Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra*, and notes on potential hybridization of these two species

Morfologická a cytologická variabilita druhů *Spergularia echinosperma* a *S. rubra* s ohledem na jejich potenciální hybridizaci

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Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra* and the possibility of these two species hybridizing were investigated. The plant material was collected mainly in the western- and southern-Bohemian pond basins where *S. echinosperma* is most abundant. Using flow cytometry, we found diploid and tetraploid cytotypes among plants morphologically identified as *S. echinosperma* and only tetraploid *S. rubra*. The two tetraploid cytotypes differed significantly in genome size. Both the diploid and tetraploid *S. echinosperma* and *S. rubra* also differed morphologically. The most important identification characters were stipule length together with stipule length/width ratio, seed colour, seed size and testa verrucosity. Although the morphological data suggest that tetraploid *S. echinosperma* may be a hybrid between diploid *S. echinosperma* and *S. rubra*, its genome size was significantly greater than that of a simulated allotetraploid. Since an increase in genome size following allopolyploidization is an improbable event, it is possible that other pathways were involved in the formation of tetraploid *S. echinosperma*. The nomenclature of *S. echinosperma* was also studied. Lectotypification of the name with a plant morphologically corresponding to the diploid cytotype is proposed. The morphological analysis also indicates that the holotype of *S. ×kurkae*, which was described as a putative hybrid between *S. echinosperma* × *S. rubra*, corresponds to tetraploid *S. echinosperma*.

K e y w o r d s: allopolyploidy, classification trees, discriminant analysis, flow cytometry, genome size, inter-ploidy hybridization, morphometric analysis, *Spergularia*

Introduction

There are relatively few vascular plants endemic to central Europe, especially when apomictic microspecies of genera such as *Taraxacum*, *Hieracium*, *Sorbus* and *Rubus* are not considered. One of the long-recognized central European endemics is *Spergularia echinosperma* (Čelak.) Asch. et Graebn. (*Caryophyllaceae*). It is confined to the sandy bottoms of mesotrophic freshwater reservoirs (usually fishponds) that are periodically exposed or sandy banks of large rivers. The center of its distribution is located in the southern- and western-Bohemian pond areas (Friedrich 1979, Dvořák 1990). Recently this species and many other plants inhabiting the exposed bottoms of ponds have declined in abundance due to intensification of fishpond management (Šumberová et al. 2005, 2006).

Spergularia echinosperma was described by Čelakovský (1881) as a subspecies of *S. rubra* (L.) J. Presl et C. Presl. Later, Ascherson & Graebner (1893) raised *S. echinosperma* to specific rank, which is generally accepted (e.g. Friedrich 1979, Monnier & Ratter 1993, Jäger & Werner 2002, Fischer et al. 2008). The main characters cited by Čelakovský (1881) for distinguishing *S. echinosperma* and *S. rubra* were seed colour and testa surface (black bristly seeds vs slightly verrucose brown seeds) and shape of stipules (short and widely triangular vs long and narrowly triangular). Other characters were introduced by Dvořák (1979, 1990), including leaf shape, flower pedicel length and capsule length. *Spergularia rubra* also differs from *S. echinosperma* in its ecology as it is a nearly cosmopolitan species occupying mainly human-affected habitats such as road margins or sandy paths (Friedrich 1979, Dvořák 1990).

Spergularia echinosperma and *S. rubra* are also supposed to differ in their ploidy levels, but few chromosome counts are available. *Spergularia rubra* is reported to be tetraploid ($2n = 4x = 36$) in central Europe (Dvořák 1990, Wisskirchen & Haeupler 1998), although there are also records of diploid and hexaploid plants of *S. rubra* from southern Europe (Ratter 1964, Fernandes & Leitao 1971). For *S. echinosperma*, only one chromosome count exists, which is diploid ($2n = 2x = 18$; Dvořák & Dadáková 1984).

Jage (1974) and Dvořák (1979) report the occurrence of more distinct morphotypes within *S. echinosperma*. Later, results of a more detailed study were published as a part of the *Spergularia* treatment for the Flora of the Czech Republic (Dvořák 1990). This author revealed the existence of *S. echinosperma* populations with morphological characters typical of *S. rubra* (especially seed colour and length of stipules and fruit pedicels), which he ultimately explained by inter-specific hybridization. He supposed that hybridization leads to the formation of a primary tetraploid hybrid, which he described as *S.* ×*kurkae* F. Dvořák (Dvořák 1989), accompanied by further gene introgression from *S. rubra* to *S. echinosperma*. However, the assumed tetraploid state of *S.* ×*kurkae* was documented only by a single chromosome count (Dvořák 1989), as in the case of *S. echinosperma*. With such limited data, Dvořák (1990) could not credibly infer the cytotype structure of the populations and morphotypes of the plants he studied.

The current state of knowledge of the central-European endemic *S. echinosperma* is fairly fragmentary. The chromosome numbers supporting the ploidy level difference between *S. echinosperma* and *S. rubra* and their putative hybrid *S.* ×*kurkae* are especially sparse and the morphological delimitation of *S.* ×*kurkae* and several reported morphotypes within *S.* ×*kurkae* (Dvořák 1989, 1990) are rather vague. It is obvious that *S. rubra* and *S. echinosperma* need to be revised based on an extensive screening of their morphological and cytotype variation. Therefore, we have addressed the following questions: (i) What is the cytotype structure of populations of *S. echinosperma* and *S. rubra*? (ii) What is the extent of the morphological variation and differences between particular cytotypes/species? (iii) Does the data on the morphology and genome size support the existence of hybrids between *S. echinosperma* and *S. rubra*?

Materials and methods

Plants

Five hundred and fifteen plants were collected from 27 populations of *Spergularia echinosperma* and *S. rubra* for the morphometric and flow-cytometric analyses during the years 2008 and 2009. They were collected predominantly in the southern part of Bohemia in the center of *S. echinosperma* distribution (see Appendix 1 for the exact localities and acronyms of the populations used in the text). Only mature plants with ripe capsules were collected. The numbers of plants per population ranged from 15 to 24. The only exception was the Cakov population (*S. rubra*), which consisted of only three plants. However, they occurred in a habitat atypical of *S. rubra* (an exposed pond bottom) and were therefore included in the analyses. Voucher specimens are deposited in the herbarium CBFS.

In addition, the type specimens of *S. ×kurkae* and *S. echinosperma* were included in the morphometric analyses. The holotype of *S. ×kurkae* (Czech Republic, southern Bohemia, Záblatí: southern shore of the Záblatský rybník fishpond, 425 m a.s.l.; approximate coordinates: 49°06'00"N, 14°40'00"E; 27. 6. 1942 leg. R. Kurka, CB 36098) consists of only one plant. There are two syntypes of *S. echinosperma* (Czech Republic, southern Bohemia, Protivín: at the Švarcenberský rybník fishpond near the village, 380 m a.s.l.; approximate coordinates: 49°12'28"N, 14°14'04"E; 08.1876 and 4. 9. 1880 leg. F. Čelakovský, PR 374981 and PR 374982, respectively). There are four plants on the former sheet, all of which were used for the morphometric measurements. There are eight plants on the latter sheet, of which only four are suitable for measuring morphological characters.

Cytological analyses

Flow cytometry was employed for estimating the genome size (relative fluorescence intensity) and DNA ploidy level (sensu Suda et al. 2006) of all the plants collected. We used the simplified two-step procedure of nuclear isolation and staining (Otto 1990) modified for plant tissues following the protocol of Doležel et al. (2007). Fresh leaves together with an appropriate amount of the internal standard were chopped using a razor blade in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 M citric acid, 0.5% v/v Tween 20). *Glycine max* 'Polanka' was used as the internal standard (2C = 2.50 pg, Doležel et al. 1994). The suspension was filtered through a 42 nylon mesh and after five minute incubation at room temperature 1 ml of staining solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), fluorochrome 4',6-diamidino-2-phenylindole (DAPI; 4 μg/ml) and β-mercaptoethanol (2 μl/ml) was added. The staining took 1–2 min at room temperature. The samples were run on a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp. Fluorescence intensity of 5000 particles was recorded and the sample/standard ratio of fluorescent intensities and coefficients of variation (CV) of the peaks were calculated. Only analyses with coefficients of variation below 5% were accepted. Due to the low quality of the histograms and presence of endopolyploidy, each individual of *S. echinosperma* was analysed separately. For *S. rubra*, it was possible to use pooled samples of up to 5 individuals. Only analyses enabling precise estimation of the relative fluorescence were used for statistical comparisons of the genome size (150 samples with 237 plants), while the poor quality samples were used only for assessing the ploidy level.

