Intraspecific differentiation of *Sparganium erectum* in the Czech Republic: molecular, genome size and morphometric analysis

Vnitrodruhová diferenciace *Sparganium erectum* v České republice: molekulární a morfometrické analýzy a stanovení velikosti genomu

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Aquatic and wetland plants tend to be very phenotypically plastic, which accounts for the taxonomic difficulties in many groups. In the genus *Sparganium*, which comprises about 14 species, numerous taxa at different ranks are described. The classification of the genus is based on generative characters on the fruit, which are less influenced by the environment than vegetative characters. Nevertheless, the intraspecific division of *Sparganium erectum* poses problems, especially the existence of several intraspecific taxa along with intermediate individuals. In this study we examined four European subspecies of *S. erectum* (subsp. *erectum*, subsp. *microcarpum*, subsp. *neglectum* and subsp. *oocarpum*) from 64 populations in the Czech Republic. A combination of multivariate morphometrics, AFLPs and genome size estimation allowed us to confirm the current subspecies classification and investigate putative intraspecific hybridization. Four genetic groups with different genome sizes corresponding to the subspecies were found. Morphological characters that were described in previous studies correlated with these genetic groups and thus affirmed the classification. The most important characters for subspecies differentiation were width and length of fruit, style length, length of the upper part of the fruit and constriction in the middle part of the fruit. In addition, admixed individuals between the genetic groups were recorded. The hybrid origin of subsp. *oocarpum* was confirmed, being derived from the crossing of subsp. *erectum* and subsp. *neglectum*. Finally, three other hybrid combinations were detected, suggesting recent hybridization: subsp. *erectum* × subsp. *microcarpum*, subsp. *microcarpum* × subsp. *neglectum*, and subsp. *erectum* × subsp. *oocarpum*.

K e y w o r d s: AFLP, Czech Republic, genome size, hybridization, model-based clustering, multivariate morphometrics, *Sparganium*

Introduction

Aquatic environments are highly dynamic, variable and heterogeneous over a relatively small scale (Sculthorpe 1967). Wetland and aquatic plants are generally well adapted to changing habitat conditions (fluctuations in the level, turbidity or temperature of the water), which are frequently connected with considerable variation in morphology that may complicate taxonomic differentiation (Barrett et al. 1993, Kaplan 2002a, Santamaria 2002). Sizes of vegetative organs, such as the length and width of leaves, may vary depending on habitat conditions, which make them unreliable for identifying species.

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Similarly, juvenile and sterile stages of some species (and sometimes even genera) cannot be distinguished (Cook & Nicholls 1986).

Intraspecific morphological variation has also influenced taxonomic concepts in the genus *Sparganium* L. Although different authors recognize numerous taxa at various ranks within this genus (Ostenfeld-Hansen 1897, Graebner 1900, Cook 1980), most of these taxa probably represent only phenotypic variability of a single taxon. This is due to the use of unstable vegetative characters for taxonomic differentiation (Čelakovský 1896, Ascherson & Graebner 1897, Čelakovský 1899, Graebner 1900, Hegi 1936, Casper & Krausch 1980). The last taxonomic monograph on *Sparganium* is mainly based on the morphological characters of drupe-like fruit that vary little and recognizes about fourteen species (Cook & Nicholls 1986, 1987). In the Czech Republic, four species of *Sparganium* are recognized: *S. angustifolium* Michx., *S. erectum* L., *S. emersum* Rehmann, and *S. natans* L. (Kaplan 2002b). The taxonomy of *S. erectum* is reported by Haasová (1997). Although characters such as the absolute length and width of the fruit are less convenient for identification, their ratios have some differentiation value. The colour of the upper and lower parts of the fruit also appears to be useful for delimitating infrageneric taxa (Cook & Nicholls 1987, Kaplan 2002b).

The present worldwide classification of *S. erectum* is based on the morphology of the fruit and distinguishes five subspecies: *S. erectum* subsp. *erectum*, *S. e.* subsp. *oocarpum* (Čelak.) Domin, *S. e.* subsp. *neglectum* (Beedy) K. Richt., *S. e.* subsp. *microcarpum* (Neumann) Domin, and *S. e.* subsp. *stoloniferum* (Graebn.) H. Hara (Cook 1980, Cook & Nicholls 1987). Plant material of the Asian subsp. *stoloniferum* was not available to us, so we included only the four subspecies occurring in Europe in this study. In addition, intermediate individuals between subspecies are found and mixed populations of individuals of different subspecies are reported, which indicates possible hybridization between subspecies. *Sparganium erectum* subsp. *oocarpum* is thought to be of hybrid origin, the putative parental taxa being *S. e*. subsp. *neglectum* and *S. e.* subsp. *erectum* (Cook 1961). Moreover, a previous study of herbarium specimens and morphology of the fruit found subsp. *microcarpum* to be very variable morphologically and revealed that many individuals are intermediate between subsp. *microcarpum* and subsp. *neglectum* (Haasová 1997). Therefore, this author suggests merging both subspecies into one taxon.

To determine the intraspecific variation in *Sparganium erectum* and, if possible, propose taxonomic conclusions, we decided to use a combination of classical (morphometric analysis) and molecular methods, such as Amplified Fragment Length Polymorphisms (AFLPs) and flow cytometry (FCM). This combination of methods is a powerful tool for assessing intraspecific variation and unravelling the origins of hybrids (Gobert 2002, Guo et al. 2006, Španiel et al. 2011, Píšová et al. 2017, Lepší et al. 2019, Popelka et al. 2019, Silbernagl & Schönswetter 2019) or hybrid lineages within a species (Daneck et al. 2011). The AFLP method generates a large number of loci distributed throughout a genome and does not require any previous sequence knowledge (Vos et al. 1995). Bayesian clustering in STRUCTURE classifies samples into genetic groups (Pritchard et al. 2000, Evanno et al. 2005) and allows subsequent determination of admixed individuals (putative hybrids; Paszko & Nobis 2010, Ciotir et al. 2017, Píšová et al. 2017). Determining genome size using flow cytometry is a simple and rapid method, which is widely used in studies of biosystematics (Doležel et al. 2007, Slovák et al. 2009, Loureiro et al. 2010, Kolář et al. 2013, Linder et al. 2017) and facilitates the determination of hybrids

(Bureš et al. 2004, Vít et al. 2014, Prančl et al. 2018). However, these methods have not previously been used for studying the genetic relationships and hybridization in *Sparganium*. We did not investigate chromosome numbers because they are reported to be uniform across the whole genus (2n = 30; Löve & Löve 1948, 1956, Cook & Nicholls 1986). The origin of new taxa via hybridization not accompanied by whole-genome duplication and an increase in ploidy level is described as speciation by homoploid hybridization (Rieseberg 1997). The establishment of a new homoploid hybrid taxon requires hybrid segregants to be isolated by a sterility barrier or some external isolation mechanism (e.g. by geographic or ecological isolation; Buerkle et al. 2000, Gross & Rieseberg 2005).

To investigate homoploid hybridization in *S. erectum* and reassess its current classification, we addressed the following questions: (i) Does the genetic pattern or variation in genome size within *S. erectum* allow the delimitation of clearly separated groups that could be used as a basis for intraspecific taxonomic classification? (ii) Are genetic variation and variation in genome size correlated with morphological variation? (iii) What morphological characters are most correlated and can they be used for the delimitation of intraspecific taxa? (iv) Is *S. erectum* subsp. *oocarpum* of hybrid origin? What are its parental taxa? (v) Should the subspecies *neglectum* and *microcarpum* be merged into a single taxon or distinguished as two distinct taxa and which characters can be used for their identification?

Materials and methods

Plant material

Altogether 276 individuals of four subspecies of *Sparganium erectum* (subsp. *erectum*, subsp. *microcarpum*, subsp. *neglectum* and subsp. *oocarpum*) from 64 natural populations in the Czech Republic were collected in 2007–2008 (Appendix 1). The subspecies were preidentified in the field using identification keys (Cook & Nicholls 1986, Kaplan 2002b) and the populations in Appendix 1 are listed according to this preliminary identification. Differences between field identification and resulting determination based on AFLP data and genome size are discussed later. Typical habitats of *S. erectum* in the Czech Republic are fishponds, river banks and other wetlands in river floodplains, which correspond to the distribution of the sites sampled predominantly along the Labe, Vltava and Lužnice rivers and their tributaries. Most plants were sampled in central and southern Bohemia, and one population was sampled in southern Moravia (Electronic Appendix 1AB). Material for AFLP analyses (altogether 276 leaf samples), morphological analyses (1770 samples of fruit from 276 individuals) and flow cytometry (276 fresh leaves) was collected, using the following procedures. For AFLP analyses, part of a young leaf was dried immediately in silica gel and another part was used for the estimation of genome size by flow cytometry (up to five individuals per population, depending on population size). For morphometric measurements, five ripe fruit were randomly chosen from a mixture of all the fruit on each individual. When putatively mixed or intermediate populations were found, the number of individuals sampled for AFLPs, FCM and morphometric analyses was increased to 15 per population to ensure a more thorough examination. Because *S. erectum* is a rhizomatous clonal plant, only individuals (ramets) located 10 m

apart were collected to minimize sampling the same clone several times. Even so, clonality of plants was further checked by genotyping of individuals based on AFLP data and any clones were removed from all subsequent analyses. Voucher specimens of plants are deposited in the herbarium at the Faculty of Sciences, Charles University in Prague (PRC).

AFLP analysis

Total genomic DNA was extracted from silica-dried leaf material using CTAB isolation buffer following the protocol of Doyle & Doyle (1987). Final DNA pellets were dissolved in 100 µl of 1×TE buffer. The DNA concentration was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and the DNA was diluted to 100 $ng \cdot \mu l^{-1}$.

