A morphometric study and revision of the *Asplenium trichomanes* group in the Czech Republic

Morfometrická studie a revize komplexu *Asplenium trichomanes* v České republice

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A detailed cytogeographic and morphometric study of the *Asplenium trichomanes* group in the Czech Republic is presented. We detected diploid (2n = 72), tetraploid (2n = 144) and hybrid triploid plants (2n = 108). Based on the morphometric study, four intraspecific taxa are recognized. These taxa correspond to the four subspecies of *A. trichomanes* (*A. t.* subsp. *trichomanes*, *A. t.* subsp. *quadrivalens*, *A. t.* subsp. *pachyrachis* and *A. t.* subsp. *hastatum*) distinguished in the floras of western, southern and northern Europe. Triploid plants were determined as *A. t.* nothosubsp. *lusaticum* (*A. t.* subsp. *trichomanes* × *A. t.* subsp. *quadrivalens*). The individual morphological characters used for determining subspecies are evaluated and a determination key presented.

K e y w o r d s: Central Europe, cytotypes, ferns, flow cytometry, DNA ploidy level, taxonomy

Introduction

In Europe, *Aspleniaceae* is the family with the largest number of species within the *Pteridophyta*. The genus *Asplenium* L. comprises several taxonomically critical species complexes, including the *Asplenium trichomanes* group, which shows complicated patterns of minor morphological and significant karyological variation. The evolutionary history and relationships among taxa in this group have been intensively studied in W, S and N Europe (Lovis 1964, Tigerschiöld 1981, Reichstein 1984, Nyhus 1987, Rasbach et al. 1990, 1991, Bennert & Fischer 1993, Jessen 1995, Vogel et al. 1998, 1999a, 1999b, Hilmer 2002). However, morphological variation and the distribution of these taxa are insufficiently known in C and E Europe, as they are often not adopted in local floras or checklists (Futák 1966, Křísa 1988, Mirek et al. 1995, Ciocârlan 2000, Kubát et al. 2002, Fischer et al. 2005). The reasons for ignoring the taxa within *Asplenium trichomanes* group are (i) the lack of diagnostic morphological characters, (ii) frequent co-occurrence at their localities, and (iii) hybridization among the taxa (see Fig. 1).

The aims of this study were to: (i) determine DNA ploidy levels within the *Asplenium trichomanes* group in the Czech Republic, (ii) analyse morphological variation in the group and (iii) compare recognized morphological units with morphological characteristics of the taxa known from the literature and (iv) evaluate discriminating ability of the morphological characters studied.

Summaries of habitat preference of individual taxa from the *Asplenium trichomanes* group and of their distribution in the Czech Republic are presented in another paper (Ekrt 2008).

Taxonomic survey of the *Asplenium trichomanes* group in Europe

The *Asplenium trichomanes* group includes cytologically and ecologically distinct taxa with almost worldwide distribution, which are obviously still undergoing active evolution (Lovis 1973, 1977). These taxa are usually distinguished at the subspecific level (Reichstein 1984, Viane et al. 1993, Frey et al. 1995).

The ploidy differentiation (diploid to hexaploid level) in the *Asplenium trichomanes* group was discovered in the second half of the 20th century (Manton 1950). Diploid, triploid, tetraploid, and hexaploid cytotypes are known from Europe (Reichstein 1981, Nyhus 1987, Bennert & Fischer 1993, Jessen 1995, Hilmer 2002). In C Europe, five subspecies (two at the diploid and three at the tetraploid level) of the *Asplenium trichomanes* group are recognized, sharing minor variation in morphology but differing mostly in ecology (Lovis et al. 1989, Bennert & Fischer 1993).

Two diploid (2n = 2x = 72) taxa, *Asplenium trichomanes* L. subsp. *trichomanes* and *A. t.* subsp. *inexpectans* Lovis, are known. *Asplenium trichomanes* subsp. *trichomanes* is an obligate calcifugous plant, growing only on siliceous or serpentine rocks (Meyer 1962, Rothmaler 1963, Reichstein 1981, 1984). *Asplenium trichomanes*, the nominate subspecies, was described from Scandinavia by Linné (1753). *A. t.* subsp. *inexpectans*, which was described from Austria (Langenbrucke), is a rare, strictly calciphilous taxon, growing on limestone and dolomite rocks (Lovis 1964, Reichstein 1981, 1984).

Tetraploid cytotypes (2n = 4x = 144) are relatively polymorphic. At present, four taxa of this ploidy level are distinguished throughout Europe. Three of them occur in C Europe, with *A. t.* subsp. *quadrivalens* D. E. Mey. being the most common taxon (Meyer 1962, Lovis 1964, Reichstein 1984). This subspecies occurs on both calcareous and siliceous rocks, as well as on man-made habitats (walls, quarries) and is described from Germany (Bavaria, Ruhpolding) (Meyer 1962). Autotetraploid origin of this taxon is assumed, because of the chromosomal behaviour of *A. t.* subsp. *trichomanes* (Bouharmont 1972, Reichstein 1981).

The other tetraploid taxon, *A. t.* subsp. *pachyrachis* (Christ) Lovis et Reichst., is quite rare throughout Europe (Lovis & Reichstein 1985). It grows in crevices in steep overhanging limestone, dolomite or calcareous sandstone rocks and very sporadically on man-made walls (e.g., old castles). This subspecies was in the past recognized also at the species level, mainly due to its typical habitus and biotope specificity [*Asplenium csikii* Kümmerle et Andrasovszky from Albania, (Kümmerle 1922)]. Originally, it was described by Christ (1900) from Switzerland (St. Maurice).

Asplenium trichomanes subsp. *hastatum* (Christ) S. Jess. is also described from Switzerland (Lugano) by Christ (1900) and recently was revived by Jessen (1995). This taxon inhabits shady limestone gorges, dolomitic rocks or walls and is known at present only from W, C and E Europe (Jessen 1995).

The last European tetraploid taxon, with a relatively conspicuous morphology, is *A. t.* subsp. *coriaceifolium* H. Rasbach, K. Rasbach, Reichst. et H. W. Bennert. It grows only on dry limestone rocks in Mallorca and S Spain (Rasbach et al. 1990, 1991).

Hexaploid cytotypes (2n = 6x = 216) are the highest known ploidy level among the European members of the *Asplenium trichomanes* group. Hexaploids are known from Macaronesia (*A. t.* subsp. *maderense* Gibby et Lovis) and also from several localities in Europe. First European hexaploid type was recorded (but not formally described) in Belgium

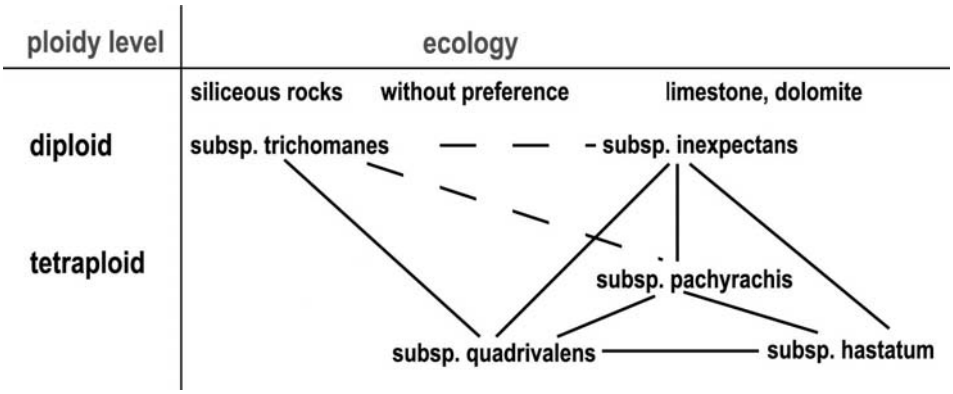

Fig. 1. – Diagram showing recently distinguished taxa of the *Asplenium trichomanes* complex in the C and W Europe based on their hybridization relationships and ecological preferences. Spontaneous hybridization (—); artificial hybridization (---). Compiled according to Bennet & Fischer (1993), Cubas et al. (1989), Jessen (1995), Lovis & Reichstein (1985), Reichstein (1981) and Vogel et al. (1998).

and France (Bouharmont 1968). This cytotype is supposed to have arisen by autopolyploidization from a triploid (2n = 3x = 108) hybrid *A. t.* subsp. *quadrivalens* × *A. t.* subsp. *trichomanes* [*A. t.* nothosubsp. *lusaticum* (D. E. Meyer) Lawalree], but the cytological data were never published (Rasbach et al. 1991). Another hexaploid type of *A. trichomanes*, probably of different origin than the previous one, was discovered and cytologically confirmed (Bennert et al. 1989) in S Spain. The origin of this hexaploid type from a triploid hybrid, *A. t.* subsp. *coriaceifolium* × *A. t.* subsp. *inexpectans* (*A. trichomanes* nothosubsp. *malacitense* H. Rasbach, K. Rasbach, Reichst. et H.W. Bennert) (Rasbach at al. 1990, 1991) was confirmed by isoenzyme analysis (Bennert & Fischer 1993).

About nine hybrid combinations among the individual taxa of the *Asplenium trichomanes* group are known (see Fig. 1). Most of these hybrids are of natural origin with two only produced under artificial conditions (Reichstein 1981, Lovis & Reichstein 1985, Cubas et al. 1989, Bennert & Fischer 1993, Jessen 1995, Vogel et al. 1998). Depending on the ploidy level of the parental plants, the hybrids can be diploid (only artificially induced ones), triploid, or tetraploid. Hybrids can be identified easily by their aborted spores and intermediate morphology (Reichstein 1981, Lovis & Reichstein 1985, Jessen 1995).

Material and methods

Plants used in this study

Results presented here are based both on a field study and examination of herbarium specimens. Forty-six localities were sampled in the Czech Republic and one in Slovakia during 2000-2004 (see Appendix 1 for the list of localities). Our sampling strategy was as follows: (1) to explore various habitat types (such as limestone, siliceous or serpentine rocks, or man-made walls) and record the occurrence of the taxa on diverse substrates over a large spatial scale, (2) investigate the large limestone regions in the Czech Republic,

where the occurrence of rare taxa was expected and (3) sample all the morphologically different types at each locality. The number of samples per locality varied from 5 to 20, reflecting the population size, abundance and variation of the plants.