The same method, but with the fluorochrome propidium iodide (PI) together with RNaseIIa (both at a final concentration of 50 µg/ml) replacing DAPI in the staining solution, was used for estimating the genome size of an additional set of plants. Since the PI fluorochrome intercalates evenly between the DNA base-pairs, it can be used to assess the total content of DNA in mass units (Doležel et al. 2007). *Lycopersicon esculentum* 'Stupické polní rané' (2C = 1.96 pg, Doležel et al. 1992) was used as the internal standard. The samples were run on a Partec CyFlow SL flow cytometer (Partec GmbH, Münster, Germany) equipped with a 532 nm (green) diode-pumped solid-state laser (100 mW output). Plants grown from seeds in a growth chamber from three populations per species/cytotype were analysed (Appendix 1). Three plants from each population were used for the analysis; each plant was repeatedly measured on three different days. Relatively high coefficients of variation of up to 6.4% were accepted if the repeated measurements resulted in a consistent genome size. If the difference between individual measurements of one individual exceeded 2%, additional measurements were performed and the most outlying measurement was discarded.

To confirm the FCM results, chromosome counts were carried out on three plants of each species and cytotype (populations Malobor, Smrzov, and StHlina) using a rapid squash method. The apical root meristems of germinated seedlings were pre-treated with a saturated water solution of p-dichlorobenzene (3 h, room temperature), fixed in a 3:1 mixture of 96% ethanol and glacial acetic acid overnight at 4°C, macerated in 1:1 mixture of 96% ethanol and hydrochloric acid for 1 minute, and stained with lacto-propionic orceine. The chromosomes were counted using a light microscope at a magnification of 1000×.

Morphometry

In total, 13 quantitative and 6 derived ratio characters were used for the analyses (Table 1). Diagnostic characters reported by Dvořák (1979, 1990) and other important characters based on our field experience were included. The seed colour of the sampled plants, one of the important characters for traditional species delimitation used by Czech authors (Dostál 1989, Dvořák 1990, Hrouda 2002), was also recorded. However, as the colour was difficult to score, it was not used in the statistical analyses. Unfortunately, it was not possible to include floral characters since flowers were not present on plants with ripe seeds. Three randomly selected leaves, stipules and capsules from one of the primary stems were measured and the average values used. One seed was collected from three randomly chosen capsules from the lower part of the main inflorescence. Seed dimensions and papilla length were measured on light microscope photographs (40× magnification) using tpsDig 2.12 (Rohlf 2008). Papilla shape (PapRat) was expressed as the ratio of the width of the upper part (head, usually broad in *S. echinosperma*) and that of the lower part of the papilla (neck; Fig. 1). Papillae without a head wider than its neck were assigned the value 1. The density of papillae (PapNum) was expressed as the number of papillae visible on one quarter of a seed's circumference (Fig. 1).

The data were processed by multivariate statistical analyses. Characters that deviated most from a normal distribution in each of the pre-defined groups were log-transformed (Table 1).

Table 1. – Morphological characters used in the morphometric analyses and summary of their values for *Spergularia rubra* (249 individuals), *S. echinosperma* tetraploids (184 individuals) and *S. echinosperma* diploids (61 individuals). The numbers denote (minimum–) 10th percentile/**mean**/90th percentile (–maximum). Characters log-transformed prior to the CDA analysis are marked with an asterisk.

Acronym	Character [units]	*S. echinosperma* diploid	*S. echinosperma* tetraploid	*S. rubra*
CapsLeng*	capsule length [mm]	(1.9–)2.5/**3.0**/3.5 (–4.1)	(2.6–)3.0/**3.6**/4.3 (–5.5)	(2.3–)3.0/**3.5**/4.0 (–4.6)
FrPedLen*	length of the fruit pedicel adjacent to the capsule [mm]	(1.7–)4.2/**6.0**/8.1 (–12.5)	(2.0–)4.1/**6.9**/10.5 (–23.7)	(1.5–)2.3/**3.6**/5.6 (–8.4)
InterLen*	length of the internode adjacent to the measured leaf [mm]	(5.9–)8.5/**12.0**/15.8 (–26.8)	(2.2–)4.8/**11.1**/19.0 (–33.5)	(1.3–)2.8/**7.7**/16.3 (–28.4)
Int-Leaf*	internode length/leaf length ratio	(0.78–)0.94/**1.35**/1.78 (–2.49)	(0.37–)0.65/**1.03**/1.52 (–2.77)	(0.24–)0.54/**0.98**/1.46 (–2.86)
LeafLeng*	leaf length [mm]	(4.1–)5.6/**9.4**/14.6 (–18.8)	(2.5–)5.5/**11.0**/17.9 (–24.7)	(3.0–)4.5/**7.7**/14.1 (–24.8)
LeafRat*	leaf length/width ratio	(9.8–)13.3/**20.5**/30.2 (–45.3)	(5.0–)11.3/**18.9**/28.3 (–49.4)	(6.6–)9.2/**13.8**/20.0 (–32.5)
LeafWidt*	leaf width [mm]	(0.3–)0.3/**0.5**/0.6 (–0.7)	(0.3–)0.4/**0.6**/0.8 (–1.1)	(0.2–)0.4/**0.6**/0.8 (–1.2)
LengSeed*	seed length [µm] (Fig. 1)	(394–)409/**451**/484 (–514)	(439–)479/**535**/586 (–642)	(415–)469/**517**/567 (–636)
PapHei*	papilla height [µm] (Fig. 1)	(16–)18/**20**/23 (–26)	(16–)21/**25**/29 (–35)	(12–)15/**18**/21 (–25)
PapNum	number of papillae on one quarter of the seed circumference (papillae density)	(10–)12/**15**/17 (–20)	(7–)8/**11**/14 (–16)	(3–)5/**7**/9 (–12)
PapRat*	ratio of the papilla upper part (“head”) width and papilla lower part (“neck”) width (papilla shape)	(1.04–)1.13/**1.29**/1.48 (–1.78)	(1.09–)1.23/**1.49**/1.84 (–2.25)	(1.00–)1.03/**1.15**/1.29 (–1.44)
Ped-Cap*	pedicel/capsule length ratio	(0.68–)1.41/**2.02**/2.69 (–3.68)	(0.70–)1.26/**1.90**/2.89 (–5.39)	(0.40–)0.65/**1.02**/1.52 (–2.37)
PlHeight*	height of the longest stem [cm]	(3–)3/**6**/10 (–12)	(1–)2/**7**/12 (–23)	(3–)6/**10**/16 (–31)
SeedCol	seed color	black	black	brown
SeedRat*	seed length/width ratio	(1.22–)1.25/**1.35**/1.44 (–1.55)	(1.17–)1.25/**1.33**/1.41 (–1.57)	(1.10–)1.23/**1.30**/1.39 (–1.54)
StemsNum*	number of stems	(1–)1/**4**/9 (–19)	(1–)1/**5**/9 (–19)	(2–)5/**18**/34 (–63)
StpLt*	stipule length [mm]	(1.0–)1.1/**1.4**/1.6 (–1.8)	(1.3–)1.7/**2.2**/2.8 (–4.0)	(2.1–)2.9/**3.5**/4.3 (–4.9)
StpRT*	stipule length/width ratio	(0.48–)0.67/**0.86**/1.16 (–2.0)	(0.75–)0.98/**1.31**/1.68 (–2.09)	(1.43–)1.81/**2.34**/2.85 (–4.04)
StpWd	stipule width [mm]	(0.7–)1.3/**1.7**/2 (–2.3)	(0.8–)1.4/**1.7**/2.1 (–2.5)	(1.0–)1.3/**1.6**/1.9 (–2.4)
WidtSeed*	seed width [µm] (Fig. 1)	(267–)310/**337**/373 (–405)	(283–)362/**405**/452 (–491)	(315–)361/**401**/451 (–501)