AFLP analysis (Vos et al. 1995) was done using the AFLP Core Reagent Kit I (Invitrogen) and the AFLP Pre-Amp Primer Mix I (Invitrogen) following the manufacturer's instructions as modified by Záveská et al. (2011) and further modified to yield the procedure described below. Total genomic DNA (~100 ng) was double-digested for 3h at 37 °C with 0.5 U each of *Eco*RI and *Mse*I restriction enzymes (Invitrogen) and 1 µl of a 5× reaction buffer (Invitrogen) in a total volume of 5 µl. Subsequently, adaptors were ligated for 3h at 37 °C by adding 4.8 µl of an adaptor/ligation solution (Invitrogen) and 0.2 U of T4 DNA ligase (Invitrogen) to the digested DNA (total volume 10 µl). The preamplification mixture (total volume 5 µl) contained 0.5 µl of restricted/ligated DNA, 4.0 µl of Pre-Amp Primer Mix I, 0.5 µl of 10× buffer for RedTaq JumpStart (Sigma) and 0.1 U of RedTaq JumpStart DNA polymerase (Sigma). After preamplification, the DNA was 10× diluted with ddH_2O. Three primer combinations were used for selective amplification: *Eco*RI-ACT-(6-FAM)/*Mse*I-CAT, *Eco*RI-AAG-(HEX)/*Mse*I-CTC and *Eco*RI-ACC-(NED)/*Mse*I-CAT. The reaction mixture for selective amplification contained 2.3 µl of the diluted preamplification mixture, 1 µl of a 10× buffer for RedTaq, 0.2 µmol dNTP, 0.5 pmol of an *Eco*RI-selective fluorescence-labelled primer, 2.5 pmol of an *Mse*I-selective primer and 0.2 U of RedTaq JumpStart DNA polymerase (Applied Biosystems) in a total volume of 10 µl. The selected amplification products were electrophoresed on an ABI 3100 Genetic Analyzer (Applied Biosystems) with GeneScan ROX 500 (Applied Biosystems) as a size standard in the DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague. In total, 276 samples from 64 populations were analysed. The whole AFLP procedure was repeated for 8% (22) of the samples and the error rate was estimated by comparing identical samples (Bonin et al. 2004).

Molecular data analyses

GeneMarker v1.8 (SoftGenetics LLC, PA, USA) was used for analysing AFLP data and transferring them into a binary data matrix. Only unambiguous bands were used for subsequent analyses; faint bands were excluded. Three *Eco*RI/*Mse*I AFLP primer combinations generated 125 AFLP markers, ranging in size from 100 to 500 base pairs. Problematic loci were then removed from the AFLP matrix. Further, clonality (the number of genotypes, G) based on the initial number of individuals (Nind) in each population was detected using R 3.5.2 (R Development Core Team 2008) and script AFLPdat (Ehrich 2006, the threshold applied was equal to error rate, see Results). The resulting matrix of

genotypes without clones was used in subsequent analyses. In addition, the genetic variation of each population was estimated by calculating Nei's gene diversity (He), percentage of polymorphic loci (Pp) and Simpson's diversity index (D, which express the probability that two randomly sampled individuals are genotypically different) using the R script AFLPdat (Ehrich 2006).

Bayesian non-hierarchical clustering in STRUCTURE 2.3.2.1 (Pritchard et al. 2000) using a Markov chain Monte Carlo (MCMC) algorithm and an admixture model with correlated allele frequencies was used to define the AFLP groups, to confirm the hybrid origin of subsp. *oocarpum* and assess the degree of admixture among subspecies. Ten replicates for each K = 1–10 were used to determine the stability of the results. The burn-in length of 100,000 generations and an additional 1,000,000 generations of MCMC chains after burn-in were run on the Metacentrum VO infrastructure (https://metavo.metacentrum.cz, Falush et al. 2007). The output files were summarized in the R-script Structure-sum-2009 (Ehrich et al. 2007) to determine the optimal number of clusters (K) based on the similarity coefficients between the runs (ΔK; Evanno et al. 2005, Nordborg et al. 2005). Graphical outputs for selected Ks were generated using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) and Distruct (Rosenberg 2004). Samples with low admixture (up to 15%) were considered members of one of the K AFLP groups and served as predefined groups in morphometric analyses, whereas highly admixed individuals (more than 15%, i.e. genetically intermediate between the AFLP groups), were passively projected onto ordination diagrams in subsequent analyses (i.e. PCoA, PCA, CDA; Píšová et al. 2017).

To explore the distribution of genetic variation within and among species and populations, two analyses of molecular variance (AMOVAs, Excoffier et al. 1992; implemented in Arlequin 3.5.1.2., Excoffier & Lischer 2010) were performed: (i) three-level analysis (among species, among populations within species and within populations) and (ii) two-level analysis for each subspecies (among populations and within populations). Only predefined AFLP groups with non-admixed individuals were used for AMOVA calculations.

Principal coordinate analysis (PCoA), implemented in Canoco 5 (ter Braak & Šmilauer 2012) was performed using Jaccard's similarity coefficients (Jaccard 1908) for the calculation of the distance matrix. In addition, a neighbour-network and a neighbour-joining tree were constructed in SPLITSTREE v.4.11.3 (Huson & Bryant 2006) and PAUP 4.0 (Swofford 2002), respectively.

To explore the dynamics of the hybridization between *S. erectum* subsp. *erectum*, *S. e.* subsp. *neglectum* (parental subspecies) and their putative hybrid *S. e.* subsp. *oocarpum* we applied the probabilistic Bayesian-based methods implemented in NewHybrids software. This approach estimated the probability that an individual was a pure parental species (A or B) or one of the different hybrid categories, i.e. F1, F2, F1×A, or F1×B (Anderson & Thompson 2002). Uninformative priors (Jeffreys) were given to both, allele frequency and admixture distributions. The analysis was run for 50,000 MCMC sweeps after 10,000 burn-in steps. The analysis was run with 104 samples, i.e. 38 samples classified as *S. erectum* subsp. *erectum*, 13 as *S. e.* subsp. *neglectum* and 53 as *S. e.* subsp. *oocarpum*.

In all graphical outputs of molecular analyses, AFLP groups are colour-coded in accordance with the presentation of our STRUCTURE results. Highly admixed individuals are marked by grey symbols.

Genome size

Estimates of genome sizes were obtained using propidium iodide flow cytometry following the simplified two-step procedure using Otto buffers described by Doležel et al. (2007). Intact leaf tissue (1 cm^2) of *S. erectum* together with an appropriate volume of the internal reference standard (*Glycine max* cv. Polanka, 2C = 2.50 pg) were chopped up using a sharp razor blade in a Petri dish containing 0.5 ml of the Otto I buffer (water solution of 0.1 mol citric acid monohydrate and 0.5% Tween 20). The crude suspension was filtered through nylon mesh (42-µm pore size) and incubated for 15 min at room temperature. After incubation, 1 ml of staining solution containing Otto II buffer (0.4 mol $Na_2HPO_4 \cdot 12\ H_2O$) and propidium iodide (final concentration 50 $\mu g \cdot ml^{-1}$), RNase IIA (50 $\mu g \cdot ml^{-1}$) and β-mercaptoethanol (2 $\mu l \cdot ml^{-1}$) was added. A Partec CyFlow SL (Partec GmbH, Münster, Germany) flow cytometer, equipped with a green diode-pumped solid-state laser (100mW, 532-nm, Cobolt Samba, Cobolt, Sweden) as the excitation light source, was used for recording the fluorescence intensity of isolated nuclei. Final histograms were evaluated using FloMax software (version 2.4d, Partec GmbH, Münster, Germany) and only analyses with coefficients of variance (CV) of the sample G1 peak below 3% were considered. DNA contents of samples were calculated based on means of peaks (Doležel et al. 2003) using the following formula: Sample 2C DNA content (pg) = (sample G_1 peak mean / standard G_1 peak mean) × standard 2C DNA amount (pg). A one-way ANOVA procedure followed by a Tukey's HSD multiple comparison test (function 'glht' as implemented in the R package multcomp; Hothorn et al. 2008) were used to determine statistical differences in genome size between seven genetic groups defined using AFLP and visualized using box plots.

Morphometric analyses

Because of the high phenotypic plasticity of the vegetative parts of the plants, we decided to analyse characters only on generative parts (namely fruit) in our morphometric analyses because they appear to be more stable (Cook & Nicholls 1986, Haasová 1997, Kaplan 2002b). The fruit are known to change in size and colour during maturation, so only ripe and fully developed fruit were collected. Altogether, 14 morphological characters were used in the primary analysis (Table 1, Fig. 1) out of which four were ratios describing the shape of the fruit typical of each subspecies. Five fruit from each individual (genotype) were measured as replicates and the mean for each character was used in subsequent analyses. Means and ratios of characters are commonly used for analysing morphological measurements (Humphries et al. 1981, Brochmann 1992, Fici 2001, Morozowska et al. 2011). For every character basic descriptive statistics (mean, SD, min. and max.) were calculated and boxplots were created in R. However, the characters 'sterility of fruiting heads' and 'shoulder between upper and lower part' were invariable within groups and could not be used in the discriminant analyses (Marhold 2011). The remaining 12 characters were tested for normality by the Shapiro-Wilk statistic (characters that were not normally distributed were log-transformed; Table 1) and a non-parametric Spearman correlation coefficient was computed to determine the correlations of morphological characters (both using R version 3.4.0, R Foundation for Statistical Computing, Vienna, Austria).

To gain a preliminary insight into the overall variation in morphology, principal component analysis (PCA) was performed using the MorphoTools suite of R scripts

Table 1. – List of morphological characters used in morphometric analyses of the fruits of four subspecies of *Sparganium erectum* (see Fig. 1 for graphical explanation). Invariable characters that were excluded from the multivariate analyses are marked with asterisk.