Herbarium vouchers are kept in PRC, some duplicates in CB and also in the private herbarium of the first author. Plant species nomenclature follows Frey et al. (1995), excluding that of the *Asplenium trichomanes* group, for which the authorities are given when first mentioned in the text.

Flow cytometry

All the plants analysed by flow cytometry were also included in the morphometrical studies. Whole plants with rhizomes were stored in plastic bags at 4°C; their DNA ploidy level was determined within seven days.

DNA ploidy level was estimated using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) and the two-step procedure of nuclei isolation, originally described by Otto (1990) and partially modified by Suda & Trávníček (2006). In total, 340 samples from 47 localities (about 5–10 plants per locality) were analysed. Diploid sample of *Asplenium trichomanes*, verified by chromosome counting (locality 21, sample 46-5, n = ca 36^{II} – counted by V. Jarolímová), was used as an internal standard. Approximately 50 mg of tissue from the leaves (without sporangia) of fresh plants were chopped together with the leaf tissue of the internal standard plant, using a fresh razor blade, in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a nylon mesh (42 µm). After an incubation period (10–15 min at room temperature with occasional shaking), the staining solution containing 1 ml Otto II buffer (0.4 M Na_2HPO_4 . 12 H_2O), fluorochrome (4 µg/ml DAPI) and ß-mercaptoethanol (2 µg/ml) was added. The staining lasted 1–2 min. The cytometer was adjusted so that the fluorescence of G0/G1 nuclei of the diploid *A. trichomanes* subsp. *trichomanes* was localized on channel 200. Fluorescence of at least 4000 nuclei was recorded and the coefficient of variation for each analyzed plant was calculated.

Morphometry

We used 463 plants for the multivariate analyses of morphological characters (*A. trichomanes* subsp. *trichomanes* – 43 plants; *A. t.* subsp. *quadrivalens* – 329 plants; *A. t.* subsp. *pachyrachis* – 50 plants; *A. t.* subsp. *hastatum* – 41 plants). In this study, all plants from each locality are analysed as independent observations in order to prevent the creation of mixed population samples (Lovis & Reichstein 1985, Jessen 1995, Stark 2002). Only plants with developed sori were collected. Plants with completely aborted spores (potential hybrids) were not included in the analysis (30 plants).

Twenty-two morphological and micromorphological characters were measured (Table 1) on fertile plants collected in the field. All diagnostic characters presented in the literature, as well as additional, potentially useful characters were included in our study. Spore size was measured using a light microscope at a magnification of 1000×, with a precision of 1µm. Spore size (exospore length) is the average of the measurements of 20–30 untreated air dry spores from each plant. Annulus length was measured at a magnification of 100×, with a precision of 10 µm. Untreated dry sporangia were examined using a light microscope and whole orange-brown bold cells of stretched/bent annulus were measured on an

Table 1. – Morphological characters used in the multivariate analyses (PCA and LDA).

Acronym	Character
anulen	mean sporangium annulus length [μm] / mean of 5 annuli
clspor	sporangium annulus type stretched (0), bent (1)
diaur	pinnae not auriculate (0), pinnae biauriculate (1)
edge	pale margin of pinnae, absent (0) vs. present (1)
enpilen	terminal pinna length [mm]
enpiwid	terminal pinna width [mm]
form	fronds pressed agains the substrate (0), fronds upright (1)
hairpin	glandules of dorsal side of pinnae, absent (0), present (1)
ind1pi	number of sori on the lowest pinna
int7/8	distance between the pinnae at 7/8 of lamina [mm]
lam	lamina length [mm]
lamend	lamina not tapered (0), lamina tapered (1) at terminal part
overpi	pinnae not overlapping (0), pinnae overlapping (1)
pi1/2len	pinna length at 1/2 of lamina [mm]
pi1/4len	pinna length at 1/4 of lamina [mm]
pisum	number of pinna pairs per lamina
rhaes	rachis type: erect or arched (0), rachis sigmoidal (1)
rhaled	rachis wings without distinct papillas (0), vs. with distinct papillas (1)
rhawid	rachis width in the middle of lamina [mm] / mean of 5 laminas
scalen	rhizome scales length [mm] / mean of 5 scales
scaur	rhizome scales appendage, absent (0) vs. present (1)
sporlen	spore length [μm] / mean of 20–30 exospores

annulus. Complete, untreated scales, separated from the terminal part of rhizome (central part of leaf rosette) using tweezers, were measured using a light microscope, at a magnification of 25×, with an accuracy of 125 μm. Rhizome scale appendages were measured in a similar manner, with only multicellular appendages considered. Rachis width was measured in the middle, using a light microscope at a magnification of 25×, with an accuracy of 125 μm. Dimensions of annulus, scales and rachis are averages of five independent measurements per individual. Pale margin was recorded as present only if the leaf had more than three continuous rows of hyaline cells at its margin.

DNA ploidy level was used, in combination with the spore length, annulus type, and growth form to classify plants into four subspecies. Consequently, these characters were not included in the discriminant analyses used to define the groups. But these characters were used (except DNA ploidy level) in the principal component analysis, which is descriptive.

Data analysis

Prior to running multivariate analyses, quantitative data were log-transformed [$x' = ln\ (x + 1)$] to bring their distribution closer to the normal distribution. Qualitative characters were coded as binary (dummy) variables.

(1) Principal components analysis (PCA) was applied to the primary data matrix containing all recorded morphological characters. PCA based on a correlation matrix was used. PCA provided an insight into the overall pattern of variation and uncovered morphological discontinuities among the taxa studied. For PCA, CANOCO for Windows (ter

Braak & Šmilauer 2002, Lepš & Šmilauer 2003) was used and the results were visualized using CanoDraw for Windows 4.0 (ter Braak & Šmilauer 2002).

(2) Linear discriminant analysis (LDA; Klecka 1980, Krzanowski 1990) was used to find morphological characters giving the best separation of the a priori distinguished taxa. To understand, which morphological characters contribute to individual splits separating the four taxa, several discriminant analyses were performed on different subsets of the whole data set. In the first step (i), DNA ploidy level and spore size, which differs greatly between ploidy levels (see point 5 below), defined the two groups of diploids and tetraploids and consequently, DNA ploidy level and length of spores (*sporlen*) were excluded as predictors in this LDA. In the second step (ii), tetraploid plants were classified according to whether the sporangium is stretched and bent (and the *clspor* character was not used as a predictor). Finally, (iii) tetraploid plants with stretched sporangia were grouped according to their growth form (and the *form* character was therefore excluded). To survey overall data variability, another LDA was computed distinguishing all four taxa, and with all the grouping characters (DNA ploidy level, length of spores – *sporlen*, type of annulus – *clspor*, and the type of growth form – *form*) excluded as canonical predictors. The linear discriminant function was then calculated and its predictive ability tested by cross-validation. Computations of discriminant analyses were carried out in the STATISTICA 5.5 software package (StatSoft 1998).

(3) Summary statistics were used to compare taxa. Within each group the mean, standard deviation, minimum and maximum values, and 5% and 95% percentiles were computed for selected characters important for taxa determination (Appendix 2).

(4) Differences in the taxonomically interesting characters among taxa were evaluated by an analysis of variance (ANOVA); post-hoc comparisons among taxa were carried out by Tukey's honest significant difference test.

(5) ANOVA analyses were also used to test the strength of the relationship between ploidy level and the morphological characters commonly reported to differ between ploidy levels, using the plants with ploidy level verified by flow cytometry. Both the summary statistics and ANOVA analyses were computed using STATISTICA 5.5 (StatSoft 1998).

Results

Flow cytometry

Flow cytometry analysis detected diploid, triploid and tetraploid plants (Fig. 2, Table 2, Appendix 1). A total of 29 diploid plants were found at nine localities. Only three triploid plants were found, each at a separate locality. The majority of the plants analysed were tetraploids (308 plants), found at 42 localities. The coefficient of variation ranged from 1.3% to 2.6% for the analysed diploid plants, from 1.9% to 2.6% in the triploid plants, and from 1.6% to 3.7% in the tetraploid plants. Diploid and tetraploid plants co-occurred at two localities (nos 32 and 46). At another three localities (12, 26, 35; Fig. 3), diploid, triploid and tetraploid individuals co-occurred. No hexaploid cytotype was detected.

Principal components analysis (PCA)

PCA revealed clear morphological differentiation among individual plants (Fig. 4). The ordination diagrams in Fig. 4A and Fig. 4C visualize by different symbols the four taxo-

Table 2. – Summary of flow cytometry characteristics of the ploidy levels in the *Asplenium trichomanes* group. N – number of samples; 2C ratio ± s.d. – mean somatic relative nuclear DNA content (sample/standard ratio of samples ± standard deviation; diploid *A. t.* subsp. *trichomanes* was used as internal standard = 1); 2C min/max – minimum and maximum values of 2C ratio; CV– range values of coefficient of variance of sample peaks.

