Jihočeská univerzita v Českých Budějovicích

Přírodovědecká fakulta

Diplomová práce

A cytological, morphometric, and ecological study of *Spergularia echinosperma* in the Czech Republic and its comparison with a closely similar species *S. rubra*

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České Budějovice, 2009

Kúr P. (2010): A cytological, morphometric, and ecological study of *Spergularia echinosperma* in the Czech Republic and its comparison with a closely similar species *S. rubra*. MSc. Thesis, in English. – 27 pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation:

In the present study, I dealt with morphological, cytological, and ecological research on a rare Central-European species *Spergularia echinosperma* and its comparison with a similar weedy species *S. rubra*. Existence of two cytotypes of *S. echinosperma* significantly differing in their morphology was revealed, as well as distinct morphological differences between the two species were found. Moreover, the analyses revealed one possibly hybridogenous population. In addition, both the species and the cytotypes were also proven to display different germination behavior, which I correlate with their individual ecological adaptations.

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5. 1. 2010

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Podpis

Poděkování:

Především děkuji školiteli Milanu Štechovi za všeobecnou pomoc a podporu v průběhu celé práce. Dále pak patří můj velký dík Petrovi Kouteckému a Pavlu Trávníčkovi za výraznou pomoc při laboratorních analýzách vzorků, Alžbětě Manukjanové a Elišce Vicherové za podání pomocné ruky při přípravě klíčících experimentů a Vlastě Jarolímové za pokus o zasvěcení do tajů karyologických metod. Souhrnný dík rovněž patří pracovníkům několika rybářství za jejich spolupráci při hledání vhodných lokalit pro sběr vzorků, stejně jako Pavlíně Bukáčkové ze Správy CHKO Žďárské vrchy za pomoc s vytipováním lokalit na území chráněné krajinné oblasti.

Vlastním textem předkládané práce je rukopis určený ke zveřejnění v odborném periodiku.

Abstract:

Morphological variation, cytotype structure and germination ecology of a rare Central-European species *Spergularia echinosperma* was examined and compared with a common, morphologically similar species *S. rubra*. Material from 27 localities in the Czech Republic was sampled. The evaluation was done using morphometric multivariate and flow cytometry analyses. An occurrence of two cytotypes, diploid (2n = 2x = 18) and tetraploid (2n = 4x = 36), was revealed in *S. echinosperma*, with diploids being considerably rarer than tetraploids. Most of the populations were cytotypically uniform while two of them displayed co-occurrence of the both cytotypes. On the contrary, only tetraploids were found in *S. rubra* in the studied region. Genome size turned out to differ completely between the species, being applicable as an independent character for their distinguishing. Moreover, morphometric analyses revealed a relatively clear separation between both the species and the cytotypes of *S. echinosperma*. Seed color was found to differ fully between the species. Another relatively reliable character was stipule length/width ratio, which turned out to be quite a good predictor for distinguishing of both the species and the cytotypes within *S. echinosperma*. Other acceptable characters were height of the papillae on seed testa for distinguishing between the species, and seed length and papillae density differing significantly between the *S. echinosperma* cytotypes. Based on the results, a tentative key for the identification of the species and cytotypes is presented. The analyses also identified a morphologically transitional population, possibly of hybrid origin. In addition, distinct differences in germination ecology of the species were revealed. The germination behavior corresponded well to expectations based on species life histories and habitat preferences. While the weedy species *S. rubra* germinated rapidly to high percentages without any special treatments, *S. echinosperma*, a species of temporarily exposed pond bottoms, germinated slowly and possessed strong primary innate dormancy, for whose breaking a cold stratification with subsequent fluctuating temperature was required. Slight differences in the germination behavior of the cytotypes were also detected, which are speculated to possibly account for the relative scarcity of the diploids.