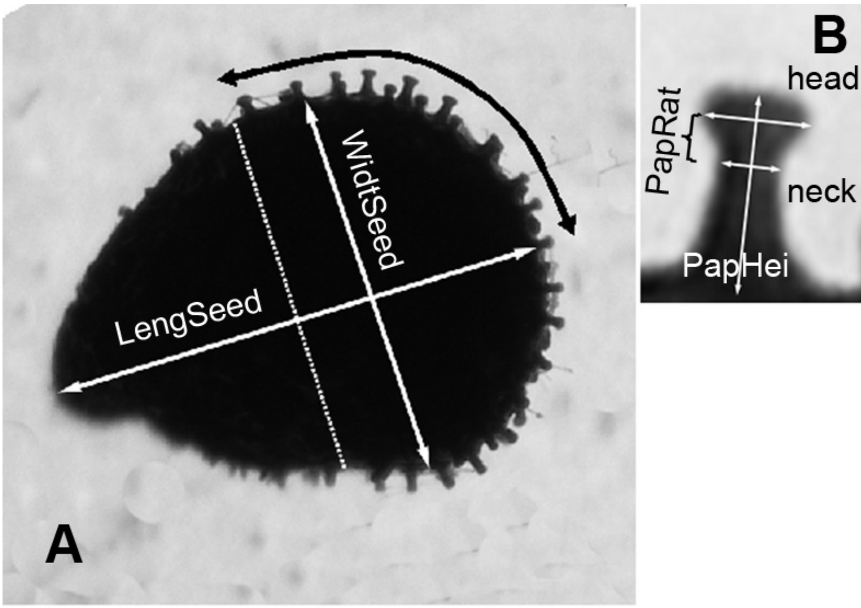

Fig. 1. – Characters measured on the seeds (A) and surface papillae (B). The black curved line specifies the part of the seed circumference where the density of papillae was determined. The longitudinal border of this part is a plane halving the vector of maximal seed length and perpendicular to it (indicated by a dotted line). The character PapRat was computed by dividing the width of the papilla head by the width of the neck.

The plants were divided into two groups based on seed colour, black vs brown, corresponding to the species *S. echinosperma* and *S. rubra*, respectively. The black-seeded plants were additionally divided into two groups based on the flow cytometry data (see Results). One population (Veselsky), however, could not be unambiguously assigned to either of the groups since its seeds were dark brown rather than black or brown. Therefore, it was excluded from the analyses. To find out which characters significantly separated the groups, canonical discriminant analysis (CDA) with forward selection of characters was applied. The type specimens and plants from the Veselsky population were projected to the ordination space as passive samples. The threshold significance level was set to $\alpha = 0.05$ and a Monte-Carlo permutation test (999 permutations) used. The analysis was carried out in CANOCO for Windows 4.5 (ter Braak & Šmilauer 2002). The predictive ability of the selected characters was subsequently tested by classificatory discriminant analysis based on the posterior group membership probabilities in the statistical package R 2.11.0 (R Development Core Team 2010). Cross-validation using each population as a leave-out unit was used (the lda function from the MASS package). The herbarium specimens and plants from the Veselsky population were classified using classification rules based on the other populations with known ploidy levels. The percentage of misclassified samples in each group served as a measure of the predictive ability.

We also reanalysed the data by classification trees that represent a non-parametric alternative to the classificatory discriminant analysis. The essential difference between these two methods is that classification trees, instead of using all characters together, create a hierarchical classification based on univariate splits that can then be visualized as an easily interpretable tree diagram (Breiman et al. 1984). Although this approach has not been widely used in plant taxonomy, it is suitable for analyzing taxonomic data (e.g. Joly & Bruneau 2007, Depypere et al. 2009). We used the function rpart (package rpart) implemented in the R statistical package (R Development Core Team 2010). The minimum split parameter (minsplit) was set to 1 and the initial complexity parameter (cp) to 0.001. A cross-validation using the populations as the leave-out subsamples was used to assess the optimal tree complexity, instead of random subsamples as implemented in the original method (Venables & Ripley 2002). The resulting tree was selected on the basis of the 1-SE rule (Venables & Ripley 2002).

Results

Cytological analysis

Two groups with different genome sizes were discovered among black seeded plants morphologically determined as *Spergularia echinosperma*. Because the chromosomes are very small (typically < 1 μm) we were able only to roughly estimate the number of chromosomes. However, this was sufficient to identify one cytotype as diploid (2n = ca 18) and the other as tetraploid (2n = ca 36) (hereafter referred to as "diploid *S. echinosperma*" and "tetraploid *S. echinosperma*"). Only diploids were found at three localities and only tetraploids at nine localities, and at two localities there was a mixture in which diploids were in the minority (frequencies of 5% and 30% in the Cky and Driten populations, respectively). Only tetraploids were recorded in the populations of *S. rubra*.

The tetraploid cytotype of *S. echinosperma* has a larger genome than tetraploid *S. rubra*. The mean difference was 7.8% using DAPI staining and 8.3% using PI staining (Fig. 2, Table 2). The monoploid (1Cx) genome size of diploid *S. echinosperma* is larger by 5.3% (DAPI staining) or 3.2% (PI staining) than that of tetraploid *S. echinosperma* (Fig. 2, Table 2). We were able to demonstrate these differences in the genome sizes of the three cytotypes using simultaneous flow cytometry analysis (Fig. 3). The mean somatic (2C) genome sizes based on PI staining and converted into mass of DNA is 0.63 pg for diploid *S. echinosperma*, 1.22 pg for tetraploid *S. echinosperma* and 1.12 pg for *S. rubra*. The genome sizes of the plants from the Veselsky population fall within the range of tetraploid *S. echinosperma*. In *S. rubra* (population Luznice) we found one individual that had a genome size that was 2.5% smaller (PI staining).

Morphometry

Marginal effects of all characters in the CDA were highly significant (P < 0.001). Forward selection identified 12 characters that contributed most to the separation of the groups (Table 3, Fig. 4). Both *Spergularia rubra* and the cytotypes of *S. echinosperma* were clearly differentiated from each other. The tetraploid *S. echinosperma* was morphologically intermediate between the diploid cytotype and *S. rubra*. Plants from the Veselsky population, assigned to tetraploid *S. echinosperma* based on genome size, were markedly closer to *S. rubra* (Fig. 4). The position of the plants of the Cakov population, which were collected from the exposed bottom of a pond, was at the edge of the morphological

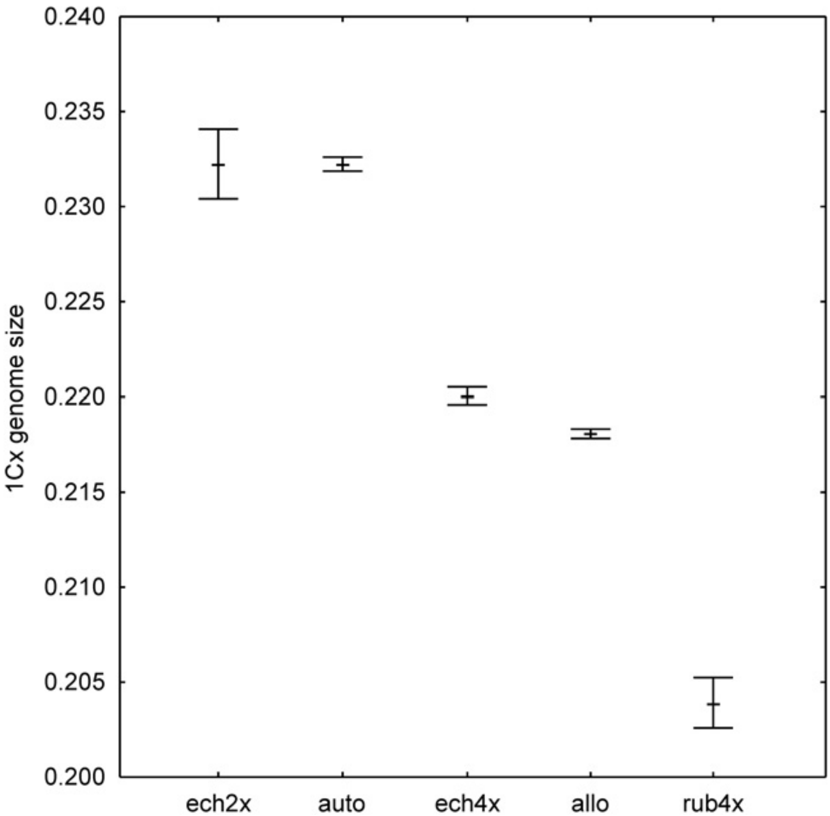

Fig. 2. – Box-and-whisker plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x), *S. rubra* (rub4x), a hypothetical *S. echinosperma*-*S. rubra* allopolyploid (allo) and hypothetical *S. echinosperma* autopolyploid (auto), expressed in terms of a ratio with the 1C value of the standard *Glycine max*.