	Transformation
Infructescence character	
*Sterility of fruit heads	
Fruit characters	
Peduncle	Untransformed
Length of fruit (without style) (mm)	log10 (x)
Width of fruit (mm)	Untransformed
Length of the lower part of fruit (mm)	Untransformed
Length of the upper part of fruit (mm)	log10 (x)
Length of style (mm)	Untransformed
*Shoulder – shoulder between the upper and lower part of fruit	
Number of angles	Untransformed
Constriction in the middle of fruit: 0 - absent, 1 – present	Untransformed
Length of fruit/ width of fruit	Untransformed
Length of the upper part of fruit/ length of the lower of fruit	Untransformed
Length of style/ length of fruit	log10 (x)
Length of style/ width of fruit	Untransformed

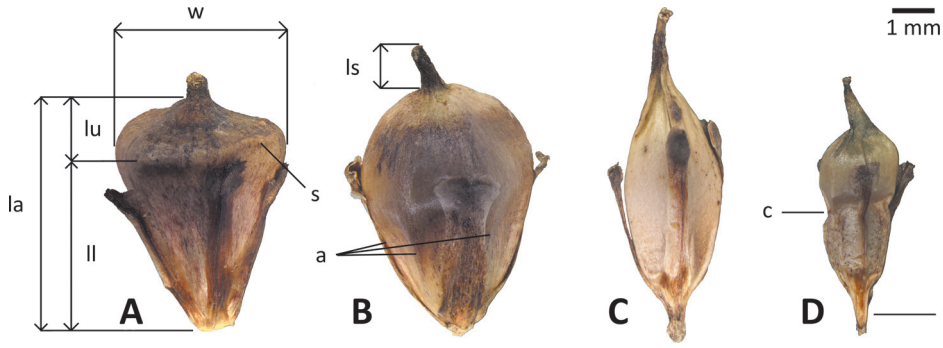

Fig. 1. – Morphological characters of fruit: (A) *Sparganium erectum* subsp. *erectum*; (B) *S. e.* subsp. *oocarpum*; (C) *S. e.* subsp. *neglectum*; (D) *S. e.* subsp. *microcarpum*. Scale bars: 1 mm. a – number of angles; c – constriction; la – length of fruit; ll – length of the lower part of fruit; lu – length of upper part of fruit; ls – length of style; p – peduncle; s – shoulder between upper and lower parts; w – width of fruit. Photo S. Píšová.

(Koutecký 2015). The AFLP groups were visualized using colours according to the STRUCTURE analysis and highly admixed individuals were presented by grey symbols. Subsequently, a canonical discriminant analysis (CDA) with groups predefined by AFLP (without admixed individuals) was used to determine variation among these groups and find the most important characters for their differentiation. Highly admixed individuals were passively projected onto the ordination diagram afterwards. Another CDA analysis was employed to differentiate between two morphologically overlapping groups, subsp. *microcarpum* and subsp. *neglectum*. Finally, a classificatory discriminant analysis was used to verify the accuracy of the classification of the individuals into the predefined groups. Determination of highly admixed individuals was performed using predefined groups as a training dataset.

Results

AFLPs

Altogether, 103 AFLP loci were unambiguous, of which 91 (88.4%) were polymorphic. For 22 replicated samples, the error rate was 2.0% and the average number of loci per individual was 66.0.

Maximum genetic diversity was recorded in population D (He = 0.18, %poly = 27.2%) and La (He = 0.17, %poly = 17.5%) whereas minimum genetic diversity was recorded in populations Ba, L, Rz and V (He = 0.03; %poly = 2.9%, 4.9%, 5.8% and 6.8%, respectively), see Appendix 1. Genotyping of populations revealed that the initial data set of 276 individuals included 34 clones, thus the rest, 242 genotypes, were used in subsequent analyses.

The STRUCTURE analysis generated consistent results only for K = 2 and K = 4. For greater K values and K = 3 the results did not converge towards the same outcome (Electronic Appendix 2AB, Electronic Appendix 3). The solution with K = 2, separated two groups: The first group morphologically corresponds to *S. erectum* subsp. *erectum* and subsp. *microcarpum* (120 samples) and the second group to subsp. *oocarpum* and subsp. *neglectum* (66 samples). K = 3 resulted in two different outcomes for the classification of subsp. *oocarpum* (53 samples). These individuals were either not distinguished from subsp. *neglectum* (Fig. 2A, K = 3b) or apportioned approximately 50:50 to subsp. *erectum* and subsp. *neglectum* (Fig. 2A, K = 3a). The result with the greatest ΔK and a stable solution using K = 4 discriminated four genetic groups, which correspond to the four subspecies distinguished in the literature: *S. e.* subsp. *erectum* (38 samples), subsp. *oocarpum* (53 samples), subsp. *microcarpum* (82 samples) and subsp. *neglectum* (13 samples). To define individual groups and deal with admixtures, we used the ad hoc setting (up to 0.15 assignment probability of clearly placed samples in one of the four genetic groups). Moreover, part of the samples was found to have a higher admixture (i.e. with at least 0.15 assignment probability to more than one group), indicating hybridization between these genetic groups (Fig. 2A: subsp. *erectum* × subsp. *oocarpum*, 3 samples; subsp. *erectum* × subsp. *microcarpum*, 34 samples; and subsp. *microcarpum* × subsp. *neglectum*, 19 samples).

Principal coordinate analysis (PCoA) confirmed the results of the STRUCTURE analysis. The four subspecies of *S. erectum* were clearly differentiated in ordination space with admixed individuals in an intermediate position between their parental taxa (the first and second PCoA axes explained 25.5% and 14.8% of the variability, respectively; Fig. 2B). Comparable results were obtained using the neighbour-net network analysis (Fig. 2C) and neighbour-joining tree (Electronic Appendix 4), both of which separated the subspecies and the hybrid individuals between them.

All but one sample of subsp. *erectum*, subsp. *neglectum* and subsp. *oocarpum* included in the analysis by NewHybrids were classified with very high probability to be either one of the parental subspecies or F1 hybrids. All samples classified as *S. e.* subsp. *oocarpum* were estimated to be F1 hybrids between subsp. *erectum* and subsp. *neglectum*. The only exception was sample Hp33, which with a 71% probability was assigned to subsp. *neglectum* and 29% probability to a back-cross between a F1 hybrid and subsp. *neglectum* (Electronic Appendix 5).

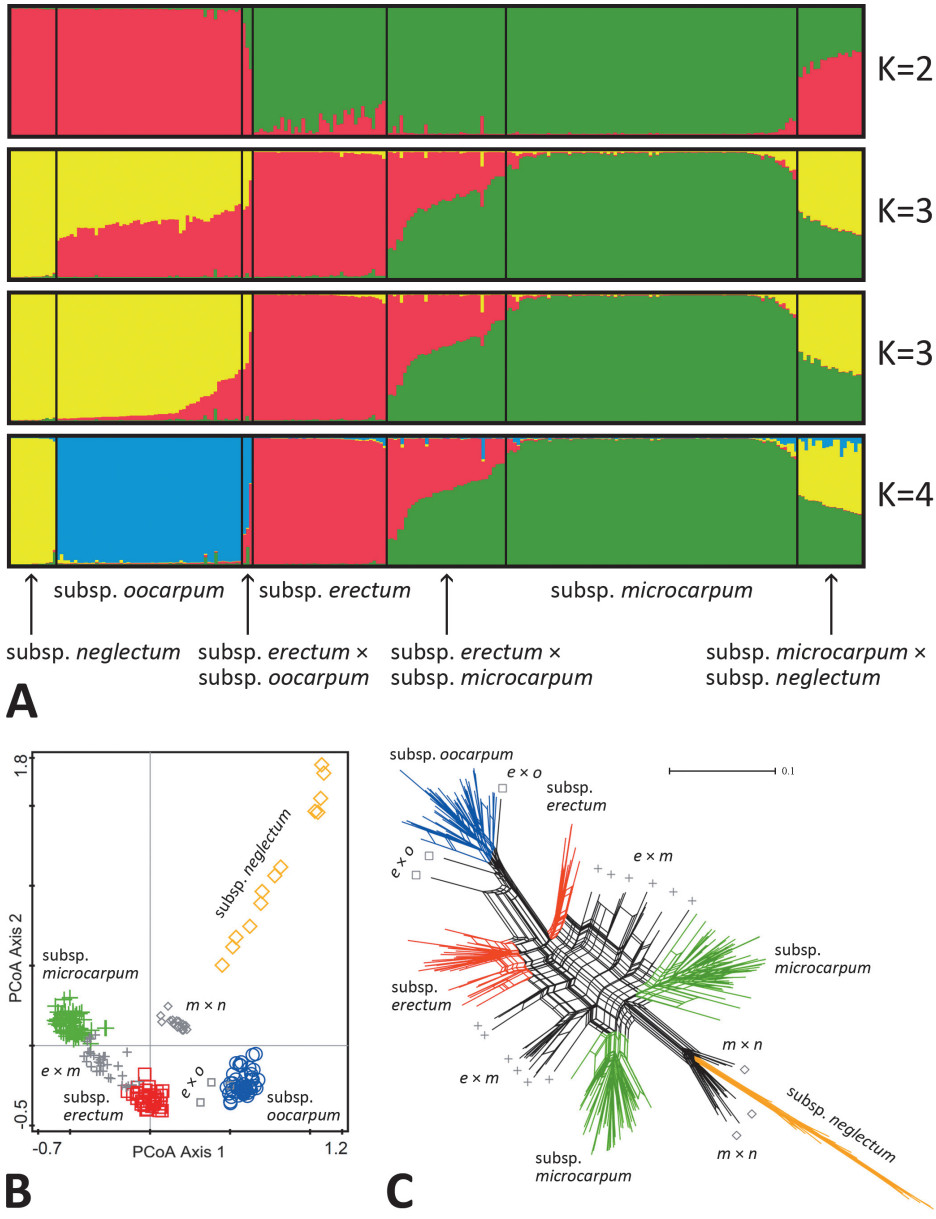

Fig. 2. – Results of molecular analyses of 242 individuals of *Sparganium erectum* based on 103 AFLP loci: (A) Bar plot showing Bayesian assignment probabilities using software STRUCTURE for two, three and four clusters (K = 2–4). The genetic groups (containing individuals with < 15% admixture with another group) associated with subspecies were recorded plus intermediate individuals between them. (B) Principal coordinate analysis (PCoA) using Jaccard's similarity coefficient. The first and second axis explained 25.5% and 14.8% of the variation, respectively. (C) Neighbour-Network. Colours indicate AFLP groups detected by STRUCTURE.

Table 2. – Analysis of molecular variance (AMOVA) of the total dataset of 187 individuals of *Sparganium erectum* (four subspecies) and separate AMOVA analysis for each subspecies. d.f. – degrees of freedom; *** $P < 0.001$.

