Taxon	Ploidy	N	Intensity of fluorescence			
			2C Min	2C Max	2C Mean±S.D.	CV (%)
D. borreri	3x	39	0.860	0.901	0.883±0.010	1.46–3.32
D. cambrensis	3x	13	0.819	0.874	0.844±0.015	1.90–3.17
D. ×critica	5x	3	1.425	1.465	1.448±0.021	1.95–2.03

Table 3. – Summary of the characteristics of genome size (2C and Cx values) in absolute units (picograms) of the taxa of the *Dryopteris affinis* group studied. N = number of samples analysed; SD = standard deviation; CV = range of values of coefficient of variance of sample peaks. Asterisk (*) = different letters indicate groups of taxa that differ significantly at P<0.001.

Taxon	Ploidy	N	2C values (pg of DNA)				Cx-value (pg of DNA)
			Min	Max	Mean±S.D.	CV (%)	Mean*
D. borreri	3x	4	25.79	26.14	26.01±0.163	1.62–2.97	8.67[a]
D. cambrensis	3x	4	25.10	25.26	25.18±0.066	2.17–2.86	8.39[b]
D. ×critica	5x	3	40.65	40.82	40.76±0.095	2.17–3.27	8.15[c]
D. filix-mas	4x	1	33.80	33.80	33.80	1.57–2.96	8.45[b]

a comparison of the same taxa based on relative genome size showed a 4.6% difference ($F = 111.3$; $P < 0.001$). Additionally the genome size of one sample of *D. filix-mas* was 33.80 pg. Monoploid genome sizes reflecting the amount of DNA for one complete set of chromosomes (1Cx-value; Greilhuber et al. 2005) were calculated as, 8.67±0.05 pg, 8.39± 0.02 pg, 8.15±0.02 pg and 8.45 pg for *D. borreri*, *D. cambrensis*, *D. ×critica* and *D. filix-mas,* respectively. All taxa (except *D. cambrensis* and *D. filix-mas*) differ significantly in Cx-values ($P < 0.001$; Table 3).

The distribution in the study area of taxa for which the genome size is known is given in Fig. 4.

Multivariate morphometrics

The main pattern of variation in characters and its relationship to the taxonomic identity of the plants was examined by principal component analysis (PCA). Genome size was used as an independent criterion for the identification of species. Two samples of *D. cambrensis* forming the upper extreme values in the analysis of relative genome size (overlapping with the extreme lower values of *D. borreri*) were included in the analysis of absolute genome size, but no overlap was detected. PCA revealed clear morphological differentiation between the species (Fig. 5). The first three principal components (axes) accounted for more than 66% (33.7%, 19.5% and 12.9%, respectively, for the first to third axis) of the total variation in the morphological characters of all specimens. The first axis is correlated with characters such as length of lamina (La), length of petiole (Pe), and lengths of pinnae and their segments (Pi, PuL, PuN). The second axis correlated with scale characters (ScC, ScL, ScW) and the ratio of lamina length to lamina width (La/2xPi; see Fig. 5).