DNA ploidy level	N	2C ratio ± s.d.	2C min/max	CV (%)
2x	29	1	1.000/1.000	1.6–2.6
3x	3	1.511±0.008	1.502/1.518	1.9–2.6
4x	308	2.053±0.053	2.000/2.142	1.6–3.7

nomic groups distinguished by the adopted determination characters (DNA ploidy level, spore length, annulus type, and growth form). The first four principal components (axes) explained more than 56% (23.9%, 14.9%, 9.5% and 8.5%, respectively, for first to fourth axes) of the total variation in the morphological characters of all specimens. The first axis is correlated with characters such as sporangium annulus type, pale margin to pinnae, rachis type, mean sporangium annulus length, growth form, lamina length and distance between the pinnae at 7/8 of lamina (*clspor*, *edge*, *rhaes*, *anulen*, *form*, *lam*, *lamend*, *int7/8*), and the second PCA axis with characters such as pinna length at 1/2 of lamina,

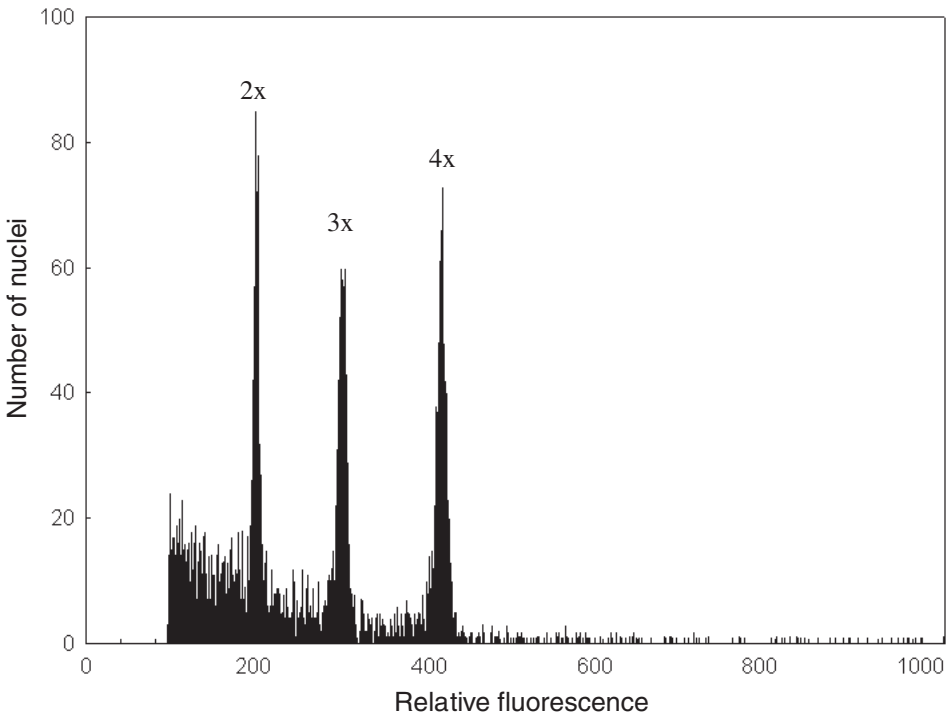

Fig. 2. – Histogram of relative DNA content obtained after analysis of DAPI-stained nuclei isolated from *Asplenium trichomanes* leaf tissues. Simultaneous analysis of diploid (Peak 1 – *Asplenium trichomanes* subsp. *trichomanes*, CV = 1.51%), triploid (Peak 2 – *A. t.* nothosubsp. *lusaticum*, CV = 1.95%) and tetraploid (Peak 3 – *A. t.* subsp. *pachyrachis*, CV – 2.46%) plants from locality 35.

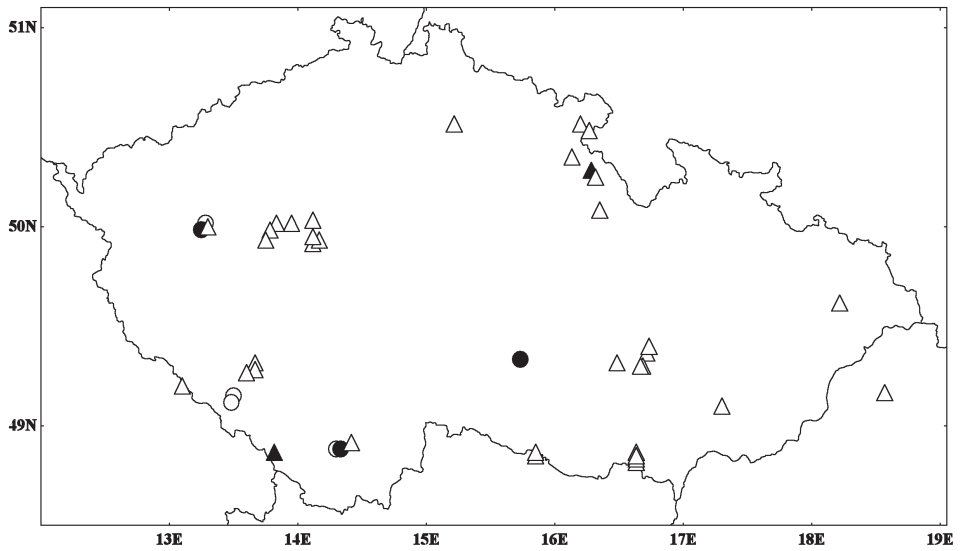

Fig. 3. – Distribution of ploidy levels of the *Asplenium trichomanes* complex in the study area: ○ localities with only diploid plants; △ localities with only tetraploid plants; ▲ localities with diploid and tetraploid plants; ● localities with diploid, triploid and tetraploid plants.

rachis width in the middle of lamina, terminal pinna length and auriculate/nonauriculate pinnae (*pi1/2len*, *rhawid*, *enpilen*, *diaur*). The third and fourth axes are very similar in the total variation explained and also in their ability to separate diploid and tetraploid plants, albeit in a slightly different manner. The third axis is uniquely correlated with the distance between pinnae at 7/8 of lamina length and the width of rachis in the middle of the lamina (*int7/8*, *rhawid*). On the other hand, the fourth axis correlates with the presence of auriculate pinnae, of overlapping pinnae and of rhizome scale appendages (*diaur*, *overpi*, *scaur*). As the rather weak separation of diploid and tetraploid plants is more visible along the fourth axis, this axis is presented in Fig. 4C and 4D.

PCA results suggest three distinct groups of plants, with only a slight overlap at their margins. Two of these groups correspond to morphologically defined tetraploid taxa *A. t.* subsp. *hastatum* and *A. t.* subsp. *pachyrachis*. The last group contains both diploid and tetraploid plants, corresponding to *A. t.* subsp. *trichomanes* and *A. t.* subsp. *quadrivalens*, respectively; these two taxa are not so clearly separated morphologically.

Discriminant analysis

Hypotheses about the pattern in variation suggested by the PCA results and cytometric analysis were tested using LDA. Three analyses were carried out to find the best discriminating characters for the groups/taxa defined at different hierarchical levels. Differences between diploid and tetraploid plants were examined in the first step. The plants not analysed by flow cytometry were classified using the length of spores, which corresponds very closely to DNA ploidy level (Fig. 5). The characters most strongly correlated with the canonical axis separating diploid and tetraploid plants were: the presence of papillas on

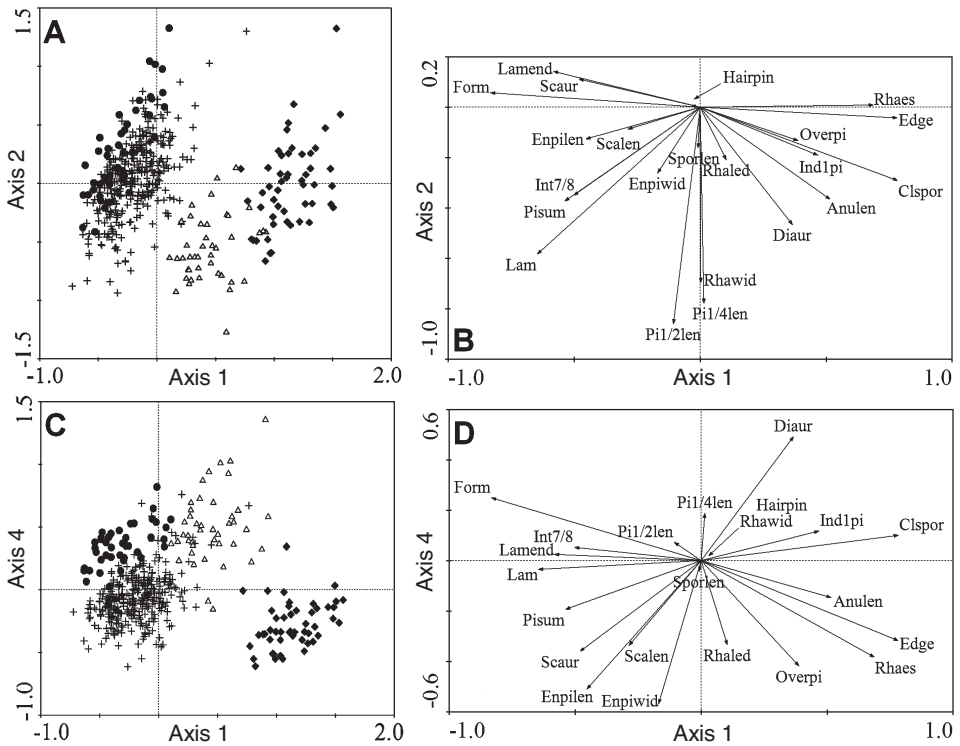

Fig. 4. – PCA ordination of specimens (A, C) and characters (B, D) of the *Asplenium trichomanes* complex (● subsp. *trichomanes*, + subsp. *quadrivalens*, △ subsp. *hastatum*, ◆ subsp. *pachyrachis*). PCA ordination for axes 1 and 2 (A, B) and axes 1 and 4 (C, D)

rachis wings (*rhaled*), length of the annulus (*anulen*), length of the terminal pinna (*enpilen*), distance between bases of the pinnae at 7/8 of the lamina length (*int7/8*), width of the terminal pinna (*enpiwid*) and the presence of scale appendages (*scaur*) (Table 3). Similarly in the second LDA (analysis of tetraploids only, classified by their annulus type), characters best correlated with the canonical axis separating *A. t.* subsp. *quadrivalens* (with stretched type of annulus) from the other taxa (with bent type of annulus) were growth form of plant (*form*), presence/absence of pale margin to pinnae (*edge*), auriculate/nonauriculate pinnae (*diaur*), tapering/nontapering lamina at the terminal end (*lamend*) and presence/absence of scale appendages (*scaur*). In the last LDA (analysis of tetraploids with bent annuli, classified by the type of growth form – *form*), the differences between *A. t.* subsp. *hastatum* (fronds upright) and *A. t.* subsp. *pachyrachis* (fronds pressed against the substrate) were examined. Characters best correlated with the canonical axis were the presence/absence of a pale margin to pinnae (*edge*), shape of rachis (*rhaes*), auriculate/nonauriculate pinnae (*diaur*), overlapping/nonoverlapping pinnae (*overpi*) and pinnae length at 1/4 of the lamina length (*pi1/4len*) (Table 2). Results of the overall LDA, with all the group-defining characters (DNA ploidy level, spore length, annulus type, and growth form) excluded as predictors, are given in Fig. 6.