Table 2. – Summary of the genome sizes of the *Spergularia echinosperma* cytotypes, *S. rubra*, and simulated auto- and allopolyploids based on DAPI staining (expressed as the ratio to the 1C value of the standard *Glycine max*) and PI staining (expressed in picograms of DNA). 2C – somatic genome size; 1Cx – monoploid g. s.; N – number of samples; SE – standard error of mean.

Taxon	PI staining			DAPI staining		
	N	Mean 2C±SE	Mean 1Cx±SE	N	Mean 2C±SE	Mean 1Cx±SE
S. echinosperma 2x	9	0.627±0.001	0.314±0.001	21	0.464±0.002	0.232±0.001
S. echinosperma 4x	9	1.217±0.002	0.304±0.001	92	0.880±0.001	0.220±0.001
S. rubra 4x	8	1.124±0.001	0.281±0.001	16	0.815±0.002	0.203±0.001
S. rubra outlier	1	1.097	0.274		–	
Hypothetical allopolyploid	72	1.190±0.001	0.297±0.001	336	0.872±0.001	0.218±0.001
Hypothetical autopolyploid	45	1.255±0.001	0.314±0.001	231	0.929±0.001	0.232±0.001

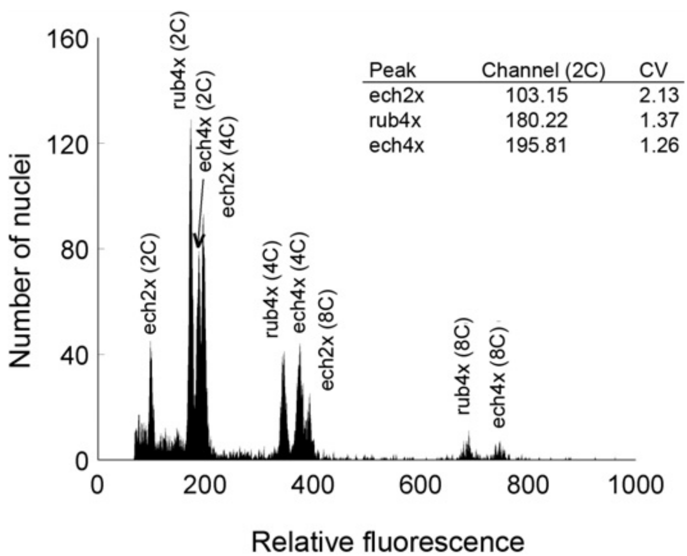

Fig. 3. – Histogram of relative fluorescence of DAPI-stained nuclei of the diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and tetraploid *S. rubra* (rub4x) corroborating the differences in the genome sizes of these three taxa. The genus *Spergularia* displays considerable endopolyploidy with three detectable peaks for a single plant corresponding to 2C, 4C, and 8C DNA content. This allows direct comparison of diploids (4C peak) and tetraploids (2C peaks).

variability of *S. rubra* (not shown), but they did not deviate significantly from the rest of the group either in morphology or genome size.

The best predictors for all the three groups were stipule length (StpLt) and the stipule length/width ratio (StpRT). As they are correlated, only marginal effects of both characters were significant, while inclusion of one character made the conditional effect of the other insignificant. Density of papillae (PapNum) could also be used to discriminate between the three groups. Number of stems (StemsNum) and plant height (PlHeight) proved to be an effective way of discriminating mainly between *S. rubra* and both *S. echinosperma* cytotypes. Seed dimensions (LengSeed and WidtSeed) and capsule length (CapsLeng) differed between the diploids and both tetraploids. Finally, papilla height (PapHei), papilla shape (PapRat), fruit pedicel length (FrPedLen), leaf length (LeafLeng), and stipule width (StpWd) best differentiated tetraploid *S. echinosperma* from the other two groups. Values of all the quantitative characters measured are summarized in Table 1.

The predictive ability of the 12 characters selected was tested using classificatory discriminant analysis. All individuals of *S. rubra* and all but one individual of the diploid *S. echinosperma* were correctly classified. The one misclassified sample was mistaken for the tetraploid *S. echinosperma*. In the tetraploid *S. echinosperma*, the number of misclassifications was higher with three individuals erroneously classified as diploids and one individual as *S. rubra*. The overall percentage misclassified was very low, 1.0% (Table 4).

Only 82.2% of individuals from the Veselsky population were correctly classified, whereas it was 97.8% in all the other populations of tetraploid *S. echinosperma* (Table 4). The discriminant analysis assigned all the misclassified individuals from the Veselsky population to *S. rubra*.

Table 3 – Morphological characters of *Spergularia echinosperma* and *S. rubra* tested in the forward selection with their conditional and marginal effects and their correlations with axes of the canonical discriminant analysis (CorE scores). λ_A – eigenvalue representing the conditional effect of each character (when added to the already selected characters); λ_1 – eigenvalue representing the marginal effect of each character (when it is the only predictor in the model).

Character	Conditional effects			CorE scores		Marginal effects		
	λ_A	F	p	Axis 1	Axis 2	λ_1	F	P
StpLt	0.801	328.8	0.001	–0.8825	0.1497	0.801	328.8	0.001
PapHei	0.413	257.7	0.001	0.5455	0.4967	0.544	183.9	0.001
LengSeed	0.069	47.1	0.001	–0.2011	0.5418	0.334	98.6	0.001
PapNum	0.065	48.4	0.001	0.7830	–0.0389	0.654	239.0	0.001
FrPedLen	0.059	48.6	0.001	0.6068	0.2463	0.429	134.2	0.001
PlHeight	0.063	57.6	0.001	–0.3720	–0.1118	0.151	40.1	0.001
PapRat	0.019	17.7	0.001	0.5631	0.4206	0.494	161.3	0.001
StpWd	0.011	10.4	0.004	0.1834	0.1455	0.061	15.4	0.001
LeafLeng	0.010	9.4	0.001	0.3151	0.1786	0.131	34.5	0.001
WidtSeed	0.009	8.5	0.002	–0.3150	0.4765	0.326	95.9	0.001
StemsNum	0.006	6.1	0.009	–0.6597	–0.1339	0.453	144.1	0.001
CapsLeng	0.005	4.8	0.028	–0.2036	0.3382	0.156	41.5	0.001
InterLen	0.003	n.s.		–		0.158	42.1	0.001
Int-Leaf	0.003	n.s.		–		0.092	23.7	0.001
LeafRat	0.003	n.s.		–		0.186	50.4	0.001
SeedRat	0.003	n.s.		–		0.065	16.5	0.001
LeafWidt	0.002	n.s.		–		0.052	13.0	0.001
StpRT	0.002	n.s.		–		0.785	317.8	0.001
Ped-Cap	0.001	n.s.		–		0.487	158.5	0.001

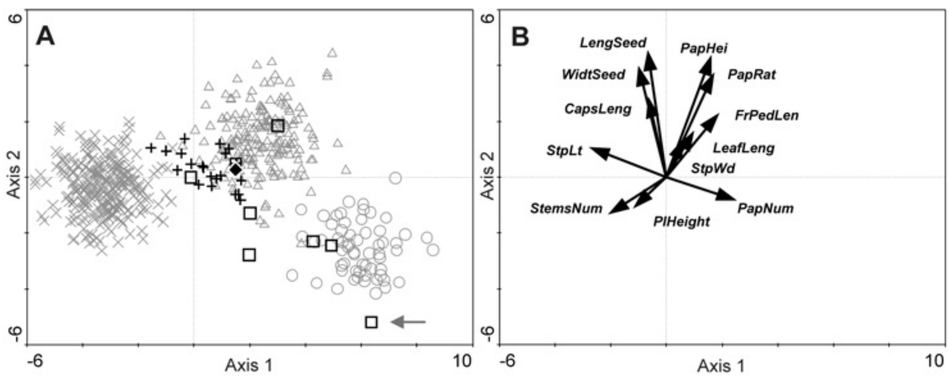

Fig. 4. – Results of CDA of individuals (A) and characters selected by forward selection (B). *Spergularia echinosperma* diploids: grey circles; *S. echinosperma* tetraploids: grey triangles; *S. rubra* tetraploids: grey X-crosses; population Veselsky: black crosses; *S. echinosperma* syntypes: black squares; *S.* ×*kurkae* holotype: black diamond. The arrow denotes the proposed lectotype of *S. echinosperma*. The two canonical axes extract 46.1% and 30.3% of the total variation among the groups.