Grouping	Source of variation	d.f.	Sum of squares	Variance components	% of total variance
4 subspecies	among subspecies	3	1557.19	11.94	64.1***
	among populations / within groups	51	705.07	3.03	16.3***
	within populations	132	483.18	3.66	19.7***
subsp. *erectum*	among populations	10	142.72	2.99	42.2***
	within populations	27	110.75	4.10	57.8***
subsp. *microcarpum*	among populations	24	346.26	3.23	45.3***
	within populations	58	225.77	3.89	54.7***
subsp. *neglectum*	among populations	3	50.55	4.41	59.0***
	within populations	9	27.60	3.07	41.0***
subsp. *oocarpum*	among populations	14	165.54	2.47	44.1***
	within populations	38	119.07	3.13	55.9***

Analysis of the molecular variation (AMOVA) of the four genetic groups corresponding to the subspecies revealed that most of the variation (64.1%) was attributed to differences between subspecies. Only 16.3% of the variation occurred within subspecies and the remaining 19.7% was distributed among individuals within populations. The second AMOVA analysis was for each subspecies separately. The greatest difference in the variation among populations (59.0%) and within populations (41.0%) was recorded in subsp. *neglectum* and the smallest difference in subsp. *erectum* (42.2% and 57.8% of the variation; Table 2).

Intraspecific variation in genome size

Flow cytometry analyses resulted in high-resolution histograms with mean CVs of G_1 peaks of *S. erectum* samples and the internal reference standards of 2.54% (range 1.37–3.24) and 1.99% (range 0.86–3.21), respectively. Intraspecific variation in genome size differed significantly between the subspecies ($P < 0.001$) and ranged from 2C = 0.98 pg to 2C = 1.20 pg (Table 3, Appendix 1). The first AFLP group, corresponding to subsp. *erectum*, had the largest genome size of 2C = 1.16±0.02 pg. The second group (subsp. *oocarpum*) had an intermediate genome size between that of the putative parental subspecies *erectum* and *neglectum* (2C = 1.08±0.02 pg; Fig. 3). The groups of subsp. *microcarpum* and subsp. *neglectum* had similar 2C genome sizes, albeit still statistically distinguishable; that of subsp. *microcarpum* was 1.02±0.02 pg and that of subsp. *neglectum* was 0.99±0.01 pg (Fig. 4A). The genome sizes of admixed individuals were either similar to the genome size of non-admixed individuals or intermediate between parental subspecies. Admixed individuals from populations Ce and R (subsp. *erectum* × subsp. *oocarpum*), however, were not assigned by the STRUCTURE analysis to subsp. *oocarpum* group yet had the same genome size of 2C = 1.08 pg. Similarly, populations Km and Vb (putative subsp. *erectum* × subsp. *microcarpum*) had the same genome size as subsp. *erectum*. On the other hand, the genome sizes of other populations were intermediate between their

Table 3. – The absolute genome sizes of *Sparganium erectum* subspecies and admixed groups measured using flow cytometry (N = 223 individuals). N – number of individuals; mean (pg) – mean value of genome size in picograms for given subspecies; S.D. – standard deviation; min. – minimum value of genome size; max. – maximum value of genome size; grouping – different letters indicate statistical differences between seven AFLP groups based on a Tukey's HSD multiple comparison test ($P < 0.001$).

Subsp.	N	Mean (pg)	S.D.	Min.	Max.	Grouping
erectum	37	1.16	0.020	1.12	1.20	A
microcarpum	71	1.02	0.018	0.99	1.07	D
neglectum	13	0.99	0.011	0.98	1.01	E
oocarpum	52	1.08	0.016	1.05	1.12	B
erectum × *oocarpum*	3	1.08	0.004	1.08	1.09	BC
erectum × *microcarpum*	29	1.10	0.031	1.04	1.15	C
microcarpum × *neglectum*	18	1.01	0.019	0.99	1.05	DE

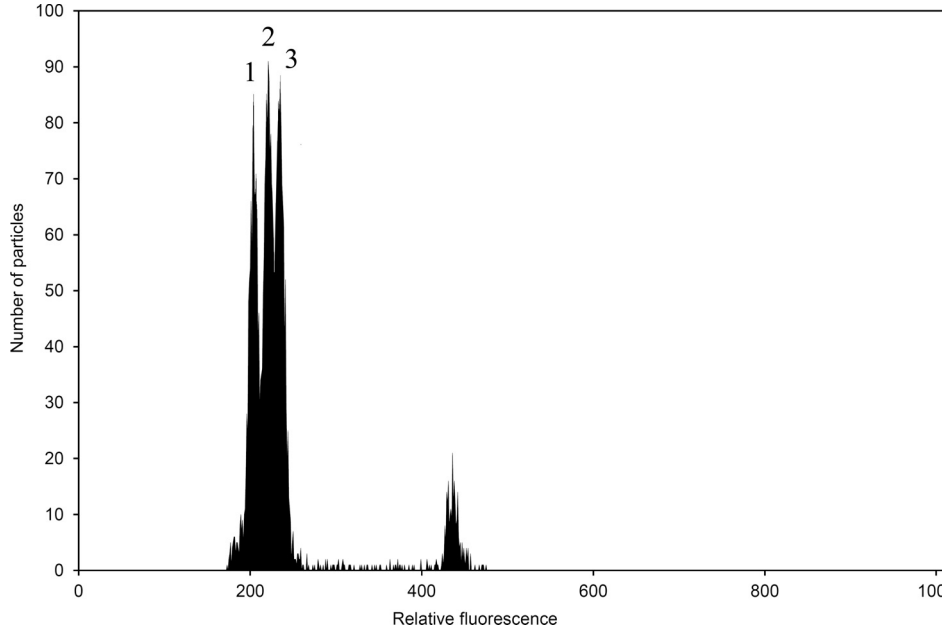

Fig. 3. – An example of a flow cytometry histogram of propidiumiodide-stained nuclei from simultaneously measured leaves of subspecies of *Sparganium erectum*. 1 – subsp. *neglectum*; 2 – subsp. *oocarpum*; 3 – subsp. *erectum*. Note the apparent intermediate genome size (2C nuclear DNA content) of subsp. *oocarpum*, which is a hybrid between subsp. *neglectum* and subsp. *erectum*.

parental taxa, such as 2C = 1.08 pg (population Za) and 2C = 1.10 pg (population Vp and Vy). Moreover, individuals from populations Dk, Ds, Ne, Ps and Tr (subsp. *microcarpum* × subsp. *neglectum*) had similar genome sizes as other individuals of subsp. *microcarpum* and individuals from population I had a genome size similar to subsp. *neglectum*.

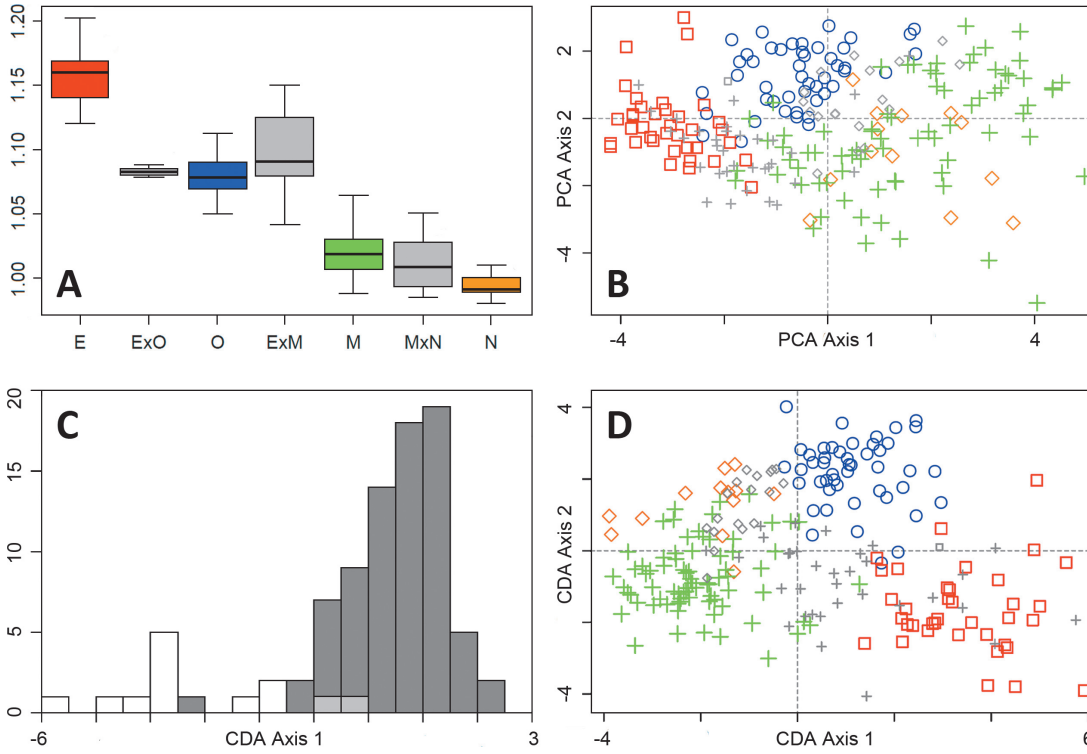

Fig. 4. – (A) Box plot showing the intraspecific distribution of genome size in *Sparganium erectum* (N = 223). The admixed groups are in grey boxes. E: subsp. *erectum*, E×O: subsp. *erectum* × subsp. *oocarpum*, O: subsp. *oocarpum*, E×M: subsp. *erectum* × subsp. *microcarpum*, M: subsp. *microcarpum*, M×N: subsp. *microcarpum* × subsp. *neglectum*, N: subsp. *neglectum*. (B) Principal component analysis (PCA) based on 12 morphological characters of the fruit of 227 individuals of *Sparganium erectum*. The first and second axis explained 42.0% and 20.6% of the variation, respectively. (C) Canonical discriminant analysis (CDA) of 77 individuals of subsp. *microcarpum* (dark grey columns) and 13 individuals of subsp. *neglectum* (white columns) with the overlap between both (light grey columns). (D) Canonical discriminant analysis (CDA) of 227 individuals of *S. erectum* based on 12 morphological characters of fruit. The first and second components explained 43.1% and 35.9% of the variation, respectively. Colours indicate AFLP groups detected by STRUCTURE and passively projected admixed individuals: □ subsp. *erectum*; ○ subsp. *oocarpum*; + subsp. *microcarpum*; ◇ subsp. *neglectum*; ▫ subsp. *erectum* × subsp. *oocarpum*; + subsp. *erectum* × subsp. *microcarpum*; ◇ subsp. *microcarpum* × subsp. *neglectum*.