V rámci této studie byla zkoumána morfologická variabilita, cytotypová struktura a ekologie klíčení vzácného středoevropského druhu *Spergularia echinosperma*. Zároveň bylo provedeno srovnání s morfologicky podobným běžným druhem *S. rubra*. Analyzované rostliny pocházely z 27 lokalit na území České republiky. Ploidní úroveň rostlin byla zjišťována pomocí průtokové cytometrie a jejich morfologická variabilita byla vyhodnocována za pomoci mnohorozměrných statistických metod. U *S. echinosperma* byl zaznamenán výskyt dvou cytotypů, diploidního (2n = 2x = 18) a tetraploidního (2n = 4x = 36), přičemž diploidi byli znatelně vzácnější než tetraploidi. Většina populací byla cytotypově uniformní, avšak ve dvou z nich byl zaznamenán výskyt obou cytotypů pohromadě. Naproti tomu, u druhu *S. rubra* byli ve zkoumané oblasti nalezeni pouze tetraploidní jedinci. Analýzy průtokovou cytometrií odhalily, že velikost genomu je spolehlivým znakem pro odlišení tetraploidů obou druhů. Morfometrické analýzy rovněž prokázaly signifikantní rozdíly jak mezi druhy, tak mezi cytotypy *S. echinosperma*. Spolehlivým znakem odlišujícím druhy se ukázala být barva semen. Kromě toho byl relativně spolehlivým znakem poměr délky ku šířce palistů, který dobře odlišoval nejen oba druhy, ale i cytotypy v rámci *S. echinosperma*. Dalším docela dobrým znakem pro odlišení druhů byla výška bradavek na osemení, hustota bradavek a velikost semen se naopak zřetelně lišily mezi cytotypy *S. echinosperma*. Na základě výsledků morfometrických analýz byl sestaven orientační klíč k určování druhů a cytotypů. Analýzy však rovněž odhalily jednu

morfologicky přechodnou populaci, která vykazovala hodnoty znaků intermediární mezi S. *echinosperma* a *S. rubra*. Vysvětlením zjištěného charakteru populace by mohl být její hybridní původ. Vedle morfologických rozdílů byly mezi druhy rovněž za pomoci experimentu zjištěny rozdíly v klíčení. Průběh klíčení odpovídal předpokladům založeným na rozdílu v ekologii druhů. Zatímco semena plevelného druhu *S. rubra* klíčila rychle a ve velkém množství bez potřeby jakéhokoli zásahu, *S. echinosperma*, druh dočasně obnažených rybničních den, vykazovala silnou primární dormanci, pro jejíž překonání byla potřebná chladná stratifikace s následným denním střídáním teplot. Navíc byly zjištěny i drobné rozdíly v průběhu klíčení mezi cytotypy *S. echinosperma*, které by potenciálně mohly mít za následek relativní vzácnost diploidního cytotypu.

Introduction

Spergularia (sand-spurrey, family *Caryophyllaceae*) is a nearly cosmopolitan genus of predominantly halophilous herbs of coastal salt marshes. It is distributed from temperate to subtropical climatic zones of the both hemispheres and on all continents except Antarctica (Friedrich 1979, Hartman & Rabeler 2005, Monnier & Ratter 1993). However, it reaches its greatest diversity in the temperate climatic zone of South America and the Mediterranean region (Ratter 1976, Rossbach 1940). Despite its nearly worldwide distribution, the genus has been taxonomically studied only to a limited extent. This is also reflected in the fact that estimates of the total count of species range from ca 20 (Dvořák 1990, Friedrich 1979) up to about 60 (Hartman & Rabeler 2005, Bittrich 1993).