Table 3. – The five best correlated characters in the three LDAs, which represent the three steps in the differentiation among four taxa. Factor = canonical coefficients of the linear discriminant function.

Step 1		Step 2		Step 3	
Characters	Factor	Characters	Factor	Characters	Factor
rhaled	–0.812	form	0.348	edge	–0.600
anulen	–0.304	edge	–0.287	rhaes	–0.499
int7/8	0.206	diaur	–0.257	diaur	0.403
enpiwid	–0.142	lamend	0.213	overpi	–0.193
scaur	–0.112	scaur	0.211	pi1/4len	0.184

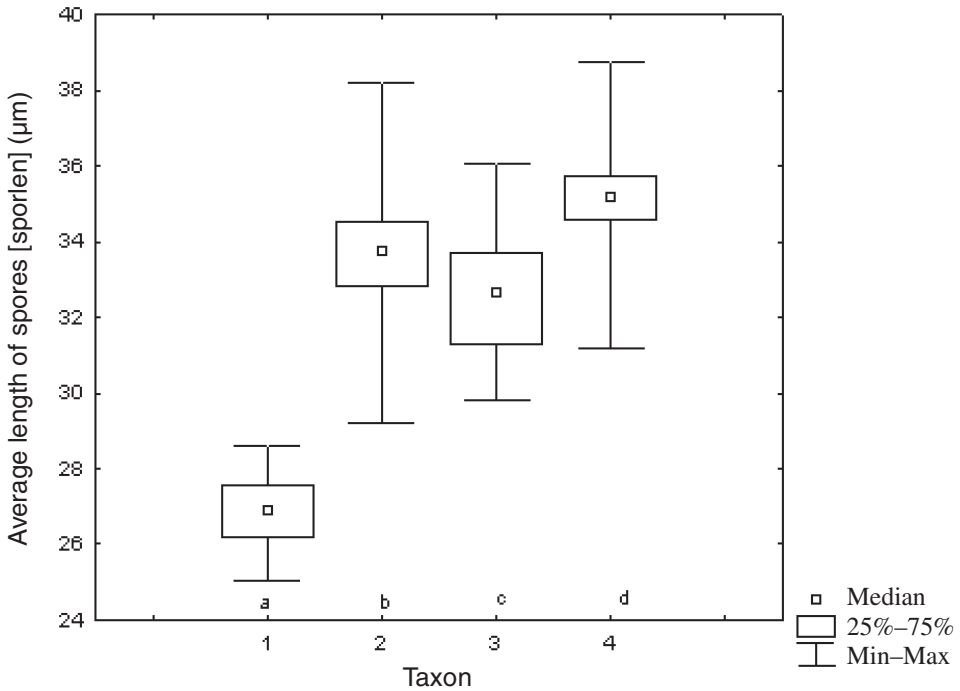

Fig. 5. – Box & whisker plot of one-way ANOVA ($F = 328.5$, $P < 0.001$) of the mean spore length (*sporlen*) of individual taxa of the *Asplenium trichomanes* group in the Czech Republic. 1 – *A. t.* subsp. *trichomanes* (diploid), 2 – *A. t.* subsp. *quadrivalens* (tetraploid), 3 – *A. t.* subsp. *pachyrachis* (tetraploid), 4 – *A. t.* subsp. *hastatum* (tetraploid). Letters at the bottom indicate the results of the Tukey HSD test, taxa labelled with the same letter do not differ significantly ($P > 0.01$). Taxa were determined based on the DNA ploidy level, spore length, annulus type, and growth form used in the discriminant analysis.

Classification function (linear discriminant function) was calculated for all the taxa examined. Classificatory precision of this function was estimated using cross-validation, and posterior probabilities of mis-classification were obtained. All the taxa studied were classified correctly in more than 93% of cases. Posterior probabilities for individual taxa are given in Table 4.

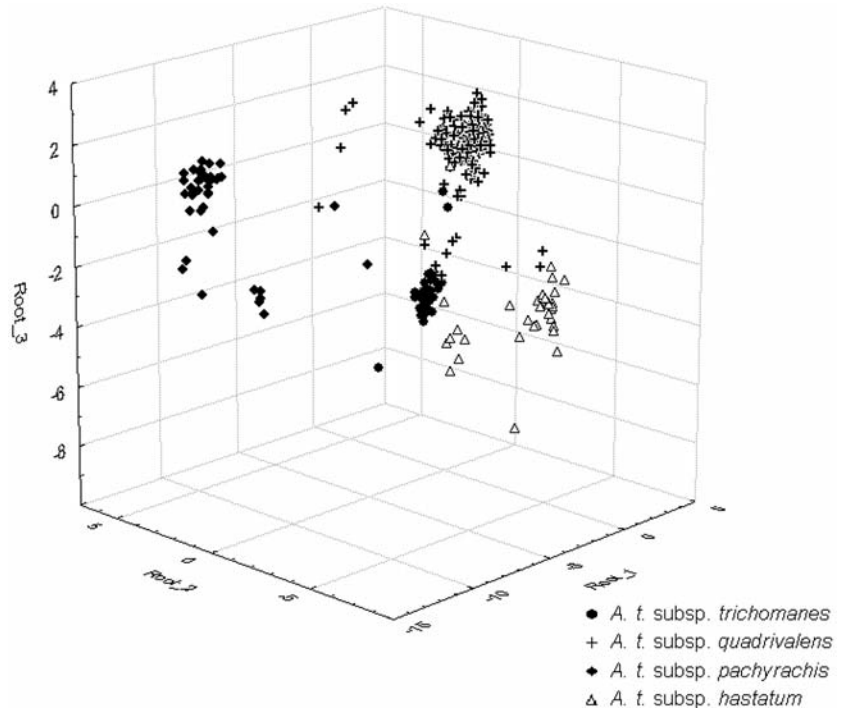

Fig. 6. – Linear discriminant analysis of individual plants of all four subspecies of the *Asplenium trichomanes* complex. The characters *sporlen*, *clspor*, *form* and ploidy level were used for delimiting particular groups of taxa and were excluded from this analysis.

Table 4. – Cross-validation results for the LDA using the full dataset of the *Asplenium trichomanes* complex. Predicted group membership refers to the percentage of observations entering cross-validation, classified in the particular group.

Actual group	Predicted group membership			
	subsp. *trichomanes*	subsp. *quadrivalens*	subsp. *pachyrachis*	subsp. *hastatum*
subsp. *trichomanes*	93.0%	7.0%	0.0%	0.0%
subsp. *quadrivalens*	1.8%	96.0%	1.2%	0.9%
subsp. *pachyrachis*	0.0%	0.0%	100.0%	0.0%
subsp. *hastatum*	0.0%	2.4%	0.0%	97.6%

Discussion

Recognized taxa

Three different values of DNA amount obtained by flow cytometry correspond with the three ploidy levels recorded among the plants. Nevertheless, the number of chromosomes was verified only for the diploid level because of the difficulty of counting of chromosomes at higher ploidy levels. For this reason, we consider diploids, triploids and

tetraploids in terms of DNA ploidy level (Suda et al. 2006). Tetraploids are the most frequent DNA ploidy level in the study area, which is probably also the case in other parts of Europe. Based on morphological characters and DNA ploidy level, four types were distinguished in the Czech Republic, largely corresponding to the subspecies recognized in other European regions (Reichstein 1984, 1997, Viane et al. 1993, Frey et al. 1995, Jessen 1995). Diploid type corresponds to *A. t*. subsp. *trichomanes*. Distribution of this subspecies is scattered and restricted to siliceous and serpentine rocks. In C Europe, another diploid taxon *A. t*. subsp. *inexpectans* is rarely recorded in Slovakia and Austria (Lovis 1964, Derrick et al. 1987, Bennert et al. 1989, Jessen 1991). In the Czech Republic, this taxon has not been discovered, in spite of the revision of a large number of specimens from Czech herbaria (Ekrt 2008). This taxon usually grows on limestone and dolomite rocks. In the Czech Republic, such habitats are very infrequent, so it is still possible that subsp. *inexpectans* is unrecorded due to its rarity.

Tetraploid *A. t.* subsp. *quadrivalens* was found to be the most common subspecies of *A. trichomanes* in the Czech Republic. This finding fully corresponds to the situation in other parts of the distribution area of the *A. trichomanes* group (Nyhus 1987, Hilmer 2002, Stark 2002). *Asplenium t.* subsp. *quadrivalens* occurs on both siliceous and calcareous rocks, and is also very frequent in secondary habitats (e.g., man-made walls, quarries). The other two tetraploid taxa, *A. t.* subsp. *pachyrachis* and *A. t.* subsp. *hastatum*, are very rare in the Czech Republic because of their dependency on limestone rocks. They are more common in neighbouring countries, where large limestone regions occur, e.g. in Slovakia and Austria (Jessen 1995).

Diagnostic value of morphological characters

Multivariate analysis of morphological characters demonstrated that the members of *A. trichomanes* group can be distinguished, but multiple characters are needed for reliable determination. Many morphological characters show considerable variation, as in other polyploid complexes of the *Asplenium* genus, such as the *A. obovatum* (Steinecke & Bennert 1993, Herrero et al. 2001) or *A. lepidum* groups (Brownsey 1976). High character variability complicates determination of the taxa in such complexes and consequently leads to their unclear treatment in local floras in certain countries (Futák 1966, Křísa 1988, Mirek et al. 1995, Ciocârlan 2000, Kubát 2002). Our multivariate morphometric analysis shows that the most similar taxa are the diploid *A. t.* subsp. *trichomanes* and the tetraploid *A. t.* subsp. *quadrivalens*. Their very close relationship was mentioned by Bouharmont (1972), who proposed that subsp. *quadrivalens* had probably arisen from subsp. *trichomanes* by autopolyploidization. There are many morphological characters common to both taxa. Characters which are most useful for their distinction are those depending strongly on the DNA ploidy level, e.g. spore size or annulus length. The evolutionary relationships between *A. t.* subsp. *pachyrachis* and *A. t.* subsp. *hastatum* have not been clarified. Molecular markers or nuclear DNA content might be useful tools in future studies and provide a better understanding of the evolution of the *Asplenium trichomanes* group.