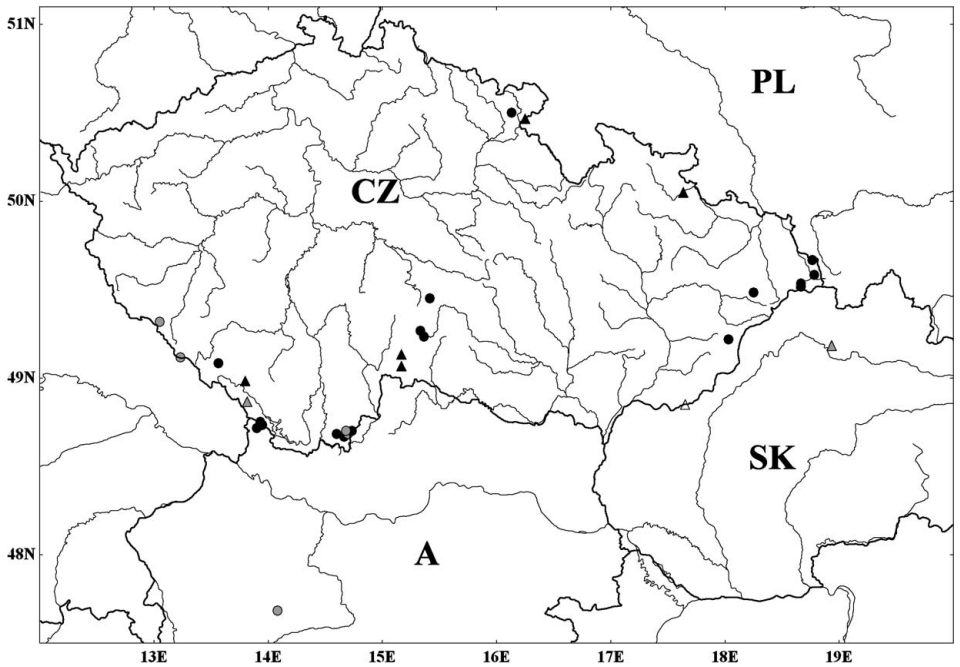

Fig. 4. – Distribution of sites from which samples of *Dryopteris borreri*, *D. cambrensis* and *D.* ×*critica* were collected and their identity confirmed by flow-cytometry. CZ – Czech Republic, SK – Slovakia, A – Austria, PL – Poland; ● *D. borreri*, ▲ *D. cambrensis*, ● *D. borreri* and *D. cambrensis*, ▲ *D. borreri* and *D.* ×*critica*, △ *D.* ×*critica*.

The best characters revealed by LDA using forward selection (see Table 4) are the number of pairs of segments in the basal half of the pinna in the central part of lamina (PuN) and the scale width at the petiole-base (ScW). Additional diagnostic characters were the type of lamina (narrow vs. broad – i.e., the ratio of lamina length to twice the pinna length; La/2xPi) and the length of segments or lobes (pinnules) in the central part of the pinna in the central part of the lamina (PuL). Petiole length (Pe) and pinna length (Pi) were excluded from the analysis because of their strong correlation (≥ 0.9) with the length of the lamina (La).

The classification function (linear discriminant function) was calculated for both species. Only the four most diagnostic characters (Table 4) were included. Classificatory precision of this function was estimated using cross-validation and posterior probabilities of misidentification obtained. Both species studied were identified correctly in 100% of cases. A taxonomic key was compiled based on the four most diagnostic characters, plus stomatal length and viability of spores (see below).

Box and whisker plots based on real values were constructed for the two most diagnostic characters (Fig. 6).

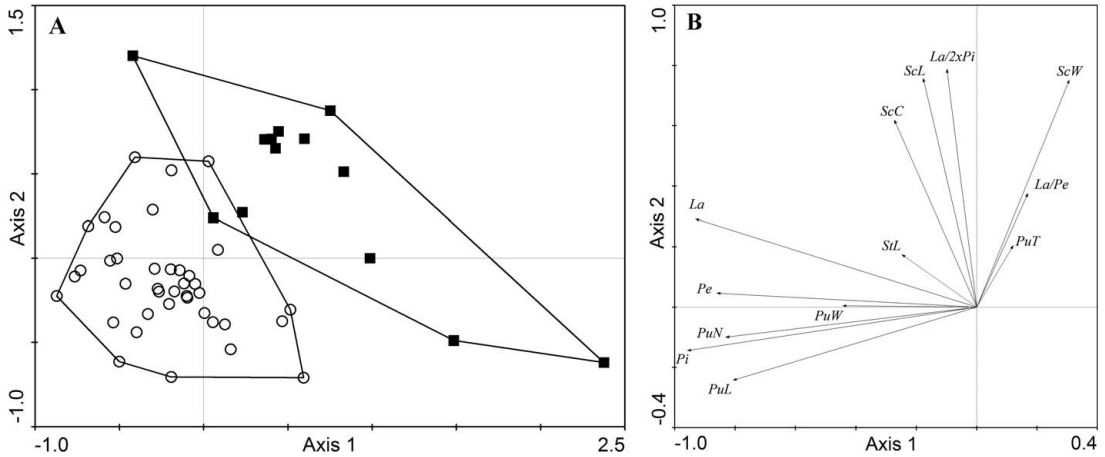

Fig. 5. – PCA ordination of specimens (A) and characters (B) of species of the *Dryopteris affinis* group (■ *D. cambrensis*; ○ *D. borreri*). The first and second components (axes) account for 33.7% and 19.5% of the variability, respectively.

Table. 4. Summary of the linear discriminant analysis (forward selection) of the morphological characters of *Dryopteris borreri* and *D. cambrensis*. Only characters significant at $P < 0.05$ were included.

Character	Step	F	P-level
PuN	1	64.68	$P < 0.001$
ScW	2	34.91	$P < 0.001$
La/2xPi	3	11.14	0.0016
PuL	4	7.54	0.0085
PuT	5	4.90	0.0319

Key to the identification of species

The most suitable combinations of morphological characters measured in this study resulted in the following key for identifying the species of the *D. affinis* group in Central Europe. In accordance with C. R. Fraser-Jenkins' (pers. comm.) taxonomic concept, *D. cambrensis* in the study area corresponds with *D. cambrensis* subsp. *insubrica* (Oberh. & Tavel ex Fraser-Jenk.) Fraser-Jenk.