In Central Europe, the genus is represented by five species (Dvořák 1990, Friedrich 1979). *Spergularia maritima* and *S. salina* are halophytes whose distribution in the continental Central Europe is restricted to scarce inland saline areas only, and *S. segetalis* is a Western European species that reaches the east border of its distribution area in Central Europe. The remaining two species are in focus of our study. *Spergularia rubra* is the most common species of the whole genus, occupying mainly human-affected habitats like road margins or sandy field paths. On the contrary, *S. echinosperma*, a morphologically very similar (and possibly closely related, Hadač 1977) species, is a very rare one endemic to Central Europe (Dvořák 1990, Friedrich 1979). It grows exclusively on bare sandy bottoms of mesotrophic water reservoirs such as ponds, or sandy banks of great rivers. The center of its distribution is located in the South-West Bohemian pond areas. The species is strongly dependent on the pond management, especially on the length of the drying period. Summer drying (i.e. leaving the fishpond without water, usually from spring to the beginning of summer) used to be a widely practiced method for improving water quality (to stop putrefying processes) and getting rid of fish parasites in the past (Šumberová et al. 2005, Šumberová et al. 2006), but it has been vastly abandoned due to increasing intensification of fishpond management in the recent years. As a result, many plant species of exposed pond bottoms, including *S. echinosperma*, have become rare and endangered (Šumberová et al. 2005, Šumberová 2003).

Spergularia echinosperma was originally described by Čelakovský (1881) as a subspecies of *S. rubra*, from which it was distinguished primarily by black bristly seeds and short, widely triangular stipules (*S. rubra* possesses slightly verrucose brown seeds and long stipules). Later, Ascherson & Graebner (1893) moved it to the species rank, which has been generally accepted up to the present time (Friedrich 1979, Monnier & Ratter 1993). Dvořák (1979, 1990), as the only author, conducted a more detailed study of the species taxonomy and its comparison with *S. rubra* (Table 1). He noticed great morphological variability of *S. echinosperma*, often accompanied by occurrence of plants possessing some characters typical for *S. rubra*. These transitional plants, moreover, fell into several distinct morphotypes displaying diverse combinations of some traits, especially seed color and length of stipules, fruit pedicels and stems (Dvořák 1989, 1990). The author explained the existence of morphologically transitional individuals by interspecific hybridization and formally described the hybrids as *S. ×kurkae* (Dvořák 1989, Table 1). However, the research was based on a few quantitative characters and field experience of the author only, and it lacks a more exact verification.

Table 1. – Values of key morphological characters of *S. echinosperma*, *S. rubra* and their hybrid *S. ×kurkae* as they were described by Dvořák (1989, 1990).

Character [unit]	*S. echinosperma*	*S. rubra*	*S. ×kurkae*
Stem length [cm]	(2.5–)3.0–5.0(–8.0)	(2–)5–15(–27)	5–14 or 18–25
Leaf length [mm]	8–23 (lower leaves 19–35)	(5–)7–15(–30)	–
Leaf length compared to the internode length	equally long as internodes	shorter or longer than internodes	–
Stipule length [mm]	0.6–1.3 (width 1.5–1.8)	3–5	0.9–1.5 or 1.1–3.7 or 2.0–3.4
Flower (fruit) pedicel length [mm]	(4–)6–12(–16)	3–8(–19)	(8–)11–16(–19) or 2–5
Sepal length [mm]	2.2–3.5	2.2–4.5	3.0–4.7
Petal length × width [mm]	2.1–3.2 × 0.7–1.2	1.2–3.6 × 1.0–2.3	2.0–3.6 (length only)
Anther length [mm]	0.1–0.5	0.3–0.9	0.4–1.1
Pollen grain length × width [μm]	20–23 × 18–21	22–25(–26) × 21–23	–
Capsule length [mm]	2.8–3.8	2.8–5.5	3.2–4.7
Seed length × width [mm]	(0.3–)0.4–0.5(–0.6) × (0.2–)0.3–0.4(–0.5)	(0.4–)0.5–0.6(–0.7) × (0.3–)0.4–0.5(–0.6)	0.38–0.66 × 0.23–0.54
Seed color	black	brown	black to dark brown
Testa	densely covered with prickly papillae	covered with cylindrical papillae	densely covered with papillae
Papilla height [mm]	0.02–0.04	0.01–0.03	up to 0.04

In addition to morphological studies, Dvořák & Dadáková (1984) carried out chromosome counting of a limited number of *S. echinosperma* specimens. They detected only diploid plants (2n = 2x = 18), which was found useful for distinguishing this species from predominantly tetraploid *S. rubra* (2n = 4x = 36; although there have been some records of diploid and hexaploid plants out of Central Europe [Ratter 1964, Fernandes & Leitao 1971]). Additionally, Dvořák (1989) reported tetraploid state for the assumed hybrid *S. ×kurkae*. In our preliminary cytotype screening of a few *S. echinosperma* populations, however, we found most of the individuals to be tetraploid, together with a minority of diploids (Kúr 2007). Hence, the morphological variation of the species has needed to be reevaluated with regard to its cytotype structure.