Presence of qualitative characters, such as the fringed margins of rhizome scales, is typical for particular taxa. Scale appendices (*scaur*) occurred frequently in *A. t*. subsp. *quadrivalens* (> 70% of the plants), but only very rarely (about 10% of the plants) in *A. t.* subsp. *trichomanes* or *A. t*. subsp. *hastatum*, and not at all in *A. t.* subsp. *pachyrachis* (Ta-

Table 5. – Frequency of the reference state (value 1) of individual binary character across the taxa studied. Nature of the reference state is given in parenthesis following the character code in the first column.

Character	Taxon (subspecies)			
	trichomanes	*quadrivalens*	*pachyrachis*	*hastatum*
clspor (bent annulus)	0%	8.3%	100%	92.9%
diaur (pinnae biauriculate)	0%	0.9%	11.8%	97.6%
edge (pale margin of pinnae)	0%	1.2%	98.0%	7.1%
form (plant upright)	100%	100%	2.0%	83.3%
hairpin (presence of glandules)	79.5%	87.1%	100%	100.0%
lamend (lamina tapered at terminal part)	79.1%	75.5%	3.9%	14.3%
overpi (pinnae overlapping)	0%	26.1%	82.4%	16.7%
rhaes (rachis sigmoidal)	0%	8.9%	98.0%	7.5%
rhaled (prominent papillas on rachis wings)	7.0%	97.9%	93.8%	97.6%
scaur (presence of scales appendage)	14.3%	73.5%	0%	9.8%

ble 5). This character is not mentioned in previous studies. Pale leaf margin, consisting of a zone of distinct hyaline cells (*edge*), is a character typical of *A. t.* subsp. *pachyrachis* (Jessen 1999). In our study, it occurred in the majority (ca 98%) of the plants in this taxon (Table 5), but was not well developed in other taxa.

Great variation was found in characters strongly related to DNA ploidy levels (i.e., spore and annulus size). An essential character for the determination of diploid vs. tetraploid taxa is a difference in mean spore length (*sporlen*) (Fig. 6, Appendix 2). Although individual values may be variable, mean spore size of the tetraploid taxa (30–39 µm) was significantly ($F = 648.91$; $P < 0.01$) higher than that of the diploids (25–29 µm), in the analysis of plants with ploidy level verified by flow cytometry. A slightly wider range of these values for diploid and tetraploid taxa is reported in other papers (Nyhus 1987, Viane et al. 1993, Hou & Wang 2000, Hilmer 2002, Kubát 2002, Stark 2002), but these papers cite a similar separation in spore size between diploid and tetraploid taxa. According to the results of the analysis of variance (for all plants and taxa), mean spore size seems to be different between tetraploid and other taxa ($F = 328.5$; all combinations of Tukey's HSD test $P < 0.001$). However, large overlaps in spore size prevent use of this character for determination (Appendix 2). ANOVA results suggest that the mean spore size differs significantly also between all the tetraploid taxa ($P < 0.001$ for all pairwise comparisons using Tukey's HSD test), but they cannot be distinguished reliably by this character due to the large overlap in the measurements (Appendix 2).

Annulus length (*anulen*) was also significantly different between DNA ploidy levels ($F = 171.31$, $p < 0.01$, for plants with verified ploidy level). Mean length of annuli of the tetraploid *A. t.* subsp. *quadrivalens* (260–340 µm) was significantly ($P < 0.01$) longer than those of the diploid *A. t.* subsp. *trichomanes* (220–290 µm). Similar ranges for these two taxa were found by Nyhus (1987). The highest (and widely overlapping) variation in annulus lengths (roughly 280–420 µm) was found in two tetraploid subspecies *A. t.* subsp. *pachyrachis* and *A. t.* subsp. *hastatum* (Appendix 2), which were also the only pair with non-significant difference in the Tukey HSD comparisons. Annulus length character is usually ignored by other authors.

All plants examined had two wings on the rachis. These wings consist of a large number of either enlarged or minute papillas (*rhaled*). This study found a strong relation be-

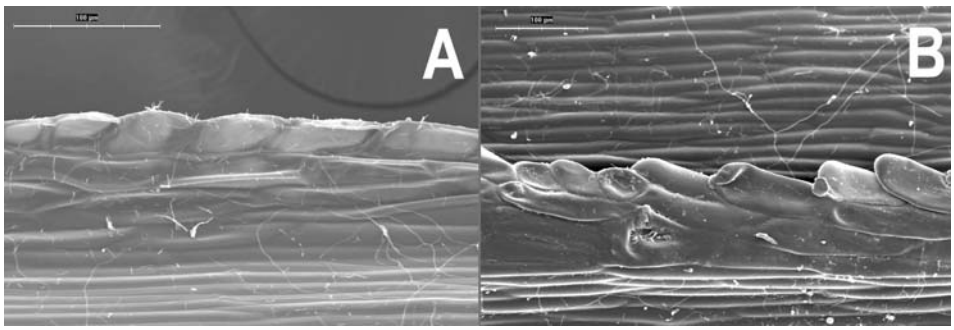

Fig. 7. – SEM pictures of the distinct flat papillas on rachis wings of the diploid *A. t.* subsp. *trichomanes* (A) and prominent papillas of the tetraploid *A. t.* subs. *hastatum* (B). Scale bars are 100 μm.

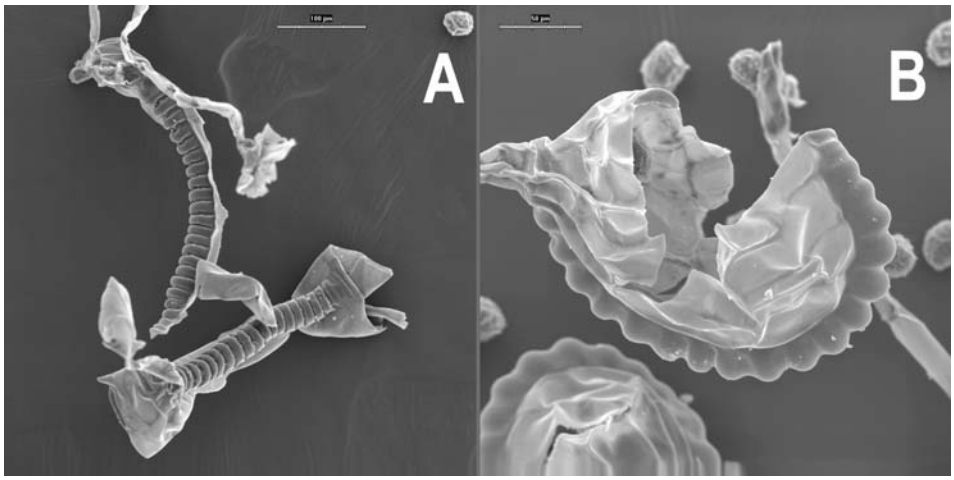

Fig. 8. – SEM pictures of the stretched type of sporangial annulus (clspor) in *A. t.* subsp. *quadrivalens*, scale bar is 100 μm (A) and of the bent type of sporangial annulus in *A. t.* subsp. *pachyrachis*, scale bar is 50 μm (B).

tween DNA ploidy level and the presence of enlarged papillas. Light, flat, minute and discreet papillas on the rachis wing are typical of the diploid *A. t*. subsp. *trichomanes* (present in ca 97% of plants), whereas the tetraploid taxa have enlarged, bulging, yellow or reddish-orange papillas on their wings (94–98% of plants; Fig. 7, Table 5). This character is not recognized in previous studies.

Another important morphological character is the shape of the annulus (*clspor*) after sporangial dehiscence. The majority of mature sporangia of *A. t.* subsp. *pachyrachis* and *A. t.* subsp. *hastatum* open at dehiscence (Fig. 8, Table 5), but later the annulus returns to its original position and becomes bent (Moran 1996). Some sporangia remain undehisced for a long time after maturation. Such sporangia resemble those of e.g., the *Asplenium lepidum* group (Brownsey 1976, 1977). This mechanism is supposed to result in a greater proportion of spores remaining within the immediate colonization area. This is considered to be an adaptive advantage for plants occupying highly specialized chasmophyte habitats

(Brownsey 1976, 1977). On the other hand, the annulus of mature sporangia of *A. t.* subsp. *trichomanes* and *A. t.* subsp. *quadrivalens* is usually stretched after the dehiscence of a sporangium. Annulus shape of mature sporangia is easily distinguished in herbarium material and living plants. Similarity of the spore dispersal mechanism in *A. t.* subsp. *trichomanes* and *A. t.* subsp. *quadrivalens* could be a consequence of a similar evolutionary history (autopolyploidization, see above). The first record of stretched/bent annulus in the *Asplenium trichomanes* group is that of Jessen (1995, 1999).

Number of chromosomes (ploidy level) is the principal character for identifying diploid and tetraploid taxa. But in various floras and keys, rhizome scale length (*scalen*) is often suggested as a character well correlated with the ploidy level and useful for diploid and tetraploid taxa discrimination (Fischer et al. 2005, Frey et al. 1995). Nevertheless, we find no clear differences in the mean length to the scales of diploid and tetraploid taxa. The difference was only marginally significant ($F = 54.62$; $P < 0.05$) for plants whose ploidy level was verified by flow cytometry. There was a strong overlap between the values for the two groups and variation ranges were also too wide (Fig. 9). The only reliable ($P < 0.001$; $F = 24.3$) difference in scale length was found between *A. t.* subsp. *quadrivalens* and the three other taxa (Fig. 9). It is worth noting that the scale lengths of the tetraploid *A. t.* subsp.