Table 4. – Summary of the classification matrices of diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and *S. rubra* (rub4x) resulting from the classificatory discriminant and classification tree analyses.

Classificatory discriminant analysis				Classification trees			
observed / predicted	ech2x	ech4x	rub4x	observed / predicted	ech2x	ech4x	rub4x
ech2x	60 (98.4%)	3 (1.6%)	0 (0%)	ech2x	61 (100%)	5 (2.7%)	0 (0%)
ech4x	1 (1.6%)	180 (97.8%)	0 (0%)	ech4x	0 (0%)	175 (95.1%)	4 (1.6%)
rub4x	0 (0%)	1 (0.6%)	249 (100%)	rub4x	0 (0%)	4 (2.2%)	245 (98.4%)

Table 5. – Posterior probabilities of classification for the *Spergularia* ×*kurkae* holotype (CB) and *S. echinosperma* syntypes (PR; the proposed lectotype marked as "lt") obtained from the classificatory discriminant analysis (ech2x – diploid *Spergularia echinosperma*, ech4x – tetraploid *S. echinosperma*, rub4x – *S. rubra*).

Specimen	Posterior probability for		
	ech2x	ech4x	rub4x
CB-36098	3.43×10^{-6}	0.99	3.31×10^{-5}
PR-374981 / 1	1.46×10^{-6}	0.99	4.58×10^{-10}
PR-374981 / 2	2.39×10^{-8}	0.69	0.30
PR-374981 / 3	1.37×10^{-6}	0.99	2.40×10^{-5}
PR-374981 / 4 (lt)	0.99	1.06×10^{-11}	5.27×10^{-24}
PR-374982 / 1	0.99	1.08×10^{-03}	5.07×10^{-13}
PR-374982 / 2	7.69×10^{-3}	0.99	2.54×10^{-5}
PR-374982 / 3	0.67	0.32	1.31×10^{-4}
PR-374982 / 4	0.99	6.34×10^{-5}	9.64×10^{-16}

The *S.* ×*kurkae* holotype was classified as tetraploid *S. echinosperma* with a nearly 100% probability (Table 5). Each of the *S. echinosperma* syntypes contained a mixture of plants classified as either diploid or tetraploid *S. echinosperma* (Table 5).

The final classification tree selected had 7 terminal nodes (complexity parameter cp = 0.011). It confirmed the high discrimination power of the two characters describing stipules, StpRT and StpLt, which distinguished both the cytotypes of *S. echinosperma* and between *S. echinosperma* and *S. rubra*. Other characters were used to discriminate the two cytotypes within *S. echinosperma*, seed length (LengSeed) and density of papillae (PapNum) and for distinguishing between tetraploid *S. echinosperma* and *S. rubra* the number of stems (StemsNum) together with density of papillae (PapNum) (Fig. 5). The overall predictive power of this model was slightly lower than that of the discriminant analysis (error rate 2.6%; Table 4). All individuals of diploid *S. echinosperma* were classified correctly. Within the tetraploid *S. echinosperma*, five individuals were erroneously classified as diploids and four as *S. rubra*. There was also a higher percentage of misclassification among *S. rubra* plants, four of which were incorrectly classified as tetraploid *S. echinosperma* (Table 4).

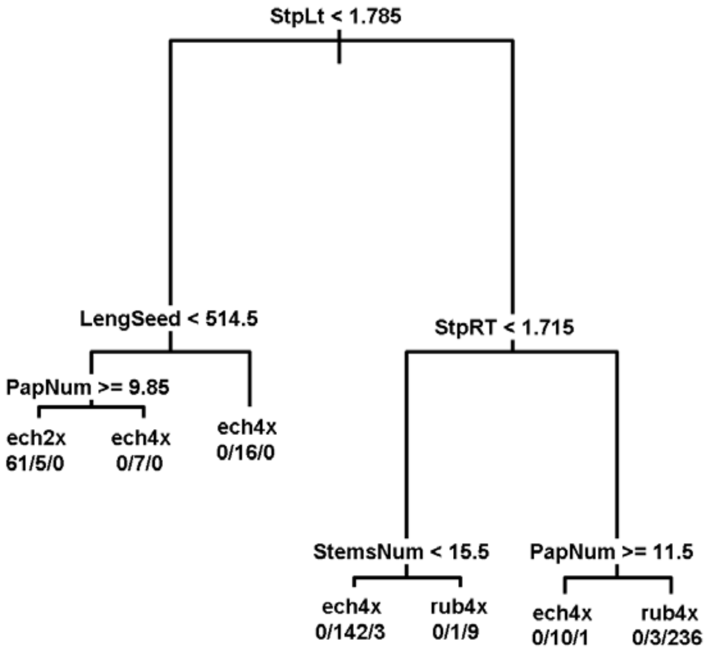

Fig. 5. – Classification tree of individuals of diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and *S. rubra* (rub4x). If a character value matches the classification rule, the determination continues to the left branch, otherwise to the right branch. Lengths of the branches correspond to the relative discriminatory powers of the respective rules. The group names at the terminal nodes indicate the predicted classification of a particular node, whereas the numbers separated by slashes indicate actual membership of samples classified to a particular node (ech2x/ech4x/rub4x).

Discussion

Ploidy levels and morphology

We found three different entities in the populations of *Spergularia echinosperma* and *S. rubra* studied. All the populations collected from outside of the exposed bottoms of ponds and one exceptional population growing on the exposed bottom of the Čakov fishpond belonged to the tetraploid cytotype of *S. rubra*. No other cytotypes were found within this species, which confirms the uniformity of *S. rubra* in central Europe (Friedrich 1979, Dvořák 1990, Wisskirchen & Haeupler 1998, Marhold et al. 2007). The occurrence of one individual with a slightly smaller genome can be most probably attributed to aneuploidy, although this was not confirmed by a chromosome count.

A diploid and a tetraploid cytotype were recorded in the other populations growing on the exposed bottoms of ponds that were identified as *S. echinosperma*. The morphometric analysis showed that the tetraploid *S. echinosperma* cytotype was significantly different from the diploid cytotype and also from *S. rubra*. The best morphological characters for discriminating between diploid *S. echinosperma*, tetraploid *S. echinosperma* and *S. rubra* were those of stipules and seeds (Fig. 4, Fig. 5, Table 3). Stipule length and stipule length/width ratio of all three entities differed (Table 1). However, the latter was more