Morphometric analyses

Most of the characters measured were not normally distributed, so a non-parametric correlation coefficient (Spearman's) was used and character values were transformed prior to subsequent analyses (Table 1). The correlation coefficients did not exceed 0.9 for any of the character pairs (Electronic Appendix 6). The strongest correlations (0.85–0.90) were recorded between two pairs of characters: length of the upper part of fruit/length of the lower part of fruit and length of the lower part of fruit; length of fruit and lower part of fruit. The largest fruit were those of the subsp. *erectum* group in which the fruit were on

average 7.3 mm long and 5.4 mm wide, with a much longer lower part and shorter upper part than in other subspecies. In contrast, the fruit of subsp. *microcarpum* (6.0 mm long, 3.2 mm wide) and subsp. *oocarpum* (5.8 mm long, but about 5.3 mm wide) were the smallest. A long style of more than 2 mm was recorded in the subsp. *neglectum* group and a constriction in the middle of fruit only in subsp. *microcarpum* group (Electronic Appendix 7, 8). Admixed individuals had intermediate characters between their parental taxa or were similar to one of them. Sterile fruit heads were recorded in subsp. *oocarpum*, which is of hybrid origin, and in the hybrid groups (subsp. *erectum* × subsp. *oocarpum*, subsp. *erectum* × subsp. *microcarpum*, and subsp. *microcarpum* × subsp. *neglectum*). A shoulder between the upper and lower part of a fruit was present only in subsp. *erectum* and its hybrids with subsp. *microcarpum*. These two characters were invariable and not included in further multivariate analyses.

Principal component analysis (PCA) based on mean values of fruit characters (227 individuals × 12 characters) revealed that the four groups of individuals defined by AFLP (representing separate subspecies, Fig. 4B) are closely related and not well separated. Individuals of *S. erectum* subsp. *erectum* and subsp. *oocarpum* were slightly separated along the first axis, which accounted for 42.0% of the variation (based on the length of the lower part of fruit, width of fruit and length of style/width of fruit), whereas individuals of *S. erectum* subsp. *microcarpum* and subsp. *neglectum* were mixed (PCA eigenvalues are presented in Table 4). In addition, passively projected admixed individuals were situated either in intermediate positions between their parental subspecies or within them.

The CDA analysis of the four groups predefined by the AFLP analysis resulted in a better separation of the subspecies. Individuals of *S. erectum* subsp. *erectum* were nearly completely separated from subsp. *microcarpum* and individuals of subsp. *oocarpum* from subsp. *neglectum* based on a combination of the first and second canonical axis (Fig. 4D). However, subsp. *microcarpum* and subsp. *neglectum* partially overlapped. Similar to the results of PCA, individuals of subsp. *oocarpum*, which is of hybrid origin, were placed between its parental subspecies (Fig. 4D). Admixed individuals of populations Za and Vp were, in accordance with the AFLP results (approximately a 50:50 admixture of both subsp. *erectum* and subsp. *microcarpum* groups), situated in intermediate positions, whereas populations Km, Vb, and Vy (with lower admixture of subsp. *microcarpum*, 20–40%) were placed within subsp. *erectum*. Populations I, Ps, Tr (subsp. *microcarpum* × subsp. *neglectum*, 50:50 admixture in AFLP) occurred in intermediate positions and populations Ds, Ne within subsp. *neglectum* and individuals from population Dk were variously distributed. The admixed individuals from population R (subsp. *erectum* × subsp. *oocarpum*, 76:24) occupied an intermediate position between subsp. *erectum* and subsp. *oocarpum*. The characters most highly correlated with the canonical axes were: width of fruit, length of style/ width of fruit and length of fruit/ width of fruit. The first three components explained 43.1%, 35.9% and 21.0% of the variance. However, the third axis did not provide a better differentiation.

Another CDA was performed in order to obtain a better insight into the similarity between the two predefined groups (subsp. *microcarpum* and subsp. *neglectum*, Fig. 4C). A canonical scatter-plot showed a partial overlap between the subspecies. The most correlated characters with the canonical axis were length of upper part of fruit, length of upper part of fruit/length of lower part of fruit and constriction in middle of fruit (Table 4).

Table 4. – Results of morphometric analyses based on 12 characters of the fruit of *Sparganium erectum*. The three highest PCA eigenvectors for the PCA analysis of 227 individuals (axes: PCA1 and PCA2), the three highest total canonical structure values for the discriminant analyses of 227 individuals (axes: CDA1 and CDA2), and 90 individuals of subsp. *microcarpum* and subsp. *neglectum* (axis: CDA M-N) are presented in bold.

Character	PCA1 (Fig. 4B)	PCA2 (Fig. 4B)	CDA1 (Fig. 4D)	CDA2 (Fig. 4D)	CDA M-N (Fig. 4C)
Peduncle	0.736	0.064	–0.358	–0.187	0.061
Length of fruit	–0.656	–0.576	0.166	–0.197	–0.149
Length of the lower part of fruit	**–0.781**	–0.519	0.329	–0.456	0.082
Length of the upper of fruit	0.467	–0.012	–0.250	0.352	**–0.454**
Length of style	0.407	–0.529	–0.191	0.149	–0.299
Width of fruit	**–0.778**	0.377	**0.702**	0.372	–0.237
Number of angles	0.327	0.617	–0.092	0.000	0.218
Constriction in the middle of fruit	0.678	0.090	–0.341	–0.283	**0.374**
Length of fruit / width of fruit	0.465	–0.759	–0.456	**–0.459**	0.084
Length of the upper part of fruit / length of the lower part of fruit	0.700	0.367	–0.258	0.438	**–0.367**
Length of style / length of fruit	0.771	–0.046	–0.314	0.313	–0.148
Length of style / width of fruit	**0.771**	–0.581	**–0.471**	–0.136	–0.043

The classificatory DA correctly assigned 87.9% of the individuals to the predefined groups and the remaining 12.1% were misclassified to other groups (Electronic Appendix 9). The lowest percentage of individuals correctly assigned was to subsp. *neglectum* (69.3%). These individuals were mostly misclassified to subsp. *microcarpum* and partly to subsp. *oocarpum*. To determine the admixed individuals, an additional analysis of the four predefined AFLP groups was used as a training set. The individuals from population R (subsp. *erectum* × subsp. *oocarpum*) were assigned either to the subsp. *erectum* or subsp. *oocarpum* group. Hybrids of subsp. *erectum* × subsp. *microcarpum* from populations Km and Vb were assigned to subsp. *erectum,* whereas individuals from population Vp were assigned to subsp. *erectum* or to subsp. *microcarpum*. Moreover, individuals from population Vy and Za were classified as subsp. *erectum* or subsp. *oocarpum* group. Finally, populations Ds, Dk, I, Ne, Ps and Tr (subsp. *microcarpum* × subsp. *neglectum*) were assigned either to subsp. *microcarpum*, subsp. *neglectum* or subsp. *oocarpum* (see Appendix 1, CDA).

Discussion

High levels of phenotypic plasticity are common in aquatic and wetland plants, and have resulted in numerous conflicting taxonomical concepts in various groups of plants (Kaplan 2002a, Santamaria 2002). Morphological variation has also complicated the taxonomy of the genus *Sparganium*. In previous studies the inclusion of vegetative parts when describing new taxa led to the description of different numbers of *Sparganium* taxa, especially at ranks below the species level (Čelakovský 1896, Čelakovský 1899, Belavskaya 1984). The classification described in Cook & Nicholls' monograph (1986, 1987) is based on more reliable generative characters. Morphological characters on fruit

seem to be stable and can be used to differentiate separate subspecies (Cook 1961). In the present study we assessed the correlation between morphological and genetic variation in *S. erectum* using a combination of molecular, morphometric and flow-cytometric data. Our results provide the first molecular information for differentiating intraspecific lineages and hybridization in this species. Our AFLP data differentiated four groups with distinct genome sizes largely corresponding to the morphological subspecies and thus confirming the reasonable intraspecific classification of *S. erectum*. Moreover, all methods (AFLP, flow cytometry and morphometric analyses) were in agreement in indicating that subsp. *oocarpum* is a hybrid between subsp. *erectum* and subsp. *neglectum* and is intermediate in terms of its genome size and morphological characters. In addition, admixed individuals were also detected among these groups, probably indicating recent hybridization. These individuals varied in their characters, ranging from those of one of their parents to the other. Only AFLP markers were able to identify parental taxa of the recent hybrids. Their genome size was often misleading and morphometric analyses were possibly affected by the altered shape and size of the fruit of the hybrids due to the partial sterility of their fruiting heads. Nevertheless, the frequency of hybridization between subspecies remains to be assessed as hybridization was not the main focus of this study.

Genome size and genetic variation

Many recent studies have used genetic analyses and estimates of genome size (Chrtek et al. 2009, Dušková et al. 2010, Chumová et al. 2015, 2017). Nevertheless, so far, only a few studies have dealt with the phylogeny of the genus *Sparganium* (Sulman et al. 2013, Ito et al. 2016) or the genetic variation in *S. erectum* (Piquot et al. 1996, Ishii et al. 2004). As intraspecific variation in *S. erectum* was not previously investigated, we focused in this study on the evaluation of genetic variation and hybridization and the detection of potential differences in genome size. The STRUCTURE analysis based on AFLP data identified four separate groups and a couple of admixed individuals among them. The first group included only individuals of subsp. *erectum* with a low admixture from the other subspecies (up to 15%, as in the study on species of *Bolboschoenus*, Píšová et al. 2017). Populations of this subspecies had the highest intrapopulation genetic variation (57.8%) and the lowest interpopulation variability (42.2%). In accordance with the STRUCTURE analysis, all such individuals were well differentiated by their genome size (2C = 1.12–1.20 pg) from all the other groups. The second group, corresponding to subsp. *oocarpum* is of hybrid origin and has an approximate 50:50 admixture from its parental taxa (subsp. *erectum* and subsp. *microcarpum*) in the solution with K = 3 in the STRUCTURE analysis. The genome size of these individuals was also intermediate (2C = 1.05–1.12 pg). Moreover, the solution with K = 4 indicated it as a separate, independent taxon (see below). The group with the majority of individuals was subsp. *microcarpum*, whose genome size ranged from 2C = 0.99 to 1.07 pg. Hybridization of this subspecies with others seems to be frequent and we found two hybrid groups of individuals with varying degrees of admixture (see below). The last group, subsp. *neglectum*, had the highest interpopulation genetic variation (59.0%) while its intrapopulation variability was low (41.0%). Its genome size ranged from 2C = 0.98 to 1.01 pg and even though it partially overlapped that of subsp. *microcarpum* the difference was statistically significant.