1a Stomata length (mean value of ca 15 stomatal guard-cells) > 58 µm, spores usually aborted with a few well developed ..... **hybrids (*D.* ×*critica*)**

1b Stomata length (mean value of ca 15 stomatal guard-cells) < 58 µm, spores usually well developed **2**

2a Number of pair of segments in the basal half of the pinna in the central part of the lamina (10–) 12–13 (–15); the largest basal petiole scales (1.0–) 2.0–2.5 (–5.0) mm wide; lamina 1.8–3× longer than wide, length of the central pinna-lobe (pinnule) in the central part of the lamina (7.0–) 10.5–15.0 (–18.0) mm ..... ***D. borreri***

2b Number of pairs of segments in the basal half of the pinna in the central part of the lamina (4–) 8–10 (–11); the largest basal petiole scales 3.0–4.5 (–6.0) mm wide; lamina 2.5–3.5× longer than wide; length of the central pinna-lobe (pinnule) in the central part of the lamina (6.5–) 7.5–9.0 (–13.0) mm ..... ***D. cambrensis***

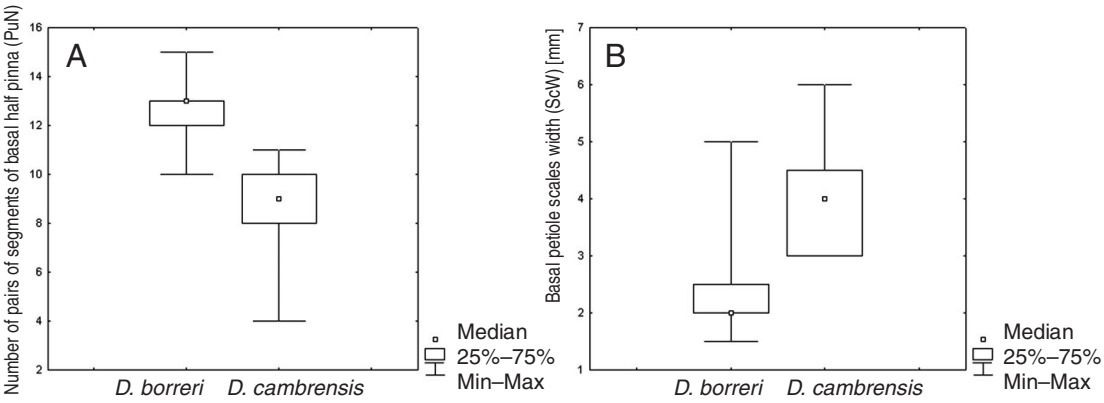

Fig. 6. – Box and whisker plots of one-way ANOVA of A – the number of pairs of segments on basal half of the pinna in the central part of lamina (PuN; F=64, P<0.001) and B –width of basal petiole scales (ScW; F=42, P<0.001).

Discussion

Geographical distribution

Various cytotypes (2x, 3x, 4x, 5x) of *Dryopteris affinis* s.l. are mentioned in the seminal book by Manton (1950) as occurring in W Europe. However, *D. affinis* s.l. was then not recognized and included within the variability of *D. filix-mas* in Central Europe (Dostál 1950, Murín & Májovský 1980, Piękoś-Mirkowa 1981). But more recently the aggregate species *D. affinis* s. l. (often cited as *D. pseudomas*) is recognized in floras of Central European countries: Czech Republic (Chrtek 1988, Kubát et al. 2002), Poland (Mirek et al. 1995) and Slovakia (P. Mráz, in preparation), or in some cases (Dostál et al. 1984) are distinguished as subspecies of the *D. affinis* group, as previously treated by Fraser-Jenkins (1980). In Austria the subspecies *D. affinis* subsp. *affinis*, *D. affinis* subsp. *borreri* and *D. affinis* subsp. *cambrensis* are widely accepted (Willner 2005). However, more recently these taxa are treated as species in the newer taxonomic concept of the genus by Fraser-Jenkins (2007). According to his current concept, *D. borreri* and *D. cambrensis* occur in the Czech Republic and Poland, while only *D. borreri* is known from Slovakia, and *D. affinis*, *D. borreri*, *D. cambrensis* and *D. pseudodisjuncta* are present in Austria.

Nevertheless, the rather restricted knowledge of the taxonomy, distribution and delimitation of taxa within the *D. affinis* group in other European floras led to many misinterpretations, neglect of and failure to recognize taxa. Our study has revealed the presence of *D. borreri* at 21 localities, *D. cambrensis* at 10 localities and pentaploid *D.* ×*critica* at three localities within the Czech Republic and its close surroundings. Whilst populations of *D. borreri* usually consist of several plants (or rarely only a single plant), *D. cambrensis* regularly only occurs as single plants. The occurrence of *D. cambrensis* in Central Europe north of the Alps is considered to be very rare, and only single plants are usually found in the central and northern part of Germany (Jessen 1984, Hilmer 1996). The single and scattered localities of *D. cambrensis* represent a distribution pattern of a species at the edge of its range, similar to that of another agamosporous fern, *D. remota* (Ekrt et al. 2007).