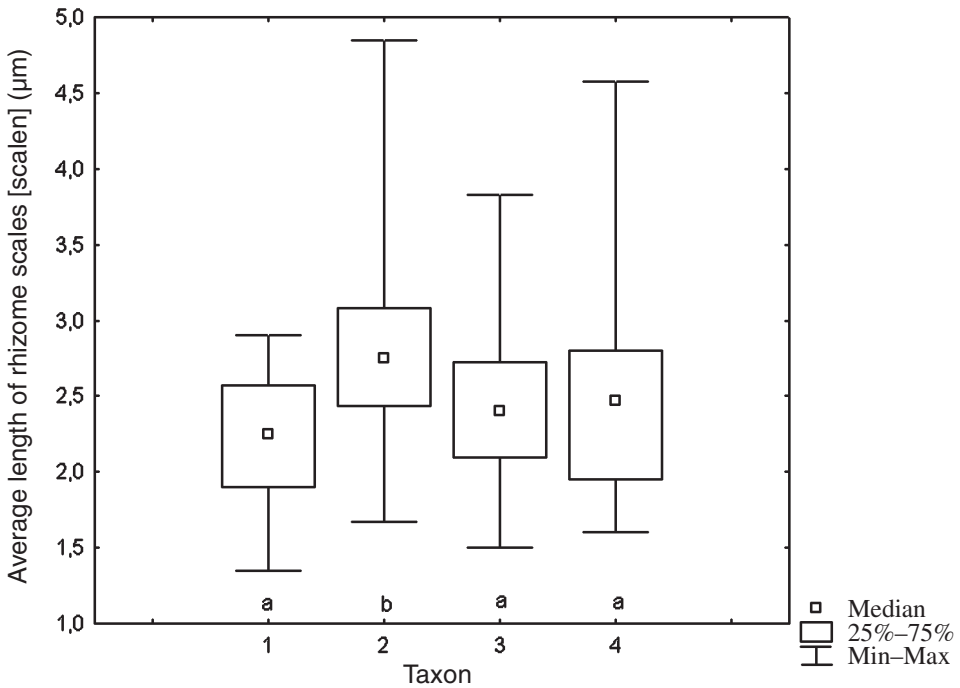

Fig. 9. – Box & whisker plot of one-way ANOVA ($F = 24.3$) of the mean rhizome scale lengths (*scalen*) of individual taxa of the *Asplenium trichomanes* group in the Czech Republic. 1 – *A. t.* subsp. *trichomanes*, 2 – *A. t.* subsp. *quadrivalens*, 3 – *A. t.* subsp. *pachyrachis*, 4 – *A. t.* subsp. *hastatum*. Letters at the bottom indicate the results of the Tukey HSD test, taxa labelled with the same letter do not differ significantly ($P > 0.01$).

pachyrachis and *A. t.* subsp. *hastatum* are often located within the intervals of the mean values usually reported for diploid taxa. Similarly, Lovis (1964) records that rhizome scale lengths are highly variable in some taxa (in diploid *A. t.* subsp. *trichomanes* and tetraploid *A. t.* subsp. *quadrivalens*) and must be therefore used with care. These taxa can be compared only if all but the largest scales are ignored, but this is not an objective approach (Lovis 1964). That rhizome scale length is unsuitable for practical determination of diploid and tetraploid taxa in the *A. obovatum* group is also reported by Steinecke & Bennert (1993).

Hybridization

Hybrids in the *Asplenium* genus are characterized by completely aborted spores and usually intermediate morphological characters (Reichstein 1981, 1984, Nyhus 1987, Jessen 1995). Plants with completely aborted spores were found also in this study. These plants occurred at localities where several taxa co-existed. Another prominent feature of these plants was their robust habitus, possibly due to an heterosis effect (Reichstein 1981). Flow cytometry revealed two DNA ploidy levels in the plants with aborted spores. Triploids were found only at three localities, where the diploid *A. t.* subsp. *trichomanes* and tetraploid *A. t.* subsp. *quadrivalens* occurred together. The joint occurrence of both subspecies and their triploid hybrid, formally called *Asplenium trichomanes* nothosubsp. *lusaticum*, is also reported from other parts of Europe (Reichstein 1981, Nyhus 1987, Stark 2002). The subsp. *trichomanes* grows only on siliceous and serpentine rocks, where the rare taxa (subsp. *pachyrachis*, subsp. *hastatum*) do not occur. For this reason, other possible triploid hybrid combinations cannot be established or they are at least very rare in the field.

Tetraploid hybrids were found at the localities where at least two tetraploid taxa co-occurred. *Asplenium trichomanes* nothosubsp. *lovisianum* S. Jess. (subsp. *hastatum* × subsp. *quadrivalens*) (17 plants from localities 9, 38, 39, 40, 41, 43, see Appendix 1) is frequent in the majority of localities of the parental taxon *A. t.* subsp. *hastatum*. On the other hand, the presence at these localities of *A. trichomanes* nothosubsp. *moravicum* S. Jess. (subsp. *hastatum* × subsp. *pachyrachis*) (four plants from localities no. 9 and 39) and *A. trichomanes* nothosubsp. *staufferi* Lovis et Reichstein (subsp. *pachyrachis* × subsp. *quadrivalens*) (five plants from localities no. 8, 28, and 44; Appendix 1) is very rare. These hybrid taxa were found only at localities where *A. t.* subsp. *pachyrachis* was present.

Determination key

The most suitable combinations of morphological characters, inferred from the results of the morphometric analyses, are used in the following key for determining the taxa of the *Asplenium trichomanes* group in the Czech Republic. Note that only the use of fertile plants will result in reliable determination.

1a Spores completely aborted.....hybrids
1b Spores fully developed.....2
2a Annulus after dehiscence of sporangium usually stretched; rachis straight or slightly curved; length of the pinnae gradually decreasing towards the apex, pinnae oblong or suborbicular, rarely auriculate.....3
2b Annulus after dehiscence of sporangium usually bent; rachis arched or sigmoidal; length of the pinnae not gradually decreasing towards the apex; pinnae triangulate, often biauriculate or deltoid.....4

3a Distance between pinnae stalks 3–7 mm (near the apex of the lamina), terminal pinna 1.5–4 mm wide; rachis wings without distinct, light yellow papillas; rhizome-scale appendages absent, mean annulus length 200–300 µm, mean exospores length 25–29 µm, diploid plants .....subsp. ***trichomanes***

3b Distance between pinnae stalks 2–4 mm (near the apex of the lamina), terminal pinna 2–7 mm wide, rachis wings usually with prominent orange papillas, some rhizome-scales with obvious appendages, mean annulus length 240–430 µm, mean exospores length 30–38 µm, tetraploid plants .....subsp. ***quadrivalens*** D. E. Mey.

4a Leaves ascending, pinnae length 6–8 mm, not imbricate, sometimes touching, lower pinnae usually biauriculate, distinct pale margin absent, rachis ± arched.....subsp. ***hastatum*** (Christ) S. Jess.

4b Leaves pressed against the substrate, pinnae length 3–7 mm, usually imbricate or touching, lower pinnae rarely biauriculate, distinct pale margin present, rachis sigmoidal.....subsp. ***pachyrachis*** (Christ) Lovis et Reichst.

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Souhrn

Prezentovaný příspěvek přináší detailní morfometrickou a cytometrickou studii skupiny sleziníku červeného – *Asplenium trichomanes* L. v České republice. Průtoková cytometrie byla použita pro analýzu ploidních úrovní rostlin ze 47 studovaných lokalit. Diploidní a tetraploidní rostliny byly nalezeny samostatně na jednotlivých lokalitách, ale také na společných lokalitách. Triploidní rostliny byly nalezeny na třech lokalitách vždy společně s diploidními i tetraploidními rostlinami. Morfometrické studium tradičně udávaných znaků a znaků nových ukazuje možné rozdělení rostlin studovaných z celého území ČR do čtyř podskupin. Tyto podskupiny lze na základě morfologických, cytologických a ekologických charakteristik ztotožnit se čtyřmi poddruhy: *Asplenium trichomanes* L. subsp. *trichomanes* (2n = 2x = 72), *A. t.* subsp. *quadrivalens* D. E. Mey. (2n = 4x = 144), *A. t.* subsp. *pachyrachis* (Christ) Lovis et Reichst. (2n = 4x = 144), *A. t.* subsp. *hastatum* (Christ) S. Jess. (2n = 4x = 144), které jsou známy i z dalších území Evropy. Podrobné rozšíření jednotlivých taxonů na území České republiky je prezentováno v samostatném příspěvku (Ekrt 2008).

Určovací klíč (pro určování taxonů z okruhu *Asplenium trichomanes* jsou nezbytné fertilní rostliny):

1a Výtrusy zcela abortované.....kříženci

1b Výtrusy vyvinuté 2

2a Prstenec po puknutí výtrusnice zpravidla napřímený; listové vřeteno vzpřímené nebo slabě obloukovitě zahnuté; délka lístků se výrazně k vrcholu čepele zkracuje; lístky obdélníkovité nebo vejčité, vždy bez ouška 3

2b Prstenec po puknutí výtrusnice zpravidla srpovitě zahnutý; listové vřeteno srpovitě zahnuté nebo esovitě prohnuté; délka lístků se k vrcholu čepele zkracuje jen nepatrně; lístky trojúhelníkovité, často ouškaté.....4

3a Vzdálenost mezi řapíčky lístků v horní části čepele asi 3–7 mm, koncový lístek 1,5–4 mm široký; křídla na vřeteni s nezřetelnými světlými papilami, oddenkové pleviny s častými přívěsky, prstenec v průměru 200–300 µm dlouhý, výtrusy (exospory) 25–29 µm dlouhé, diploidní rostliny .....subsp. ***trichomanes***

3b Vzdálenost mezi řapíčky lístků v horní části čepele asi 2–4 mm, koncový lístek 2–7 mm široký, křídla na vřeteni s výraznými zvětšenými žlutě oranžovými papilami, oddenkové pleviny bez přívěsků, prstenec v průměru 240–430 µm dlouhý, výtrusy (exospory) 30–38 µm dlouhé, tetraploidní rostliny .....subsp. ***quadrivalens*** D. E. Mey.