Nevertheless, individuals of both subspecies cannot be distinguished solely based on their genome size.

Morphological variation

Morphological characters on fruit are viewed as suitable discriminant criteria for subspecies determination in several studies (Cook 1962, Cook & Nicholls 1987, Kaplan 2002b). We compared the genetic and morphological variation in order to correlate the morphological characters with individual AFLP groups (subspecies). The first group, corresponding to *S. erectum* subsp. *erectum*, differed from the other subspecies mainly by the shoulder between the upper and lower part of the fruit, the width and length of the fruit, which is in accordance with other studies that describe its fruit as the largest and widest with a distinct shoulder. Additional characters were the ratio between the length of the style and the length of the fruit, and the ratio between the length of the style and the width of the fruit. Morphological analyses placed individuals of subsp. *oocarpum*, which is of hybrid origin, in an intermediate position between its parental taxa subsp. *erectum* and subsp. *neglectum*. The ratio between the length and width of the fruit separated it from subsp. *erectum* and the width of its fruit separated it from subsp. *microcarpum* and subsp. *neglectum*.

The third group consisted of the most variable individuals of subsp. *microcarpum* and differed in the length of style / width of the fruit and in width of the fruit, from subsp. *erectum* and subsp. *oocarpum*. An additional discriminant analysis revealed that the most important characters distinguishing subsp. *microcarpum* and subsp. *neglectum* were the constriction and number of angles delimiting subsp. *microcarpum* and style length and length of the upper part / length of the lower part of the fruit for delimiting subsp. *neglectum*. These results are in accordance with other studies that regard style length as an important character for identifying subsp. *neglectum*, and the constriction and visible angles for identifying subsp. *microcarpum* (Cook 1962, Kaplan 2002b).

Hybrid origin of Sparganium erectum subsp. oocarpum

Speciation by polyploidization is the topic of many studies (Soltis & Soltis 2009, Soltis et al. 2014, Vít et al. 2017), whereas studies dealing with homoploid speciation are rare (Lai et al. 2005, Masuelli et al. 2009, Feliner et al. 2017). Homoploid hybridization more often results in the formation of hybrid zones by introgression than in the establishment of a new hybrid taxon that needs to be promoted by ecological selection or geographic isolation (Buerkle et al. 2000, Abbott & Rieseberg 2012, Abbott et al. 2013, Yakimowski & Rieseberg 2014). Ecology of all homoploid hybrids appears to differ from that of their parental taxa (Gross & Rieseberg 2005). The parentage of putative hybrid *S. erectum* subsp. *oocarpum* was proposed by Cook (1961, 1962) on the basis of fruit morphology. This contradicts Čelakovský (1896b), who was convinced that subsp. *oocarpum* was only a variety of subsp. *neglectum*. However, the ecology of this subspecies of *S. erectum* is not well known and comparative studies (Cook 1962, Cook & Nicholls 1987) report no ecological differences between the subspecies. The distribution of the four subspecies of *S. erectum* in the Czech Republic was investigated and summarized in detail by Kaplan et al. (2015). Subsp. *erectum* and the hybrid subsp. *oocarpum* have quite similar distributions and ecology, in contrast to its second parent, subsp. *neglectum*. Both parental taxa

and their hybrid have similar distributions in the British Isles and tend to occur in the south whereas subsp. *microcarpum* occurs throughout the British Isles (Cook 1961). The intermediate position of subsp. *oocarpum* in terms of genome size (2C= 1.08 pg), genetics and morphology reported in this paper affirms its origin as a hybrid of subsp. *erectum* and subsp. *neglectum*. The NewHybrids analysis indicates that nearly all individuals of subsp. *oocarpum* are F1 hybrids. If this is true, subsp. *oocarpum* should only co-occur in mixed populations with their parents or should only be dispersed clonally, which is not the case in the Czech Republic where subsp. *oocarpum* often occurs in the absence of its parental taxa. Our sampling focused on a re-evaluation of the differentiation of the four previously distinguished subspecies. Consequently, our analyses did not determine whether subsp. *oocarpum* is a F1 hybrid or an advanced and stabilized hybridogenous subspecies. Only if sampling is focused on putative hybrids (like in the microsatellite study of *Typha* ×*glauca*; Snow et al. 2010) will the analysis distinguish between these two sorts of hybrids more safely and determine the rate of backcrossing with parental taxa.

Recent hybridization between subspecies

In addition to the previously suggested and confirmed hybrid origin of subsp. *oocarpum* another three hybrid combinations were identified. The first is *S. erectum* subsp. *erectum* × subsp. *microcarpum* (Fig. 2A), formerly reported as *S. microcarpum* × *S. ramosum* (Ostenfel-Hansen 1897), which usually has very sterile heads, as previously mentioned by Cook & Nicholls (1987). Individuals from populations Km, Vb, Vp, Vy and Za were admixed with different ratios of genetic contributions from their parents. Their fruit resembled that of subsp. *erectum*, except for population Vp, for which the fruit is morphologically intermediate between that of subsp. *erectum* and subsp. *microcarpum*. The genome size of these admixed individuals corresponds with the ratio of genetic makeup inherited from their parents. Individuals with a high percentage of their genetic makeup from subsp. *erectum* (64–77%, populations Km and Vb) had the same genome size as subsp. *erectum* (2C = 1.14 pg) whereas among genetically intermediate individuals (40–57% percentage of genetic information from subsp. *erectum*) genome size varied from intermediate (2C = 1.09 pg, population Za) to similar to subsp. *erectum* (populations Vp and Vy).

The second is subsp. *microcarpum* and subsp. *neglectum* (Fig. 2A). However, such individuals are difficult to determine. Their fruit resembles that of subsp. *microcarpum*, but are more elongate, like those of subsp. *neglectum*, and large, like those of subsp. *oocarpum*. In accordance with the observations of Cook & Nicholls (1987) these plants had partly sterile fruit heads. Individuals of this hybrid were genetically intermediate between subsp. *microcarpum* and subsp. *neglectum*. Populations Dk, Ds, Ne and Tr morphologically resemble subsp. *oocarpum*, though their genome size is similar to that of subsp. *microcarpum* (2C = 1.02 pg). In contrast, the fruit of population Ps is more similar to that of subsp. *microcarpum*. Plants from population I are morphologically intermediate between subsp. *oocarpum* and subsp. *neglectum* and have the same genome size as subsp. *neglectum* (2C = 0.99 pg). This hybrid may be what Neuman (1897) describes as *S. ramosum* f. *substerile* and Graebner (1900) reclassified as *S. ramosum* subsp. *polyedrum* var. *substerile*.

The last case of recent hybridization is that between subsp. *erectum* and subsp. *oocarpum* recorded in three admixed individuals from populations Ce and R. The only plant we were able to analyse morphometrically (population R) resembled subsp. *oocarpum* in having partially sterile fruiting heads, short and wide fruits and a similar genome size (2C = 1.08 pg). We consider the incidence of backcrosses between subsp. *oocarpum* and its parental taxa to be rarer than that recorded for the previous two hybrids, but more detailed examinations and genotyping of admixed populations is needed to determine its frequency.

Different ratios of genetic contributions from parental subspecies were recorded for the three hybrid combinations, which indicates recent hybridization. The genome sizes of individuals was either similar to that of their parental subspecies or they were similar in morphology. In contrast, we consider subsp. *oocarpum* to be a hybrid with its own characteristics (genome size, morphology and an intermediate genetic pattern). As the reproductive isolation barriers between the subspecies of *S. erectum* are apparently still not fully established, we consider these taxa to be subspecies rather than separate species. Moreover, these taxa cannot be determined in the field without ripe fruit and thus treating them as subspecies is reasonable also for practical reasons.

Several studies investigate speciation by homoploid hybridization and demonstrate different degrees of the 'intermediacy' in the hybrids. For example, natural co-occurrence of diploid *Helianthus annuus* and *H. petiolaris* has led to the formation of the reproductively isolated hybrid species *H. anomalus*, *H. deserticola* and *H. paradoxus*, whose genome sizes and habitat preferences differ from each other and from those of their parental taxa (Lexer et al. 2003, Rieseberg et al. 2003, Baack et al. 2005). A distinct ecology and distribution also facilitated the establishment of the hybrid species *Bolboschoenus laticarpus* (Hroudová et al. 2007, Píšová et al. 2017). On the other hand, a new hybrid species may be formed without strong reproductive isolation, as documented, for example for *Senecio squalidus*, which is ecogeographically isolated from its parental species after its human-mediated introduction to the British Isles from its hybrid zone on Mt Etna (James & Abbott 2005, Abbott et al. 2010). In *Carex* sect. *Vesicariae*, the origin of two hybrid taxa is reported by Pedersen et al. (2016): *Carex rostrata* var. *borealis* (*Carex rostrata* × *C. rotundata*) and *C. stenolepis* (*C. vesicaria* × *C. saxatilis*). Both hybrids have an intermediate AFLP pattern, but are closer to one of the parental species. Recently, a number of studies dealing with homoploid speciation has increased (Feliner et al. 2017, Dirmenci et al. 2018, Michálková et al. 2018) showing that this evolutionary process is more common than previously assumed.

Conclusions

In the present study, a combination of AFLPs, flow cytometry and morphometric analyses enabled us to investigate the genetic and morphological variation of *Sparganium erectum*. The results confirm the Cook & Nicholls' intraspecific classification. Four AFLP groups were identified, representing the four European subspecies, and the three hybrid groups between them. Genome size values corresponded to the delimitation of these AFLP groups. Morphological analyses identified well separated groups with partial overlaps. Both genetic and morphometric analyses confirmed that subsp. *oocarpum* is of

hybrid origin and placed it in an intermediate position between its parental taxa subsp. *erectum* and subsp. *neglectum.* Recent hybridization was detected, especially between subsp. *microcarpum* and either *erectum* or *neglectum*. Backcrosses between subsp. *oocarpum* and its parental taxa seem to be rare, however, a more detailed study is needed to improve our knowledge on hybridization dynamics in *S. erectum*. Important distinguishing characters were fruit width and length (for differentiating subsp. *erectum*), ratio between the lengths of the upper and lower part of the fruit together with fruit width (for subsp. *oocarpum*), ratio between style length and fruit width together with the ratio of fruit length to fruit width (for subsp. *microcarpum*), and finally, length of the upper part of the fruit and style length (for subsp. *neglectum*).