Genome size

In accordance with the previous treatment by Fraser-Jenkins (2007), we found and investigated two triploid taxa (*D. borreri* and *D. cambrensis*) of differing genome size and a pentaploid hybrid *D.* ×*critica* (*D. borreri* × *D. filix-mas*) in the study area.

Additionally, the genome sizes recorded (a new cytogenetic character) fill a gap in the DNA C-values database (Bennett & Leitch 2005b), which so far does not contain any data for the *D. affinis* group. Genome size equals the 1C-value for the taxa examined: *D. borreri* (13.01 pg), *D. cambrensis* (12.59 pg), *D.* ×*critica* (20.38 pg), *D. filix-mas* (16.9 pg), and these values are higher than those published for other species of *Dryopteris* listed in the database, whereas for *D. filix-mas* and *D. dilatata* 1C-values are 8.70 and 8.05 pg, respectively (Bennett & Leitch 2005b). Values in the database are much closer to monoploid genome sizes indicating the amount of DNA in one complete set of chromosomes = 1Cx-value (Greilhuber et al. 2005), not 1C-value.

The genome size of *D. borreri* and *D. cambrensis* differs by 3.02% (genome size given in absolute units) or 4.6% (relative genome size). A similar pattern of slightly differing genome sizes in closely related species are reported in another recent study, in which intermediate hybrids were also detected (Mahelka et al. 2005). Close but separate evolutionary origins of various *D. affinis* group species is also indicated by a study of their phloroglucinol derivatives (Widén et al. 1996). Based on the estimated genome composition, two thirds of the genome of the two triploid taxa is suggested to be the same and one third different.

The earliest ancestor of *D. affinis* s.s. is thought to have been a hybrid of the genome formula "OW" [*D. oreades* Fomin + *D. wallichiana* (Spreng.) Hyl.], which became fertile via agamospory. *Dryopteris borreri* ("OCW") is thought to have been an ancient hybrid species derived from diploid agamosporous *D. affinis* and the east European sexual diploid species *D. caucasica* (A.Braun) Fraser-Jenk. & Corley ("CC"), whereas *D. cambrensis* ("OOW") probably originated from diploid agamosporous *D. affinis* and the west European sexual species *D. oreades* ("OO")(Widén et al. 1996, Fraser-Jenkins 2007). The above proposed hybridizations require further molecular research but close genome composition could explain the observed low difference between genome sizes of the triploid taxa studied.

Phenotypic variation and species-specific characters

Morphological variability within the *D. affinis* group is extremely high. This variation is probably a result of ancient hybridization and maintained by isolation of small agamosporic lineages (Manton 1950, Fraser-Jenkins 2007). There are six distinct entities currently recognized as species in a recent taxonomic treatment (Fraser-Jenkins 2007), though some further adjustment and reduction in the number of species may be made (C. R. Fraser-Jenkins, pers. comm.), and many other more minor variants and types are recognized as having arisen within the species, preserved by agamosporous reproduction via spores. The majority of distinguishable types differ distinctly in morphology and geographical distribution. Some of the relatively minor variants are recognized as subspecies by Fraser-Jenkins (2007).

In contrast, alternative taxonomic treatments of Fraser-Jenkins' taxa did not accept this hierarchical taxonomic scheme and identified them as invalidly named and unranked

morphotypes (Jermy & Camus 1991, Pigott 1997, Merryweather 2002). But recently the morphotype concept has been abandoned by its advocates, including Merryweather (2007), in favour of the present hierarchical scheme, at least for most major taxa.

Studying species boundaries definable by distinct quantitative characters was a major aim of our study. Multivariate morphometric analyses were applied to the triploid taxa, *D. borreri* and *D. cambrensis,* identified on the basis of their genome size. Distinctions between taxa based on relatively qualitative standard botanical characters such as the shape of pinnae, segments, teeth, glandularity of lamina, colour of scales and similar characters are included in European keys, floras and other taxonomic publications (Dostál et al. 1984, Fraser-Jenkins 1993, Frey et al. 1995, Willner 2005, Fraser-Jenkins 2007). Some of the characters are difficult to specify. The PCA and LDA results have identified some more exact quantitative characters that can be used for identifying taxa occuring in Central Europe (see identification key above).

Owing to their distinct genome sizes, clear morphological differentiation and presumed different origin, it is suitable to treat the taxa as species. According to our findings, the taxonomic concepts of Fraser-Jenkins (2007) are the most acceptable for the taxa concerned.