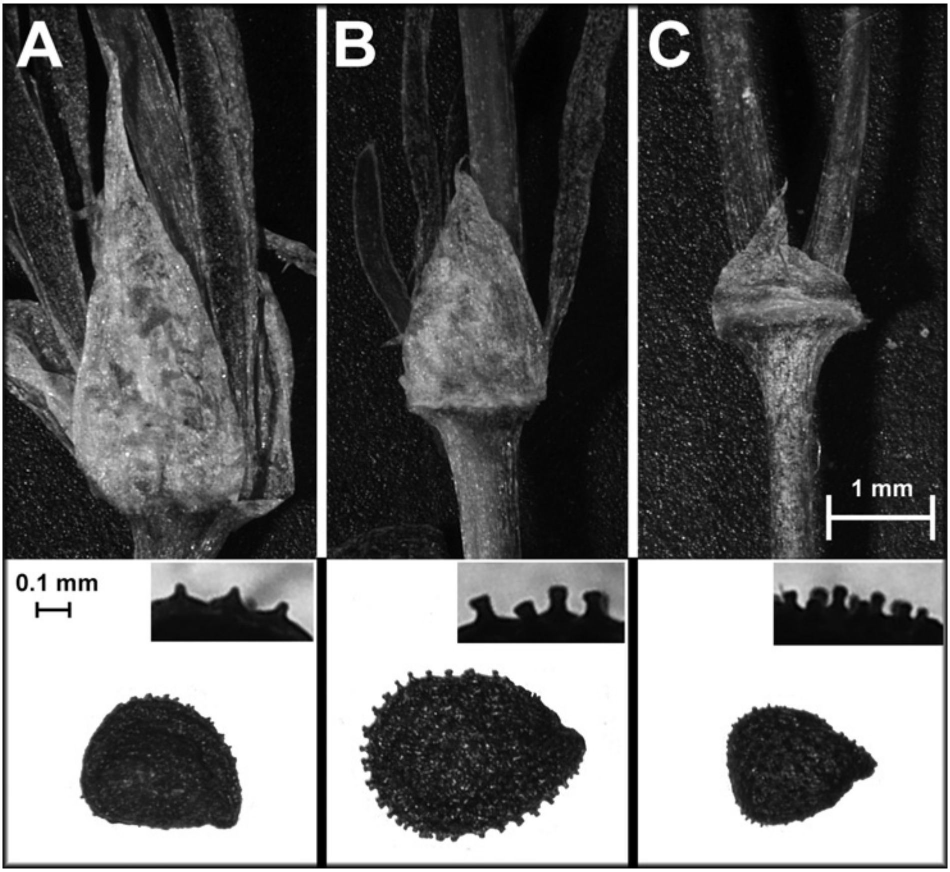

Fig. 6. – Typical stipules and seeds of *Spergularia rubra* (A), tetraploid *S. echinosperma* (B) and diploid *S. echinosperma* (C).

useful for field determination as it can be easily assessed visually. The stipules of diploid *S. echinosperma* are shorter than wide, those of tetraploid *S. echinosperma* as long as or up to 1.7× longer than wide and those of *S. rubra* more than 1.7× longer than wide (Fig. 6). Based on this single character, we were able to classify correctly 87.6% of our samples.

The seed colour is mentioned as the character that can be used to discriminate between *S. echinosperma* and *S. rubra* in the original description of *S. echinosperma* (Čelakovský 1881) and is used by some (e.g. Dostál 1989, Dvořák 1990, Hrouda 2002) but not all authors (e.g. Friedrich 1979, Monnier & Ratter 1993, Jäger & Werner 2002, Fischer et al. 2008). Our analyses confirmed that seed colour can be reliably used to discriminate between *S. echinosperma* (both cytotypes) with black seeds and *S. rubra* with brown seeds.

Other relatively reliable characters, which were less useful in the field, were seed size and testa structure. In accordance with the original description (Čelakovský 1881) and other authors (Friedrich 1979, Dvořák 1990) the seeds of *S. rubra* differ from those of *S. echinosperma* in having a low density of surface papillae, which are also considerably smaller. In addition, the *S. echinosperma* cytotypes strongly differed from each other in seed morphology. The diploids displayed significantly smaller and more densely verrucose seeds with a lower density of papillae and less pronounced papilla heads than the tetraploids (Fig. 6). Based on the results of the morphometric analyses, we compiled the following determination key for the taxa/cytotypes:

1a Seeds brown, sparsely verrucose (5–9 papillae per 1/4 of the seed circumference); stipules at least 1.7× longer than wide, at least 2.9 mm long; plants usually with more than 5 stems .... ***S. rubra***
1b Seeds black, densely verrucose (8–17 papillae per 1/4 of the seed circumference); stipules less than 1.7× longer than wide, less than 2.8 mm long; plants usually with fewer than 9 stems 2
2a Stipules shorter than wide, less than 1.6 mm long; seeds less than 0.48 mm long, density of papillae 12–17 per 1/4 of the seed circumference .... ***S. echinosperma*, diploid cytotype**
2b Stipules longer than wide, more than 1.7 mm long; seeds more than 0.48 mm long, density of papillae 8–14 per 1/4 of the seed circumference .... ***S. echinosperma*, tetraploid cytotype**

Genome size

The genome sizes of the taxa studied are the first published for the genus *Spergularia*. Their genomes are quite small, which is a common feature of the *Caryophyllaceae* (Bennett & Leitch 2010). The genome of the diploid *S. echinosperma* (2C = 0.63 pg) is even smaller than the smallest genome reported in this family so far (2C = 0.84 pg for *Polycarpaea carnosa* C. Sm. ex Buch; Bennett & Leitch 2010).

Origin of the tetraploid cytotype of Spergularia echinosperma

The tetraploid cytotype of *S. echinosperma* was morphologically intermediate between the diploid cytotype of *S. echinosperma* and (tetraploid) *S. rubra* suggesting hybrid origin. To test the hypothesis of allopolyploid origin of tetraploid *S. echinosperma*, we modelled the genome sizes of the hypothetical allopolyploids by combining two chromosome sets from each of the diploid *S. echinosperma* individuals (an unreduced gamete) with two chromosome sets from each of the *S. rubra* individuals (a reduced gamete) in our dataset (Fig. 2, Table 2). We used the data obtained from both the DAPI and PI staining. The mean genome size of the simulated allopolyploids was lower than the mean genome size of tetraploid *S. echinosperma* by 0.9% based on the DAPI and 2.2% on the PI staining. The difference was tested using a Mann-Whitney U-test in Statistica 8 (StatSoft 1998) and was significant for both the DAPI ($U = 8441$; $P < 0.001$) and PI ($U = 0$; $P < 0.001$) staining. This difference challenges the allopolyploid pathway, because it needs to assume an increase in genome size after polyploidization, which is rarely recorded (Dhillon et al. 1983, Jakob et al. 2004, Leitch et al. 2008) compared to the ubiquitous decrease in genome size.

We are aware that one-step hybridization through unreduced gametes of the diploid is not the only possibility. However, we think it is the most likely scenario. Angiosperms commonly produce unreduced gametes and this is viewed as the primary source of neopolyploid formation, especially in diploid-tetraploid crosses (Ramsey & Schemske 1998). For *Spergularia* it is reported that a few tetraploid seeds were produced by a cross between *S. maritima* (All.) Chiov. (♀, diploid) and *S. rupicola* Lebel ex Le Jolis (♂, tetraploid) (Ratter, 1976). The alternative pathway of allotetraploid formation involves an intermediate stage of (at least partly) fertile triploid progeny formed by fusion of normally developed gametes of the parental species ("triploid bridge"). These triploids can produce tetraploid offspring by selfing or backcrossing to one of the parental taxa (Bretagnolle & Thompson 1995). Though rare, this pathway of polyploid formation can be significant in diploid-tetraploid hybridization (e.g. Vardi & Zohary 1967, Anamthawat-Jónsson & Thorsson 2003, Aagaard et al. 2005, Lo et al. 2010). In *Spergularia*, nearly all triploid offspring of various diploid-tetraploid crosses are sterile and the fertility of seeds from triploid plants is very low (0.1–0.2%) (Ratter 1976). This together with the absence of triploids in wild populations (both our data and in the literature) makes the triploid bridge pathway highly improbable.