See www.preslia.cz for Electronic Appendices 1–9.

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Souhrn

Značná fenotypová plasticita, typická pro vodní a mokřadní rostliny, znesnadňuje taxonomické zpracování rodu *Sparganium*. V minulosti bylo popsáno množství variet a forem zvláště u druhu *Sparganium erectum*. Použití plastických znaků na listech se neosvědčilo, a proto je současné členění rodu založeno hlavně na tvaru a barvě nažek, což jsou znaky stabilní a specifické pro příslušné poddruhy. Celkem je rozlišováno pět poddruhů *Sparganium erectum*: subsp. *erectum*, *microcarpum*, *neglectum*, *stoloniferum* a *oocarpum*, přičemž poslední je patrně hybridogenního původu. Kromě subsp. *stoloniferum* se zbývající čtyři vyskytují také v Evropě a v České republice. Mezi poddruhy byly zaznamenány i přechodné populace, naznačující možnou hybridizaci. Pro ověření oprávněnosti současného vnitrodruhového členění tohoto druhu a zjištění míry hybridizace jsme nasbírali materiál z 64 lokalit v České republice pro morfometrické a molekulární analýzy. Genetická a morfologická variabilita byla studována za použití kombinace metody AFLP (Amplified Fragment Length Polymorphism) jako molekulárního markeru, průtokové cytometrie pro stanovení velikosti genomu a mnohorozměrné morfometrické analýzy. Výsledky dobře odlišily všechny čtyři poddruhy a odhalily přechodné jedince mezi nimi. Zjištěné genetické rozdíly a rozdíly ve velikosti genomu odpovídají současné taxonomické klasifikaci. Předpokládaný hybridní původ subsp. *oocarpum* byl potvrzen výsledky všech použitých metod, kde tento poddruh měl intermediární pozici mezi rodičovskými poddruhy *erectum* a *neglectum*. Zároveň byly zjištěny tři skupiny kříženců s přechodnými znaky, subsp. *erectum* × subsp. *microcarpum*, subsp. *microcarpum* × subsp. *neglectum* a subsp. *erectum* × subsp. *oocarpum*, naznačující v současnosti probíhající hybridizaci. Ke zpětnému křížení subsp. *oocarpum* dochází pravděpodobně jen vzácně, nicméně detailnější studie zaměřená na smíšené populace je nezbytná k porozumění míry vnitrodruhové hybridizace *S. erectum*.

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Appendix 1 – List of the populations of the subspecies of *Sparganium erectum* studied and their characteristics. AFLP group – determination of each population based on AFLP data (STRUCTURE 85%): N – *S. e.* subsp. *neglectum*, O – *S. e.* subsp. *oocarpum*, E×O – *S. e.* subsp. *erectum* × *S. e.* subsp. *oocarpum*, E – *S. erectum* subsp. *erectum*, E×M – *S. e.* subsp. *erectum* × *S. e.* subsp. *microcarpum*, M – *S. e.* subsp. *microcarpum*, M×N – *S. e.* subsp. *microcarpum* × *S. e.* subsp. *neglectum*. N_{ind} – number of individuals analysed using AFLPs; G – number of genotypes; He – Nei's gene diversity; Pp – percentage of AFLP markers demonstrating intra-population polymorphism; D – Simpson's diversity index. FCM: 2C – The average genome size of the populations of the subspecies of *S. erectum* (2C value, picograms, pg); subsp. – subspecies determination of each population based on its genome size. CDA – subspecies determination of each population based on classificatory discriminant analyses of (E – *S. e.* subsp. *erectum*, M – *S. e.* subsp. *microcarpum*, N – *S. e.* subsp. *neglectum*, O – *S. e.* subsp. *oocarpum*). ▶

Acronym	Locality and date of collection	AFLP group N	O	E×O	E	E×M	M	M×N	N_{ind}	G	He	Pp	D	FCM 2C (pg)	FCM subsp.	CDA subsp.
Sparganium erectum* subsp. *erectum																
D	Central Bohemia, the Cidlina river by the railway bridge 1.13 km NW of the village of Sány, 50°7.707'N, 15°13.945'E, 192 m, 29 VIII 2007				3				4	3	0.18	27.18	0.83	1.16	E	E, O
Do	Central Bohemia, the Cidlina river by the road bridge 400 m N of the village of Dobšice nad Cidlinou, 50°8.24'N, 15°16.00'E, 200 m, 29 VIII 2007				4				5	4	0.07	13.59	0.90	1.15	E	E
Ha	Southern Bohemia, the Přední Sax fishpond 1 km N of the village of Hamr, 49°9.77'N, 14°45.60'E, 410 m; 29 IX 2008				1				2	1	0.00	0.00	0.00	1.20	E	E
Ch	Central Bohemia, the Krčský fishpond 2.5 km NW of the town of Městec Králové, 50°13.23'N, 15°16.17'E, 210 m, 9 VII 2008				4				4	4	0.08	13.59	1.00	1.17	E	E, O
Km	Central Bohemia, the Labe river 3.1 km SW of the town of Nymburk, 50°10.37'N, 15°0.33'E, 180 m, 24 IX 2008					3			3	3	0.06	9.70	1.00	1.14	E	E
L	Central Bohemia, fire reservoir in the town of Libice nad Cidlinou, 50°7.73'N, 15°10.73'E, 190 m, 25 VIII 2007				2				5	2	0.03	4.85	0.60	1.15	E	E
O	Central Bohemia, the Kupecká ditch 760 m from the village of Oseček, 50°6.39'N, 15°8.533'E, 180 m, 14 IX 2007				5				5	5	0.08	15.53	1.00	1.14	E	E
Po	Central Bohemia, Prague, city district of Dolní Počernice, the Počernický fishpond, 50°5.08'N, 14°35.45'E, 230 m, 14 IX 2008				2				2	2	0.12	11.65	1.00	1.19	E	E
Ro	Central Bohemia, the Třebonický fishpond, 1.28 km from the town of Rožďalovice, 50°17.85'N, 15°11.01'E, 200 m, 31 VIII 2008				4				4	4	0.08	15.53	1.00	1.14	E	E
Sa	Central Bohemia, Prague, city district of Hrnčíře, the Hrnčířský fishpond, 50°0.29'N, 14°30.48'E, 290 m, 10 IX 2008				3				3	3	0.05	8.74	1.00	1.17	E	E, O
Vl	Southern Bohemia, the Vlkovský fishpond 800 m from the village of Vlkov nad Lužnicí, 49°8.72'N, 14°43.94'E, 390 m, 29 IX 2008				4				4	4	0.09	16.50	1.00	1.17	E	E
Vy	Southern Bohemia, the Dolní u Smítky fishpond behind the Rožmberk fishpond 3.37 km SE of the village of Lužnice, 49°3.07'N, 14°47.91'E, 430 m, 29 IX 2008					2			3	2	0.09	8.74	0.67	1.10	O	E, O
Vb	Central Bohemia, the Labe river by the road bridge 1 km from the					5			5	5	0.09	18.45	1.00	1.15	E	E
Vz	village of Velké Zboží, 50°9.951'N, 15°5.42'E, 184 m, 5 IX 2008		4						4	4	0.06	9.71	1.00	1.08	O	O

Acronym	Locality and date of collection	N	AFLP group O	E×O	E	E×M	M	M×N	N_{ind}	G	He	Pp	D	FCM 2C (pg)	FCM subsp.	CDA subsp.
Sparganium erectum subsp. *oocarpum*																
C	Central Bohemia, the Cidlina river near the road bridge at the village of Libice nad Cidlinou, 50°7.42'N, 15°10.84'E, 186 m, 25 VIII 2007		5						5	5	0.09	19.42	1.00	1.07	O	O
Ce	Central Bohemia, the Labe river by the railway bridge 1.70 km NE of the town of Čelákovice, 50°10.33'N, 14°46.16'E, 170 m, 14 IX 2007		3	2					5	5	0.11	24.27	1.00	1.09	O	-
E	Central Bohemia, the Steklá ditch by the Žehuňský fishpond 1.26 km W of the village of Žehuň, 50°8.18'N, 15°16.49'E, 200 m, 29 VIII 2007		5						5	5	0.09	9.71	1.00	1.07	O	O
G	Central Bohemia, the Pazderák fishpond in the village Dolní Břežany, 49°57.744'N, 14°27.73'E, 360 m, 4 IX 2007		3						4	3	0.06	10.68	0.83	1.07	O	O
H	Southern Moravia, the Bruksa pool near the Stará Dyje river 1.28 km W of the town of Břeclav, 48°45.68'N, 16°52.10'E, 157 m, 7 IX 2007		3						5	3	0.05	8.74	0.70	1.07	O	O
Ho	Central Bohemia, Prague, city district of Vysočany, the Hořejší fishpond, 50°5.975'N, 14°31.607'E, 200 m, 17 IX 2008		3						3	3	0.08	11.65	1.00	1.09	O	O
K1	Central Bohemia, the Staré Labe oxbow lake on the Labe river 1.74 km E of the town of Kolín. 50°1.56'N, 15°13.52'E, 195 m, 31 VIII 2008		4						4	4	0.05	9.71	1.00	1.08	O	O
K2	Central Bohemia, oxbow lake on the Labe river 2.20 km of the town of Kolín, 50°1.37'N, 15°13.885'E, 190 m, 31 VIII 2008		4						4	4	0.08	13.59	1.00	1.09	O	O
Ko	Central Bohemia, the Labe river by the railway bridge in the town of Kolín, 50°1.76'N, 15°12.75'E, 200 m; 31 VIII 2008		2						4	2	0.07	8.74	0.50	1.11	O	O
Ny	Central Bohemia, the Labe river by the Kamenný most bridge in the town of Nymburk, 50°11.02'N, 15°2.49'E, 180 m, 31 VIII 2008		4						5	4	0.08	14.56	0.90	1.09	O	E, O
R1	Central Bohemia, the Mrlina river by the railway bridge in the village of Křinec, 50°15.76'N, 15°8.47'E, 210 m, 13 IX 2007			1	6				6	6	0.10	23.30	1.00	1.16	E	E
R2			2						3	3	0.08	12.62	1.00	1.07	O	E
Rz	Central Bohemia, the Bučický fishpond 1.97 km NE of the town of Rožďalovice, 50°18.938'N, 15°11.447'E, 200m, 16 VII 2008		3						4	3	0.03	5.83	0.83	1.11	O	O
V	Central Bohemia, the Máčidlo fishpond in the Libický luh Nature Reserve in the town of Velký Osek, 50°6.05'N, 15°11.01'E, 180 m, 2 VIII 2008		3						5	3	0.03	6.80	0.80	1.07	O	O