Hybridization

The absence of hybrids between the triploid taxa is attributed their agamosporous reproduction (Fraser-Jenkins 1987, 2007). On the other hand, both taxa can easily hybridize with sexual species, such as *D. filix-mas,* giving rise to pentaploid progeny (Heckmann et al. 1989, Bär & Eschelmüller 1990). Our study confirmed that the frond morphology of *D.* ×*critica*, the hybrid between *D. borreri* and *D. filix-mas*, is generally very similar to that of *D. borreri*. Because of the rarity and occurrence of only single plants of *D. cambrensis,* the probability of it hybridizing with *D. filix-mas* is very low, as is the case in Germany (Bär & Eschelmüller 1990). The considerably different genomic composition could be another factor determining a low rate of hybridization between *D. cambrensis* and *D. filix-mas*.

There are only a few microcharacters, such as fertile versus aborted spores and size of the stomatal guard-cells, that can be reliably used for identify hybrids, which accords with previous studies (Schneller 1974, Vinter 1995, 2001, Fraser-Jenkins 2007). The recently published monographic treatment of *D. affinis* s.l. (Fraser-Jenkins 2007) only mentions the quality of spores (percentage aborted) as an exclusive delimiting key-character for distinguishing between hybrid and non-hybrid taxa. However, familiarity with frond morphology, pinule-shape and lobing, scale-colour etc. provide an easy means of recognizing hybrids, which can be used in the field with considerable success and subsequently confirmed based on spore-characters (C. R. Fraser-Jenkins, pers. comm.), even though they are difficult to quantify objectively. The quality of spores can sometimes be misleading since ca 5–20% of the spores of hybrids are well developed, and non-hybrid taxa produce at least some aborted spores, and very occasional exceptional plants of *D. cambrensis* (subsp. *cambrensis*, discovered in Britain and confirmed cytologically) may produce mainly aborted spores but can be recognized by their frond-morphology, which differs from that of hybrids (C. R. Fraser-Jenkins, pers. comm.). In addition, it can be difficult to identify plants with dehisced sporangia containing no or only a few spores, particularly

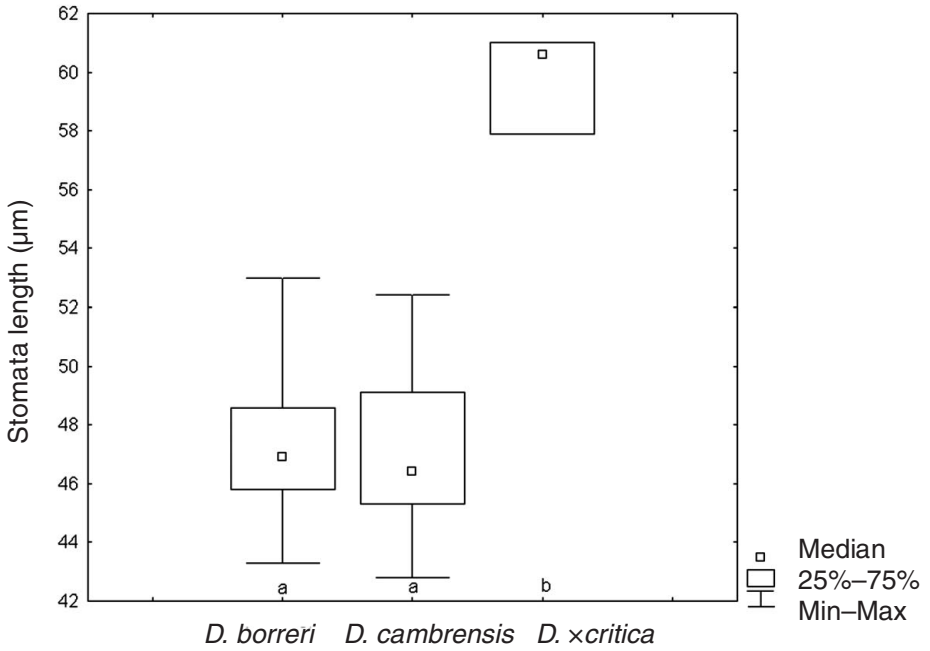

Fig. 7. – Box and whisker plot of one-way ANOVA ($F = 68$, $P < 0.001$) of the mean stomata length (StL). Letters at the bottom indicate the results of the Tukey HSD test, taxa labelled with the same letter do not differ significantly ($P > 0.01$).

old herbarium-specimens. The ambiguity resulting from the use of aborted versus well developed spores to identify hybrids is caused by variation in the success of cell-division during sporogenesis, which resulted from the agamosporic origin of the taxa in the *D. affinis* group. In sexual species of ferns, the presence of a high proportion of aborted spores is usually a very reliable way of identifying the hybrids (Wagner & Chen 1965, Reichstein 1981, Dostál et al. 1984). The best quantitative diagnostic character is undoubtedly mean stomatal length, even though it varies due to environmental factors such as water-stress, which is well known to cause inaccuracies. Stomata of the triploid taxa studied here are (43–) 45–49 (–53) µm long, whereas in the pentaploid hybrid (*D.* ×*critica*) they are much longer: 58–61 µm (Fig. 7). Similar patterns in stomatal size are recorded in previous studies (Schneller 1974, Vinter 2001).