As an alternative to allopolyploidization we also investigated the possibility that tetraploid *S. echinosperma* could be an autopolyploid derived from the diploid cytotype. We modelled the genome sizes of hypothetical autopolyploids by adding the genome sizes of each pair of *S. echinosperma* diploids in our dataset and also by doubling the genome size of each of the diploids (simulating autogamy) (Fig. 2, Table 2). The mean genome size of the hypothetical autopolyploid was greater by 5.4% based on DAPI and 3.1% based on PI staining than that of tetraploid *S. echinosperma*. There was no overlap in the genome sizes of the simulated autopolyploids and tetraploid *S. echinosperma* based on either of the methods of staining. However, this difference is relatively small and could be simply attributed to genome downsizing, which is a common phenomenon in polyploids (Leitch & Bennet 2004). Thus, it is not possible to exclude this pathway of autopolyploid formation based on the available data. The intermediate morphology of tetraploid *S. echinosperma* could result from subsequent homoploid hybridization with *S. rubra*. On the other hand, our morphometric data indicate that tetraploid *S. echinosperma* is morphologically quite homogenous and homoploid hybridization with *S. rubra* is not frequent (only the Veselsky population was conspicuously intermediate between tetraploid *S. echinosperma* and *S. rubra*).

Taxonomy and nomenclature

The tetraploid cytotype of *S. echinosperma* was more or less intermediate between diploid *S. echinosperma* and *S. rubra*. Morphological intermediacy between the "pure" *S. echinosperma* and *S. rubra* is also the attribute of the assumed hybrid *S.* ×*kurkae* according to Dvořák (1990). Indeed, discriminant analyses placed the *S.* ×*kurkae* holotype among the *S. echinosperma* tetraploids (Fig. 4, Table 5). Therefore, we conclude it was this tetraploid cytotype that Dvořák (1989) described as *S.* ×*kurkae* F. Dvořák. It is also obvious that Dvořák (1990) intended to apply the name *S. echinosperma* to the diploid cytotype. He published the diploid chromosome count as the only one for *S. echinosperma* (Dvořák & Dadáková 1984, Dvořák 1990). He even annotated, but never published, a lectotype of the name *Spergularia rubra* subsp. *echinosperma* (Fig. 7) that corresponds well with the diploids based on our results (Fig. 4, Table 5), although the original material of this name is heterogeneous and comprises both diploids and tetraploids. We therefore propose lectotypification of this name in the sense of the diploids in the present paper and we propose the same individual as F. Dvořák as the lectotype (Fig. 7).

Dvořák (1990) also reported the existence of several distinct morphotypes within *S.* ×*kurkae*. In our study, the three entities we identified were quite homogenous except for one population of tetraploid *S. echinosperma* (Veselsky) that was markedly shifted towards *S. rubra* (Fig. 4). This morphotype corresponds to one of the morphotypes described by Dvořák (1990) from the area of the Českomoravská vrchovina Highlands, characterized by the dark brown colour of its seeds and elongated stipules. Taxonomic status of this morphotype is unknown; however, its origin as a cross between tetraploid *S. echinosperma* and *S. rubra* is possible.

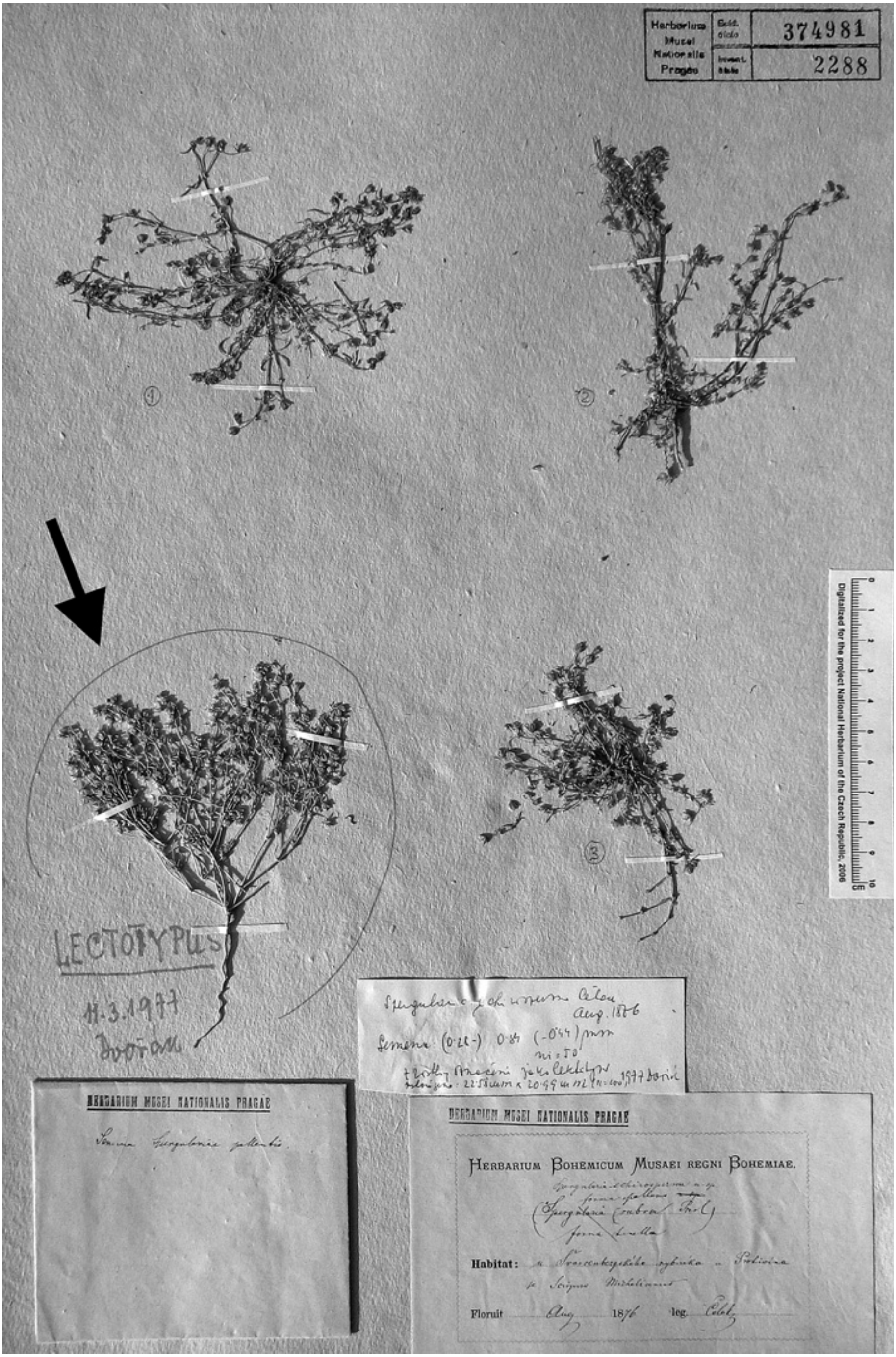

Fig. 7. – The proposed lectotype for the name *Spergularia echinosperma* (Čelak.) Asch. et Graebn., PR 374981, marked by the arrow. The text on the label reads: "*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.".

Based on current data it is not possible to designate the definitive taxonomic treatment of tetraploid *S. echinosperma*. Although its hybrid origin is strongly suggested by the morphological data, the discrepancy between the expected and observed genomes size needs further investigation. It is also unknown whether tetraploid *S. echinosperma* represents an ecologically and/or geographically well-separated entity, which would indicate it is a separate species, but this will need more extensive sampling. For now, therefore, we do not propose treating the tetraploid cytotype of *S. echinosperma* as a separate taxon.

Nomenclature of *S. echinosperma*:

Spergularia echinosperma (Čelak.) Asch. et Graebn. in Ber. Deutsch. Bot. Ges. 11: 516, 1893.

≡ *Spergularia rubra* [subsp.] b. *echinosperma* Čelak. in Prodr. Fl. Böhmen 4: 867, 1881.

Lectotype (**designated here**): “*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.”, PR 374981, left bottom individual (marked by the arrow in Fig. 7); the lectotype belongs to the diploid cytotype.