4a Listy vystoupavé, lístky 6–8 mm dlouhé, ojediněle se navzájem dotýkající, zpravidla v dolní polovině ouškaté, okraj lístků bez zřetelného světlého lemu, vřeteno ohnuté až ± srpovitě zahnuté ....subsp. ***hastatum*** (Christ) S. Jess.

4b Listy růžicovitě rozprostřené, přitisknuté k substrátu, lístky 3–7 mm dlouhé, zpravidla střechovitě se překrývající nebo dotýkající, lístky v dolní polovitě ojediněle ouškaté, okraj lístků se zřetelným světlým lemem, vřeteno zpravidla esovitě prohnuté .....subsp. ***pachyrachis*** (Christ) Lovis et Reichst.

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Appendix 1. – List of *Asplenium trichomanes* localities of the plants used in the ploidy levels analysis and multivariate study. 1 – locality number; 2 – country, region, phytogeograpical district with its number (Skalický 1988) (in parentheses is a quadrant number of the Central European grid mapping program, cf. Ehrendorfer & Hamann 1965), locality, altitude, latitude, longitude, collector, collection date; 3 – ploidy levels or chromosome numbers determined by chromosome counting; 4 – determined taxa (T = *Asplenium trichomanes* subsp. *trichomanes*; Q = *A. t.* subsp. *quadrivalens*; P = *A. t.* subsp. *pachyrachis*; H = *A. t.* subsp. *hastatum*; TxQ = *A. t.* nothosubsp. *lusaticum*; HxQ = *A. t.* nothosubsp. *lovisianum*; HxP = *A. t.* nothosubsp. *moravicum*; PxQ = *A. t.* nothosubsp. *staufferi*).

1	2	3	4
1	Czech Republic, C Bohemia, 8. Český kras (6050d): limestone debris slope over the stream in the N part of the Koda reserve, ca 700 m SW of the railway station of Srbsko village, ca 320 m, 49°55'58"N, 14°07'8"E, leg. L. Ekrt, 6. X. 2002.	4x	Q
2	Czech Republic, C Bohemia, 8. Český kras (6050d): limestone rocks of the mouth of Císařská rokle gorge in Koda reserve, ca 500 m SSE of the railway station of Srbsko village, ca 230 m, 49°55'53"N, 14°07'59"E, leg. L. Ekrt, 6. X. 2002.	4x	Q
3	Czech Republic, C Bohemia, 8. Český kras (6051c): limestone rocks over the road from Karlštejn village to Srbsko village, ca 1.5 km E of the Karlštejn village, ca 210 m, 49°56'N, 14°10'E, leg. L. Ekrt, 6. X. 2002.	4x	Q
4	Czech Republic, C Bohemia, 8. Český kras (6050b): limestone rocks over the road to Svatý Jan pod Skalou village, ca 400 m N of the Hostim village, ca 210 m, 49°57'50"N, 14°07'51"E, leg. L. Ekrt, 6. X. 2002	4x	Q
5	Czech Republic, C Bohemia, 8. Český kras (6050b): limestone rocks, ca 250 m SW of the Svatý Jan pod Skalou village, ca 200 m, 49°57'56"N, 14°07'47"E, leg. L. Ekrt, 6. VIII. 2002.	4x	Q
6	Czech Republic, E Bohemia, 15b. Hradecké Polabí (5662b): plaener rocks over the Metuje river, ca 300 m SSE of the railway station of the Nové Město nad Metují town, ca 280 m, 50°21'01"N, 16°08'30"E, leg. L. Ekrt, 29. IX. 2002.	4x	Q
7	Czech Republic, S Moravia, 16. Znojemsko-brněnská pahorkatina (6664d): small limestone cave in the Malhostovická pecka reserve, ca 1 km SW of the Malhostovice village, ca 300 m, 49°19'35"N, 16°29'40"E, leg. L. Ekrt, E. Hofhanzlová, 23. VIII. 2004.	4x	H, Q
8	Czech Republic, S Moravia, 17b. Pavlovské kopce (7165d): limestone rocks in the Kočičí skála reserve, ca 1.2 km SE of the Bavory village, ca 345 m, 48°49'N, 16°38'E, leg. L. Ekrt, 5. IV. 2002.	4x	Q, P, PxQ
9	Czech Republic, S Moravia, 17b. Pavlovské kopce (7165b): limestone rocks under the Sirotčí hrádek ruins, ca 0.4 km NW of the Klentnice village, ca 430 m, 48°50'N, 16°38'E, leg. L. Ekrt, 5. IV. 2002.	4x	H, P, Q, HxP, HxQ
10	Czech Republic, S Moravia, 17b. Pavlovské kopce (7165b): Martinské stěny limestone rocks, ca 1.2 km SE of the Horní Věstonice village, ca 370 m, 48°52'N, 16°38'E, leg. L. Ekrt, 5. IV. 2002.	4x	P
11	Czech Republic, S Moravia, 17b. Pavlovské kopce (6065b): limestone rocks under the Děvín hill in the Soutěska valley, ca 0.75 km SW of the Děvín hill, ca 370 m, 48°51'N, 16°38'E, leg. L. Ekrt, 5. IV. 2002.	4x	P
12	Czech Republic, W Bohemia, 28e. Žlutická pahorkatina (5545b): siliceous slate rocks over the Manětínský potok stream, ca 1.1 km S of the Brdo village, ca 375 m, 49°59'28"N, 13°15'37"E, leg. L. Ekrt, 4. IX. 2002.	2x, 3x, 4x	Q, T, QxT
13	Czech Republic, W Bohemia, 28e. Žlutická pahorkatina (5945b): siliceous slate rocks with a basic enrichment over the Střela river, ca 1.6 km E of the Kotaneč village, ca 410 m, 50°0'59"N, 13°18'32"E, leg. L. Ekrt, 4. IX. 2002.	4x	Q
14	Czech Republic, W Bohemia, 28e. Žlutická pahorkatina (5945b): siliceous slate rocks in the Střela river valley, ca 0.7 km SE of the Rabštejn village, ca 375 m, 50°01'45"N, 13°17'55"E, leg. L. Ekrt, 4. IX. 2002.	2x	T

15	Czech Republic, C Bohemia, 32. Křivoklátsko (5949d): calcareous rocks in the Kabečnice reserve, ca 200 m NE of the Žloukovice village, ca 230 m, 50°01'00"N, 13°57'32"E, leg. L. Ekrt, 7. X. 2002.	4x	Q
16	Czech Republic, C Bohemia, 32. Křivoklátsko (5949c): siliceous rocks in the W part of the Brdatka reserve, ca 2 km NE of the Křivoklát village, ca 405 m, 50°02'54"N, 14°07'47"E, leg. L. Ekrt, 7. X. 2002.	4x	Q
17	Czech Republic, C Bohemia, 32. Křivoklátsko (5949c): siliceous rocks in the W part of the Nezabudické skály reserve, ca 2.5 km SW of the Křivoklát village, ca 250 m, 50°01'21"N, 13°50'9"E, leg. L. Ekrt, 7. X. 2002.	4x	Q
18	Czech Republic, C Bohemia, 32. Křivoklátsko (6048b): calcareous rocks in the Čertova skála reserve, ca 1.5 km SE of the Hracholusky village, ca 250 m, 49°59'50"N, 13°47'30"E, leg. L. Ekrt, 7. X. 2002.	4x	Q
19	Czech Republic, C Bohemia, 32. Křivoklátsko (6048c): siliceous rocks in the Jezírka reserve, ca 2 km SSW of the Skryje village, ca 280 m, 49°56'52"N, 13°45'01"E, leg. L. Ekrt, 7. X. 2002.	4x	Q
20	Czech Republic, W Bohemia, 37a. Horní Pootaví (6847c): gneiss rocks over the road from Rejštejn village to Annín village, ca 1.5 km N of the Rejštejn village, ca 560 m, 49°09'21"N, 13°30'51"E, leg. L. Ekrt, 14. X. 2002.	2x	T
21	Czech Republic, W Bohemia, 37a. Horní Pootaví (6846d): gneiss rocks in the Paštecké skály reserve, ca 2 km N of the Čeňkova pila colony NNE of the Srní village, ca 600 m, 49°07'32"N, 13°29'35"E, leg. L. Ekrt, 14. X. 2002.	2x n = ca 36^{II}	T
22	Czech Republic, S Bohemia, 37b. Sušicko-horažďovické vápence (6648c): siliceous rocks with basic enrichment ca 50 m E of the Prácheň ruins, ca 1.5 km ESE of the Horažďovice village, ca 500 m, 49°19'N, 13°40'E, leg. L. Ekrt, 9. III. 2002.	4x	Q
23	Czech Republic, S Bohemia, 37b. Sušicko-horažďovické vápence (6748a): limestone rocks in the north part of Pučanka reserve, ca 300 m SW of the Hejná village, ca 530 m, 49°17'N, 13°40'E, leg. L. Ekrt, 9. III. 2002.	4x	Q
24	Czech Republic, S Bohemia, 37b. Sušicko-horažďovické vápence (6747b): limestone rocks on the SE base of Chanovec hill, ca 1.5 km SW of the Rabí village, ca 615 m, 49°16'N, 13°36'E, leg. L. Ekrt, 10. III. 2002.	4x	Q
25	Czech Republic, S Bohemia, 37k. Křemžské hadce (7151b): serpentine rocks of the Bořinka reserve, ca 1 km WNW of the railway station of Holubov village, ca 490 m, 48°53'N, 14°18'E, leg. L. Ekrt, 13. V. 2002.	2x	T
26	Czech Republic, S Bohemia, 37k. Křemžské hadce (7152a): serpentine rocks of the Holubovské hadce reserve, ca 1.4 km ESE of the railway station of Holubov village, ca 470 m, 48°53'N, 14°20'E, leg. L. Ekrt, 13. V. 2002.	2x, 3x, 4x	Q, T, QxT
27	Czech Republic, S Bohemia, 37l. Českokrumlovské Předšumaví (7052d): siliceous rocks on the left bank of Vltava river, ca 1 km SW of the Boršov nad Vltavou village, ca 410 m, 48°55'N, 14°25'E, leg. L. Ekrt, 17. XI. 2001.	4x	Q
28	Czech Republic, S Bohemia, 37l. Českokrumlovské Předšumaví (7151d), walls in the park and building of Jízdárna in area v Český Krumlov castle, ca 531 m, 48°48'45"N, 14°18'37"E, leg. L. Ekrt, E. Hofhanzlová, 4. XI. 2004.	4x	P, Q, PxQ
29	Czech Republic, N Bohemia, 55d. Trosecká pahorkatina (5457c): sandstone rocks with a basic enrichment, ca 500 m N of the Tachov colony near the Troskovice village, ca 280 m, 50°31'N, 15°13'E, leg. L. Ekrt, 9. VIII. 2002.	4x	Q
30	Czech Republic, E Bohemia, 58b. Polická kotlina (5463c): plaener rocks called Poradní skála rock in the Maršovské údolí valley, ca 1.5 km SE of the Maršov village, ca 430 m, 50°31'N, 16°12'E leg. L. Ekrt, 27. IV. 2002.	4x	Q
31	Czech Republic, E Bohemia, 58b. Polická kotlina (5563b): plaener rocks under the Bor hill, ca 1.5 km S of the Machov village, ca 580 m, 50°29'N, 16°16'E, leg. L. Ekrt, 25. IV. 2002.	4x	Q