Hybrids usually occur as scattered individuals in populations of the parental species (Bär & Eschelmüller 2006, Fraser-Jenkins 2007). We recorded the pentaploid hybrid *D.* ×*critica* very rarely in populations of *D. borreri*. A single hybrid plant was found in the valley of a small stream in the Bílé Karpaty Mts, where neither of the parental species occured. It is well known, following Manton (1950), that the low level of fertility of these apomictic hybrids is due to the occasional large, fertile spores blown from elsewhere, and C. R. Fraser-Jenkins (pers. comm.) has reported occasional small populations of hybrids probably arising from spores from a hybrid plant at certain localities. It is also well known that some hybrid plants may live for many decades, during which time the parental species might have become extinct.

Acknowledgements

We are much obliged to C. R. Fraser-Jenkins for his valuable comments and determination of *D. cambrensis* subsp. *insubrica* from our study area and for a few additions and correction of the English in this paper. Nomenclatorical part benefited from a major contribution from M. Štech, Z. Kaplan and J. Kučera. Martin Lepší and Karel Boublík kindly provided some field records of *D. cambrensis*. Jan Košnar kindly improved our English, Tony Dixon edited the final version of the manuscript. The study was supported by the grant no. 206/07/0706 from the Grant Agency of the Czech Republic and institutional research grants AV0Z60050516 (Academy of Sciences of the Czech Republic), MSM 0021620828 (Ministry of Education, Youth and Sports of the Czech Republic) and 206/05/0970 (Czech Science Foundation).

Souhrn

Dryopteris affinis agg. představuje v Evropě taxonomicky komplikovanou skupinu agamosporických druhů. Tento příspěvek přináší morfometrickou a cytometrickou studii skupiny z 27 lokalit nacházejících se zejména v České republice a okrajově v Polsku, Slovensku a Rakousku. Na základě studia ploidních úrovní a velikostí genomu pomocí průtokové cytometrie a na základě studia morfologických znaků, byly ověřeny dva triploidní (2n = 123) druhy *D. borreri* a *D. cambrensis* a jeden pentaploidní (2n = 205) kříženec *D.* ×*critica* (*D. borreri* ×*D. filix-mas*). Oba studované triploidní druhy se vzájemně liší velikostí genomu. Z území České republiky byl zároveň poprvé spočítán počet chromozómů u *D. borreri*, *D. cambrensis* a *D. filix-mas*. Jednotlivé taxony nejsou dosud v květenách České republiky, Slovenka a Polska rozlišovány. Zatímco *D. borreri* představuje roztroušeně se vyskytující druh, *D. cambrensis* je velmi vzácným druhem rostoucím zpravidla v ojedinělých exemplářích. Jednotlivé izolované výskyty představují typické rozšíření druhu na okraji svého areálu. Na základě studia kvantitativních morfologických znaků byly nalezeny vhodné znaky na determinaci jednotlivých druhů:

1a Průduchy větší než 58 µm (průměrná hodnota za ca. 15 buněk), výtrusy značně nepravidelné, převážně abortované ..... **kříženci (*D.* ×*critica*)**
1b Průduchy menší než 58 µm (průměrná hodnota za ca. 15 buněk), výtrusy převážně vyvinuté 2
2a Bazální polovina lístku ve středu čepele s (10–) 12–13 (–15) páry úkrojků; největší bazální pleviny na řapíku (1,0–) 2,0–2,5 (–5,0) mm šir., světle hnědé; čepel 1,8–3,0× delší než širší; středový úkrojek lístku v centrální části čepele (7,0–) 10,5–15,0 (–18,0) mm dl. ..... ***D. borreri***
2b Bazální polovina lístku ve středu čepele s (4–) 8–10 (–11) páry úkrojků; největší bazální pleviny na řapíku 3,0–4,5 (–6,0) mm šir., čepel 2,5–3,5× delší než širší; středový úkrojek lístku v centrální části čepele (6,5–) 7,5–9,0 (–13,0) mm dl. ..... ***D. cambrensis***

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Received 14 March 2009
Revision received 8 July 2009
Accepted 24 July 2009