Acronym	Locality and date of collection	AFLP group N	O	E×O	E	E×M	M	M×N	N_{ind}	G	He	Pp	D	FCM 2C (pg)	FCM subsp.	CDA subsp.
Vo	Central Bohemia, the Bačovka stream along the main road in the town of Velký Osek; 50°6.06'N, 15°11.30'E, 180 m, 2 VIII 2008		3						4	3	0.05	7.77	0.83	1.07	O	O
Sparganium erectum* subsp. *microcarpum																
Al	Central Bohemia, Prague, city district of Dolní Počernice, the Aloisov fishpond, 50°6.30'N, 14°33.95'E, 230 m,14 IX 2008						3		3	3	0.06	8.74	1.00	1.03		M, N
Ba	Central Bohemia, the Pomezní rybník 1.5 km NW of the village of Bratronice, 50°4.64'N, 13°59.96'E, 400 m, 4 X 2008						2		2	2	0.03	2.91	1.00	1.02		M
Br	Central Bohemia, the U paseky fishpond 1.67 km NW of the village of Bratronice, 50°4.54'N, 13°59.69'E, 400 m, 4 X 2008						3		3	3	0.06	9.71	1.00	0.96		O, M
Db	Central Bohemia, Prague, city district of Dobřejovice, the Dobřejovický stream by the Na Návsi bus stop, 49°58.902'N, 14°34.752'E, 330 m, 11 IX 2008						2		2	2	0.13	12.62	1.00	1.01		M
Dk	Northern Bohemia, the Robečský stream by the railway bridge near the Máchovo jezero fishpond in the town of Doksy, 50°34.201N, 14°39.37'E, 270 m, 12 IX 2008							5	5	5	0.06	10.68	1.00	1.02		O, M
Ds	Northern Bohemia, the Robečský stream below the Čepelský fishpond in the town of Doksy, 50°33.669'N, 14°39.503'E, 280 m, 12 IX 2008							2	2	2	0.04	3.88	1.00	1.02		M
F	Northern Bohemia, small wetland by farm in the village of Císařský 1.14 km W of the town of Šluknov, 51°0.06'N, 14°25.95'E, 400 m, 2 IX 2007						3		4	3	0.06	8.74	0.83	1.02		M
Hp1	Central Bohemia, Prague, city district of Horní Počernice (Svépravice), wetland by the Eliška fishpond, 50°5.95'N, 14°36.87'E, 230 m, 7 IX 2008						5		5	5	0.05	9.71	1.00	1.02		M, N
Hs	Central Bohemia, the Chumava stream in the village of Hostomice, 49°49.397'N, 14°2.619'E, 340 m, 12 IX 2008						1		2	1	0.00	1.94	0.00	0.99		M
I	Southern Bohemia, the Malý Jordán fishpond 3 km N of the city of Tábor, 49°26.14'N, 14°40.08'E, 430 m, 9 IX 2007							5	5	5	0.06	13.59	1.00	0.99		M, N, O
J	Southern Bohemia, the Loucký fishpond in the town of Černovice, 49°22.71'N, 14°57.74'E, 630 m, 12 IX 2007						3		3	3	0.05	7.77	1.00	1.01		M, N
Jo	Northern Bohemia, the Jordán stream by the road from the town of Doksy to the village of Břehyně, 50°34.27'N, 14°40.81'E, 260 m, 12 IX 2008						3		3	3	0.08	12.62	1.00	1.04		M, O

Acronym	Locality and date of collection	AFLP group N	O	E×O	E	E×M	M	M×N	N_{ind}	G	He	Pp	D	FCM 2C (pg)	CDA subsp.
K	Central Bohemia, small pond by the road from the town of Karlštejn to the village of Krupná, 49°55.491'N,14°9.902'E, 300 m, 29 VII 2008						4		5	4	0.07	11.65	0.90	1.00	M
La	Central Bohemia, the W bank of the Pánovka fishpond 1.83 km S of the village of Lány, 50°6.50'N, 13°56.67'E, 400 m, 30 IX 2008						2		2	2	0.17	17.48	1.00	1.00	N, O
M	Central Bohemia, the Bojovský stream by Zámecký fishpond in the town of Mníšek pod Brdy, 49°52.124'N, 14°15.454'E, 390 m, 17 VIII 2007						3		5	3	0.07	11.65	0.70	1.01	M
Ma	Central Bohemia, Prague, city district of Dolní Počernice, the Svépravický stream by Vidlák fishpond, 50°5.72'N, 14°34.33'E, 220 m, 14 IX 2008						2		2	2	0.04	3.88	1.00	1.02	M
N	Eastern Bohemia, the Závidkovický stream 1.13 km from the village of Nová Ves u Světlé nad Sázavou, 49°38.92'N, 15°25.27'E, 430 m, 19 VIII 2007						3		4	3	0.08	12.62	0.83	1.03	E, M
Ne	Southern Bohemia, the Nežárka river below the weir in the village of Hamr, 49°9.45'N, 14°45.995'E, 420 m, 29 IX 2008							2	2	2	0.08	7.77	1.00	1.04	O
P	Central Bohemia, the Botič stream by the bridge below the Průhonice Castle in the village of Průhonice, 50°0.10'N, 14°33.36'E, 290 m, 24 VIII 2007						5		5	5	0.07	13.59	1.00	1.02	M
Pl	Eastern Bohemia, forest wetland by the Bolevecký stream near the Strženka fishpond on the N edge of the city of Plzeň, 49°47.28'N, 13°21.82'E, 350 m, 16 IX 2007						3		4	3	0.08	12.62	0.83	0.99	M
Ps	Central Bohemia, Prague, city district of Dolní Počernice, stream by the Počernický fishpond, 50°5.25'N, 14°35.09'E, 230 m, 14 IX 2008							3	3	3	0.10	14.56	1.00	1.00	M
Q	Central Bohemia, the Lomnický stream by the Štičí fishpond in the village of Mirošovice, 49°54.44'N, 14°42.41'E, 341 m, 15 IX 2007						5		5	5	0.10	21.36	1.00	1.03	M
Re	Central Bohemia, small forest fishpond in the Prameny Klíčavy Nature Reserve 5.85 km W of the town of Nové Strašecí, 50°8.892'N, 13°49.102'E, 420 m, 13 IX 2008						2		2	2	0.11	10.68	1.00	1.03	M
S	Central Bohemia, streamlet 320 m E of the forest path 1.78 km NW of the village of Sychrov u Dobříše, 49°46.64'N, 14°6.41'E, 440 m, 22 VIII 2007						3		5	3	0.05	6.80	0.70	1.00	M

Acronym	Locality and date of collection	AFLP group N	O	E×O	E	E×M	M	M×N	N_{ind}	G	He	Pp	D	FCM 2C (pg)	CDA subsp.
Sr	Central Bohemia, small fishpond by the Bubovický stream 1.34 km NE of the village of Srbsko, 49°56.64'N, 14°9.07'E, 400 m, 6 X 2008						2		4	2	0.07	7.77	0.50	1.04	–
St	Central Bohemia, the Novostrašecký fishpond 1.70 km W of the town of Nové Strašecí,; 50°9.120'N, 13°52.58'E, 410 m; 13 IX 2008						1		2	1	0.00	0.00	0.00	1.07	M
T	Central Bohemia, the Louže pond in the village of Třebotov, 49°58.437'N, 14°17.62'E, 360 m, 8 VIII 2007						12		15	12	0.09	25.24	0.94	1.02	M
Tr	Southern Bohemia, the Prostřední stoka channel near Třeboň railway station, 49°0.39'N, 14°46.62'E, 430 m, 28 IX 2008							1	3	1	0.00	1.94	0.00	1.02	M
U	Central Bohemia, Prague, city district of Újezd nad Lesy, the Blatovský fishpond in the village of Blatov, 50°4.79'N, 14°38.38'E, 245 m, 25 VII 2007					6	6		14	12	0.11	33.98	0.97	1.02	E, M
Vp	Central Bohemia, Prague, city district of Šeberov, the Kovářský fishpond, 50°0.88'N, 14°30.96'E, 290 m, 8 IX 2008					14			15	14	0.08	22.33	0.99	1.10	E, M, O
X	Central Bohemia, Prague, city district of Černý most, the Chvalka stream by the slip-road, 50°6.144'N, 14°35.171'E, 230 m, 15 IX 2007						2		4	2	0.06	5.83	0.50	0.99	M
Y	Central Bohemia, Prague, city district of Černý most, the Svépravický stream among the slip-roads, 50°5.938'N, 14°35.628'E, 230 m, 15 IX 2007						3		5	3	0.05	8.74	0.70	1.01	M
Za	Central Bohemia, the Berounka river at Zadní Třebáň railway station, 49°55.16'N, 14°12.22'E, 200 m, 6 X 2008					4			5	4	0.08	14.56	0.90	1.08	E, M, O
Sparganium erectum* subsp. *neglectum															
Hp2	Central Bohemia, Prague, city district of Horní Počernice (Svépravice), the Eliška fishpond, 50°5.87'N, 14°36.67'E, 230 m, 7 IX 2008	3							3	3	0.04	5.83	1.00	1.00	M, N, O
Hp3	Central Bohemia, Prague, city district of Horní Počernice (Svépravice), the Xaverovský fishpond II, 50°6.15'N, 14°35.83'E, 230 m, 7 IX 2008	5							5	5	0.09	18.45	1.00	1.00	N, O
W	Central Bohemia, the wetland by the Pšovka river, 1 km SE of the village of Hleďsebe; 50°21.40'N, 14°34.32'E, 200 m, 17 IX 2007	2							5	2	0.04	4.85	0.40	0.98	M, N
Z	Central Bohemia, the Pšovka river, Lhotka u Mělníka, 50°22.20'N, 14°33.10'E, 210 m, 17 IX 2007	3							3	3	0.04	5.83	1.00	0.99	N