32	Czech Republic, E Bohemia, 59. Orlické podhůří (5663d): siliceous mica schist rocks cca 1.2 km SW of the Šediviny village, ca 560 m, 50°17'57"N, 16°17'32"E, leg. L. Ekrt, 29. IX. 2002.	2x, 4x	Q, T
33	Czech Republic, E Bohemia, 59. Orlické podhůří (5763b): walls of the Nový hrad (Klečkov) ruins, ca 3.5 km NE of the Skuhrov nad Bělou village, ca 480 m, 50°15'11"N, 16°19'20"E, leg. L. Ekrt, 29. IX. 2002.	4x	Q
34	Czech Republic, E Bohemia, 63a. Žambersko (5964a): walls of the Litice ruins near the Litice nad Orlicí village, ca 445 m, 50°5'7"N, 16°21'6"E, leg. L. Ekrt, 22. IX. 2002.	4x	Q
35	Czech Republic, SE Bohemia, 68. Moravské podhůří Vysočiny (6660c): gneiss rocks over the Brtnice river, ca 650 m S of the railway station of Přímělkov village, ca 435 m, 49°20'17"N, 15°44'25"E, leg. L. Ekrt, 19. VIII. 2004.	2x, 3x, 4x	Q, T, QxT
36	Czech Republic, S Moravia, 68. Moravské podhůří Vysočiny (7161a): gneiss rocks ca 200 m SW of the Hardeggská vyhlídka lookout, ca 2.8 SSW of the Čížov village, ca 320 m, 48°51'23"N, 15°51'35"E, leg. L. Ekrt, 24. VII. 2002.	4x	Q
37	Czech Republic, S Moravia, 68. Moravské podhůří Vysočiny (7161a): siliceous debris ca 1.5 km W of the Čížov village, ca 415 m, 48°52'56"N, 15°51'6"E, leg. L. Ekrt, 24. X. 2002.	4x	Q
38	Czech Republic, S Moravia, 70. Moravský kras (6666a): limestone rocks of the Pustý žleb gorge, ca 250 m NNE of the crossway Pod Salmovkou, W of the Ostrov u Macochy village, ca 420 m, 49°22'33"N, 16°43'24"E, leg. L. Ekrt, 22. VII. 2002.	4x	H, Q, HxQ
39	Czech Republic, S Moravia, 70. Moravský kras (6666a): limestone rocks of the Pustý žleb gorge, ca 500 m NNE of the crossway Pod Salmovkou, W of the Ostrov u Macochy village, ca 440 m, 49°22'N, 16°43'E, leg. L. Ekrt, 22. VII. 2002.	4x	H, P, Q, HxP, HxQ
40	Czech Republic, S Moravia, 70. Moravský kras (6666c): limestone rocks over the entrance to the Býčí skála cave, ca 2.2 km W of the Habrůvka village, ca 350 m, 49°18'N, 16°41'E, leg. L. Ekrt, 22. VII. 2002.	4x	H, P, HxQ
41	Czech Republic, S Moravia, 70. Moravský kras (6566c): limestone rocks near the entrance to the Sloupsko-Šošůvská jeskyně cave in the Sloup village, ca 465 m, 49°24'38"N, 16°44'19"E, leg. L. Ekrt, 22. VII. 2002.	4x	Q, HxQ
42	Czech Republic, S Moravia, 70. Moravský kras (6666c): limestone rocks of the Jáchymka cave in the Josefovské údolí valley, ca 2 km SW of the railway station of Adamov town, ca 300 m, 49°18'N, 16°40'E, leg. L. Ekrt, 5. V. 2001.	4x	H
43	Czech Republic, SE Moravia, 77c. Chřiby (6869d): walls of the Buchlov castle, ca 6.5 km NNW of the Buchlovice town, ca 500 m, 49°6'28"N, 17°18'40"E, leg. L. Ekrt, 23. VII. 2002.	4x	H, HxQ
44	Czech Republic, NE Moravia, 84a. Beskydské podhůří (6375c): walls in the deer-park of Hukvaldy ruins area, ca 30 m of the entrance, ca 100 m SE of the church of the Hukvaldy village, ca 355 m, 49°37'22"N, 18°13'22"E, leg. L. Ekrt, E. Hofhanzlová, 24. VIII. 2004.	4x	P, Q, PxQ
45	Czech Republic, S Bohemia, 88a. Královský hvozd (6744d): siliceous rocks with a basic enrichment, near the peak Grosser Osser (Ostrý) hill, under the chalet, ca 4.3 km ENE of the Lam village, ca 1 276 m, 49°12'12"N, 13°06'38"E, leg. L. Ekrt, 14. X. 2002.	4x	Q
46	Czech Republic, S Bohemia, 88b. Šumavské pláně (7148b): siliceous rocks with a basic enrichment in the Stožecká skála reserve, ca 100 m SW of the Stožecká kaple chapel, ca 1.7 km N of the Stožec village, 960 m, 48°52'26"N, 13°49'18"E, leg. L. Ekrt, 15. X. 2002.	2x, 4x	Q, T
47	Slovakia, 13. Strážovské vrchy (6877a), Súlovské skály, limestone conglomeration rocks, ca 2 km SE of the Jablonové village, ca 425 m, 49°10'01"N, 18°34'33"E, leg. L. Ekrt, 28. IX. 2004.	4x	P

Appendix 2. – Results of exploratory data analysis of subspecies of the *Asplenium trichomanes* complex: 1 – *A. t.* subsp. *trichomanes*, 2 – *A. t.* subsp. *quadrivalens*, 3 – *A. t.* subsp. *pachyrachis*, 4 – *A. t.* subsp. *hastatum*. For character abbreviations see Table 1.

Character	Group	S.D	Minimum	5% percentile	Mean	95% percentile	Maximum
anulen	1	20.95	206	222	249.63	290	306
(μm)	2	25.00	240	262	298.29	340	430
	3	41.84	158	284	341.98	400	410
	4	37.77	292	300	337.30	424	434
enpilen	1	2.00	2	3	5.91	9	12.5
(mm)	2	1.80	2	3.5	6.24	9.5	13
	3	1.39	1.5	3	4.44	6.5	8
	4	2.08	1	1.5	4.51	9	9
enpiwid	1	0.90	1	1.5	2.44	4	5
(mm)	2	1.47	1	2	3.75	6.5	10
	3	1.48	1	1.5	3.59	6	7
	4	1.51	1	1	3.41	6	7
ind1pi	1	0.47	0	0	0.13	2	2
	2	0.37	0	0	0.08	1	2
	3	1.05	0	0	0.91	3	3
	4	1.41	0	0	0.79	4	6
int7/8	1	1.55	1	2.5	4.60	7.5	8
(mm)	2	0.93	0.5	2	3.15	4.5	7
	3	0.72	1	1.5	2.39	3.5	4.5
	4	0.66	1.5	2	2.77	4	4.5
lam	1	50.14	43	47	126.81	197	234
(mm)	2	38.16	6.5	65	118.01	186	248
	3	23.44	16	34	68.06	118	125
	4	30.41	44	56	106.81	154	188
pi1/2len	1	1.06	2.5	3	4.59	6.5	7
(mm)	2	1.23	2.5	3.5	5.20	7	11
	3	1.34	1.5	3	5.01	7	8.5
	4	1.02	4	6	6.99	8	9.5
pi1/4len	1	0.96	2	2.5	3.65	5	6
(mm)	2	1.13	2	2.5	4.16	6	8
	3	1.25	1.5	2	4.08	6.5	7
	4	1.44	3.5	4	6.21	8.5	9
pisum	1	7.09	9	12	22.60	31	40
	2	5.42	9	16	24.23	33	38
	3	3.99	8	11	17.82	25	26
	4	4.36	10	13	21.10	27	28
rhawid	1	0.09	0.22	0.25	0.39	0.51	0.61
(mm)	2	0.15	0.07	0.30	0.40	0.54	0.60
	3	0.07	0.23	0.31	0.43	0.55	0.56
	4	0.09	0.28	0.29	0.46	0.58	0.63
scalen	1	0.45	1.35	1.48	2.21	2.83	2.90
(mm)	2	0.45	1.68	2.08	2.76	3.47	4.85
	3	0.48	1.50	1.6	2.41	3.3	3.83
	4	0.61	1.60	1.68	2.48	3.63	4.58
sporlen	1	0.91	25.03	25.47	26.91	28.23	28.60
(μm)	2	1.44	29.23	31.56	33.77	36.43	38.20
	3	1.58	29.80	30.23	32.69	35.33	36.07
	4	1.38	31.20	32.47	35.18	36.67	38.73