Appendix 1. – List of localities of taxa of the *Dryopteris affinis* group used in flow cytometry and morphometric study: no. – locality number; locality – country, phytogeograpical district (only for localities in the Czech Republic) with its number (Skalický 1988), locality, latitude, longitude (coordinate system WGS-84), altitude, collector, collection date (a field locality code is given in parentheses); n – number of plants examined using flow cytometry; taxa(n) – the number of taxa identified (bor = *Dryopteris borreri*; cam = *D. cambrensis*; ×cri = *D.* ×*critica*); fil = *D. filix-mas*, reference sample). Taxa with an asterisk (*) were checked using chromosome counts and taxa with the symbol (#) were analysed to determine their absolute genome size.

No.	Locality	n	Taxa(n)
1	Czech Republic, 33. Branžovský hvozd: Liščí, foothill of Jezvinec hill, c. 1.8 km NE of the village centre, 49°19′19.7″N, 13°03′42.6″E, c. 590 m alt., leg. L. Ekrt 15. X. 2008, (JEZ).	4	bor(2)+(1)#, cam(1)* #
2	Czech Republic, 58e. Žaltman, Rokytník, vinicity of old military fortification near the forest edge, c. 500 m NNE of the Maternice hill, 50°30′22″N, 16°08′07″E, c. 530 m alt., leg. L. Ekrt 14. X. 2004, (ROK).	1	bor(1)
3	Czech Republic, 67. Českomoravská vrchovina: Střížovice, ancient stony wall at the edge of alder forest c. 850 m SE of the village centre, 49°08′12.0″N, 15°10′01.8″E, 540 m alt., leg. L. Ekrt 26. V. 2008, (KUN).	1	cam(1)
4	Czech Republic, 67. Českomoravská vrchovina: Ústí near Humpolec, edge of small stream in alder forest, c. 1 km SSE of the village centre, 49°27′52″N, 15°25′03″E, 590 m alt., leg. L. Ekrt 15. IX. 2007, (UST).	1	bor(1)
5	Czech Republic, 74b. Opavská pahorkatina: Brantice, c. 1.5 km SE of the village centre, 50°03′08.5″N, 17°38′15.5″E, 460 m alt., leg. L. Ekrt 17. IX. 2007, (BRA).	3	cam(3)
6	Czech Republic, 78. Bílé Karpaty lesní: Vápenky, foothill of Velká Javořina Mt., 48°51′58″N, 17°39′40″E, c. 620 m alt., leg. L. Ekrt, 18. IX. 2007, (VAP).	1	×cri(1)#
7	Czech Republic, 79. Zlínské vrchy: Lidečko, beech forest on E side of Kopec hill c. 1.9 km N of the village centre, 49°13′13″N, 18°02′48″E, c. 595 m alt., leg. L. Ekrt, 18. IX. 2007, (LID).	1	bor(1)
8	Czech Republic, 84a. Beskydské podhůří: Jablunkov, Návsí, hillside above a small stream ca 2.2 km NE of the town of Jablunkov, 49°35′30″N, 18°47′07″E, c. 430 m, leg. L. Ekrt, 17. IX. 2007, (JAB).	2	bor(2)
9	Czech Republic, 88a. Královský hvozd: Železná Ruda, Alžbětín, edge of the road near ancient village of Debrník c. 1.7 km ESE of the railway station of Alžbětín, 49°07′13.7″N, 13°14′02.4″E, 780 m alt., leg. L. Ekrt & J. Hadinec 29. VIII. 2007, (DEB).	2	bor(1), cam(1)
10	Czech Republic, 88b. Šumavské pláně: Studenec, Popelná settlement, central part of valley of Pěnivý potok stream c. 2.2 km WSW of the Popelná settlement, 49°05′27″N, 13°34′32″E, 905 m alt., leg. L. Ekrt 12. X. 2006, (PEP).	2	bor(2)
11	Czech Republic, 88d. Boubínsko-stožecká hornatina: 'Kubova Hut', side of the road of Lukenská cesta, N edge of fenced part of Boubínký prales virgin forest reserve, c. 2.8 km E of the village centre, 48°58′59″N, 13°48′41″E, 1110 m alt., leg. L. Ekrt 30. VIII. 2007, (BOU).	1	cam(1)#
12	Czech Republic, 88d. Boubínsko-stožecká hornatina: Stožec, beech forest in the Stožec reserve c. 750 m E of the peak of Stožec Mt., 48°52′56″N, 13°49′53″E, 995 m alt., L. Ekrt 13. IX. 2004, (STO).	2	bor(1)#, ×cri(1)#, fil(1)* #
13	Czech Republic, 88e. Trojmezenská hornatina: Nová Pec, old forest clearing c. 4 km SSW of the railway station of Nová Pec, 48°43′30″N, 13°54′21″E, 900 m alt., L. Ekrt & E. Ekrtová 25. IX. 2007, (PEC).	5	bor(4)+(1)*
14	Czech Republic, 88e. Trojmezenská hornatina: Nová Pec, foothill of Smrčina Mt., 48°45′27″N, 13°56′27″E, c. 880 m alt., leg. L. Ekrt, 25. IX. 2007, (SMR).	4	bor(4)
15	Czech Republic, 89. Novohradské hory: Žofín, along small stream and at the edge of the forest path c. 1.4 km W of the village centre, 48°40′30.2″N, 14°40′26.1″E, 765 m alt., leg. L. Ekrt & M. Lepší 5. IX. 2007, (PIV).	4	bor(1)+(1)#, cam(1)+(1)#
16	Czech Republic, 89. Novohradské hory: Černé údolí, edge of forest path c. 2.1 km ESE of the village centre, 48°41′35.6″N, 14°42′08.2″E, 890 m alt., leg. L. Ekrt & M. Lepší 5. IX. 2007, (POH).	2	bor(1), cam(1)#