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Souhrn

V předložené práci jsme se zabývali studiem morfologické a cytologické variability druhů *Spergularia echinosperma* a *S. rubra*. Analyzovali jsme rostliny z celkem 27 populací zejména z jižních a západních Čech, kde je druh *S. echinosperma* nejhojnější. Navíc jsme do morfometrických analýz zahrnuli typové položky druhu *S. echinosperma* a údajného křížence mezi *S. echinosperma* a *S. rubra*, popsaného jako *S. ×kurkae*. Cytometrická měření odhalila existenci dvou různých cytotypů – diploidního a tetraploidního – mezi rostlinami morfologicky odpovídajícími druhu *S. echinosperma*. U druhu *S. rubra* byl detekován jen tetraploidní cytotyp, jenž se velikostí genomu lišil od tetraploidního cytotypu *S. echinosperma*. Velikost genomu byla stanovena na 2C = 0,63 pg pro diploidy *S. echinosperma*, 2C = 1,22 pro tetraploidy *S. echinosperma* a 2C = 1,12 pg pro *S. rubra*. Všechny tři cytotypy se od sebe rovněž signifikantně lišily morfologicky. Tetraploidní cytotyp *S. echinosperma* byl nápadně intermediární mezi diploidním cytotypem a *S. rubra*. Nejdůležitějšími diskriminačními znaky jsou délka a poměr délky a šířky palistů, dále pak barva a velikost semen a rovněž také velikost a hustota jejich povrchových papil. Na základě studia morfologických znaků byl sestaven klíč na determinaci jednotlivých cytotypů:

1a Semena hnědá, řídce bradavčitá (hustota 5–9 papil na 1/4 obvodu semene); palisty alespoň 1,7× delší než široké, alespoň 2,9 mm dlouhé; rostliny obvykle s více než 5 lodyhami ....***S. rubra***

1b Semena černá, hustěji bradavčitá (hustota 8–17 papil na 1/4 obvodu semene); palisty méně než 1,7× delší než široké, kratší než 2,8 mm; rostliny obvykle s méně než 9 lodyhami 2

2a Palisty kratší než široké, kratší než 1,6 mm; semena kratší než 0,48 mm, hustota povrchových papil 12–17 na 1/4 obvodu semene ....***S. echinosperma*, diploidní cytotyp**

2b Palisty delší než široké, delší než 1,7 mm; semena delší než 0,48 mm, hustota povrchových papil 8–14 na 1/4 obvodu semene ....***S. echinosperma*, tetraploidní cytotyp**

Morfologická analýza dále potvrdila totožnost holotypu *S.* ×*kurkae* s tetraploidním cytotypem *S. echinosperma*. Dvě existující typové položky druhu *S. echinosperma* obsahují jak diploidy tak tetraploidy tohoto druhu. Vzhledem k příslušnosti jména *S.* ×*kurkae* k tetraploidnímu cytotypu proto navrhujeme lektotypifikaci jména *S. rubra* subsp. *echinosperma* Čelak. ve smyslu diploidního cytotypu. Ačkoli morfologická data svědčí o hybridním původu tetraploidního cytotypu *S. echinosperma*, velikost genomu tetraploida je signifikantně vyšší ve srovnání s hypotetickým hybridem mezi diploidy *S. echinosperma* a tetraploidy *S. rubra*, a nelze tedy vyloučit i další způsoby vzniku tetraploidů (např. autotetraploidní vznik a následná hybridizace s druhem *S. rubra*). Vzhledem k dosud nejasnému původu tetraploidního cytotypu *S. echinosperma* a nedostatku údajů o jeho ekologii a rozšíření prozatím nenavrhujeme jeho rozlišování jako samostatného taxonu.

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Appendix 1. – List of the localities of the *Spergularia echinosperma* and *S. rubra* populations used in this study together with their cytotype compositions detected by flow cytometry. Populations marked by an asterisk are those from which plants used for the measurements of the genome size using PI staining originated. The geographic coordinates are presented in WGS 84 format. ▶▶▶

Label	Locality	Latitute	Longitude	Altitude (m a.s.l.)	Number of plants	Species and cytotype
Cakov	S Bohemia, Čakov: bare bottom of the Beranov pond	48°58'51.8"N	14°19'11.5"E	420	3	*S. rubra* 4x
Cerna	Českomoravská vrchovina highlands, Černá: field path 1.7 km NW of the village	49°26'00.9"N	15°50'41.7"E	560	20	*S. rubra* 4x
Cky	SW Bohemia, Lažany: bare bottom of the Cky pond	49°21'06.9"N	13°53'28.9"E	490	20	*S. echinosperma* 4x + 2x
DolNovos	S Bohemia, Novosedly: bare bottom of the Dolní rybník pond	49°05'24.9"N	14°16'51.3"E	390	21	*S. echinosperma* 4x
Driten*	S Bohemia, Dříteň: bare bottom of the Kočínský rybník pond	49°08'56.1"N	14°21'15.0"E	460	20	*S. echinosperma* 4x + 2x
Havlic	S Bohemia, České Budějovice, Havlíčkova kolonie: lawn in a city park	48°57'43.2"N	14°28'40.8"E	400	20	*S. rubra* 4x
HorMez	Českomoravská vrchovina highlands, Horní Meziříčko: grassy playground in the village	49°09'19.0"N	15°14'29.7"E	580	19	*S. rubra* 4x
HorNovos*	S Bohemia, Novosedly: bare bottom of the Horní rybník pond	49°05'21.5"N	14°16'24.6"E	400	21	*S. echinosperma* 4x
Hurka	SW Bohemia, Záboří: bare bottom of the Hůrka pond	49°22'23.0"N	13°50'44.3"E	530	19	*S. echinosperma* 2x
Jensov*	S Bohemia, Písek: bare bottom of the Jenšovský rybník pond	49°19'35.8"N	14°06'35.0"E	400	15	*S. echinosperma* 2x
Klec*	S Bohemia, Klec: lawn in the village	49°05'49.5"N	14°44'56.6"E	420	20	*S. rubra* 4x
Knizeci	S Bohemia, Pištín: bare bottom of the Knížecí rybník pond	49°03'01.9"N	14°19'02.6"E	400	20	*S. echinosperma* 4x
Koclirov	S Bohemia, Smržov: bare bottom of the Koclířov pond	49°04'05.3"N	14°41'42.1"E	430	20	*S. echinosperma* 4x
Kozcin	SW Bohemia, Pačejov: bare bottom of the Kozčínský rybník pond	49°24'10.1"N	13°37'19.6"E	510	17	*S. echinosperma* 4x
Lhota	SW Bohemia, Horažďovická Lhota: bare bottom of the Lhota pond	49°21'30.0"N	13°40'38.6"E	470	17	*S. echinosperma* 4x
Luznice*	S Bohemia, Lužnice: road margin in the village	49°03'46.0"N	14°45'37.5"E	420	24	*S. rubra* 4x
Maj	S Bohemia, České Budějovice, Máj: sandy playground	48°59'20.2"N	14°26'08.5"E	400	20	*S. rubra* 4x
Malobor*	SW Bohemia, Sedlice: bare bottom of the Malobor pond	49°22'00.4"N	13°58'32.0"E	460	20	*S. echinosperma* 2x
Pecihradek	W Bohemia, Plzeň, Pecihrádek: field margin	49°46'06.5"N	13°24'57.0"E	330	22	*S. rubra* 4x
Pisek	S Bohemia, Písek: edge of a quarry 3 km E of the town	49°19'00.9"N	14°11'16.1"E	590	21	*S. rubra* 4x
Pracejov	SW Bohemia, Katovice: bare bottom of the Pracejovický rybník pond	49°15'18.7"N	13°50'42.0"E	420	20	*S. echinosperma* 4x
Smrzov*	S Bohemia, Smržov: bare bottom of the Vydýmač u Smržova pond	49°04'44.4"N	14°40'47.5"E	440	15	*S. echinosperma* 4x
StHlina*	S Bohemia, Stará Hlína: road margin in the village	49°02'31.9"N	14°48'36.5"E	430	20	*S. rubra* 4x
Strmilov	Českomoravská vrchovina highlands, Strmilov: crevices in square paving in the village	49°09'32.8"N	15°12'07.0"E	560	20	*S. rubra* 4x
Veselsky	Českomoravská vrchovina highlands, Nové Veselí: bare bottom of the Veselský rybník pond	49°31'17.2"N	15°54'15.2"E	560	21	*S. echinosperma* 4x
Vlkov	S Bohemia, Vlkov: sandy field margin 1.2 km NNW of the village	49°09'36.9"N	14°42'57.0"E	420	20	*S. rubra* 4x
Zavlekov	W Bohemia, Zavlekov: lawn in the village	49°20'20.5"N	13°29'36.2"E	570	20	*S. rubra* 4x