17	Czech Republic, 89. Novohradské hory, Pohorská Ves, Hojná voda virgin forest reserve, c. 1.2 km SE of the peak of Vysoká hill, 48°42′23"N, 14°45'09"E, c. 850 m alt., leg. L. Ekrt & M. Lepší, 5. IX. 2007, (HOJ).	2	bor(2)
18	Czech Republic, 89. Novohradské hory, Malonty, alder forest c. 1.7 km ENE of the village centre, 48°41'19"N, 14°36'01"E, c. 665 m alt., leg. L. Ekrt & M. Lepší, 5. IX. 2007, (MAL).	3	bor(3)
19	Czech Republic, 90. Jihlavské vrchy, Kaproun, forest below the railway station of Kaproun, 48°04'56"N, 15°10'12"E, 660 m alt., leg. L. Ekrt 30. IX. 2008, (KAP).	1	cam(1)
20	Czech Republic, 90. Jihlavské vrchy, Horní Dubenky, side of the forest road c. 2.2 km NE of the village centre, 49°16'0.7"N, 15°20'44.1"E, 695 m alt., L. Ekrt & E. Ekrtová 30. IX. 2007, (DUB).	1	bor(1)
21	Czech Republic, 90. Jihlavské vrchy, Řásná, edge of forest path, c. 2.2 km NW of the village centre, 49°14'13"N, 15°22'21'E, 690 m alt., L. Ekrt & E. Ekrtová 6. X. 2007, (RAS)	1	bor(1)#
22	Czech Republic, 99a. Radhošťské Beskydy: Dolní Lomná, along the stream below the Mionší reserve c. 1 km ESE of the Velká Polana hill, 49°31'52"N, 18°40'08"E, c. 625 m alt., leg. L. Ekrt, 18. IX. 2007, (MIO).	2	bor(2)
23	Czech Republic, 99a. Radhošťské Beskydy: Horní Bečva, Pustevny settlement, beech forest in NW part of Tanečnice Mt. c. 300 m N of Pustevny settlement, 49°29'34"N, 18°15'57"E, c. 1090 m alt., leg. L. Ekrt, 18. IX. 2007, (PUS).	2	bor(2)
24	Czech Republic, 99b. Slezské Beskydy: Nýdek, foothill of Velká Čantoryje Mt., slope leading up to a small stream c. 1.9 km NE of the village centre, 49°40'09"N, 18°46'24"E, c. 485 m alt., leg. L. Ekrt, 17. IX. 2007, (NYD).	2	bor(2)
25	Poland, Góry Stolowe, Bukowina Kłodzka, Pstrążna, edge of a small forest path from Závrchy to Pstrążna, c. 15 m SE of the state border between Poland and the Czech Republic, c. 1.2 km ESE of the village centre, 50°28′34.1"N, 16°15'12.5"E, 545 m alt., leg. L. Ekrt 9. VI. 2008, (PST).	1	cam(1)
26	Slovakia, 21b. Kriváska Malá Fatra: Krasňany, bottom part of Kúr valley c. 3.5 km SE of the church in the village of Krasňany, 49°11'34", 18°56'01", c. 605 m alt., leg. L. Ekrt, 30. IX. 2004, (KUR).	2	bor(1), ×cri(1)#
27	Austria, Totes Gebirge Mts., Hinterstoder, half way along path from the village of Hinterstoder to Priel-Schutzhaus, c. 2 km ENE of the Spitzmauer Mt., 47°41'54"N, 14°05'22"E, 1090 m alt, L. Ekrt 26. VII. 2008, (PRI).	2	bor(1), cam(1)