As stated above, the two species differ considerably in their ecology as well. *Spergularia rubra* is a weedy species widespread in ruderal habitats, where it is usually able to survive for more generations acting as a short-lived perennial (Friedrich 1979, Hartman & Rabeler 2005). On the other hand, the life cycle of *S. echinosperma* is strongly limited by usually ephemeral existence of a bare pond bottom in spring. As in the case of most other plants of exposed pond bottoms (Šumberová 2005, Salisbury 1970), a persistent seed bank is important for the species survival during the time of flooding. Therefore, we decided to supplement the morphological analyses with a comparison of germination behavior of the species. Not only are the ecological experiments useful to sketch in the differences between the species more overall, but also these two (possibly closely) related species with distinctly contrasting life histories represent a suitable model for studying adaptive significance of

species traits, which is one of the main goals of current ecological research (Lavorel & Garnier 2002). We tested differences in germination behavior as one of the most important species traits, which is essential for a species survival and is to reflect its adaptations to a particular biotope (Baskin & Baskin 1998, Fenner & Thompson 2005). In addition, knowledge of the existence of the *S. echinosperma* cytotypes allows us to assess their germination properties separately. Possible revealed differences between them may shed light on some aspects of their ecology and population dynamics.

The main objectives of this study were:

1) To carry out a complex cytotype screening of *S. echinosperma* populations to learn more about the distribution of the particular cytotypes.
2) To evaluate the morphological variation of *S. rubra* and *S. echinosperma* cytotypes to determine which morphological characters (if any) distinguish the species and cytotypes, and to clarify the existence of transitional populations.
3) To compare germination ecology of the species and cytotypes under contrasting environmental conditions.

Material and Methods

Sampling

515 plants from 27 localities of both *S. echinosperma* (14 localities) and *S. rubra* (13 localities) were sampled (both for morphometric and flow cytometry analyses) in the Czech Republic during two growing seasons in 2008 and 2009. We tried to sample an equivalent number of populations of both species. The final number of populations, however, was strongly limited by the availability of suitable dried pond bottoms at the time of *S. echinosperma* ripeness (end of May to June). The species were initially defined on the basis of seed color (*S. echinosperma* black, *S. rubra* brown). The sampling localities were situated in South and Western Bohemia and, to a lesser extend, Českomoravská vrchovina highlands (see Appendix 1 for an overview of the localities and their acronyms used in the text). Only mature individuals with ripe capsules were collected. The numbers of plants per population ranged from 15 to 24 with the exception of the population *Cakov* (*S. rubra*) where only 3 plants were found. However, this population displayed habitat occurrence untypical for *S. rubra* (bare pond bottom) and unusual morphological characteristics, so it was included too.

The holotype specimen of the assumed hybrid *S.* ×*kurkae* from the Herbarium of the South Bohemian Museum in České Budějovice (CB) was also used for the morphometric analyses. Herbarium specimens of the investigated plants are deposited in the Herbarium of the Faculty of Science, University of South Bohemia in České Budějovice (CBFS).

Chromosome counting

Several plants of *S. rubra* (population *StHlina*), *S. echinosperma* tetraploids (population *Smrzov*) and *S. echinosperma* diploids (population *Malobor*) were used for determining the chromosome numbers in order to confirm the flow cytometry results. Chromosomes were counted in actively growing cells of the apical meristem. Samples were pretreated with a saturated solution of p-dichlorbenzene (3 h, room temperature), fixed in a 3:1 mixture of ethanol and acetic acid overnight and stained with lacto-propionic orceine. The number of chromosomes was ascertained using a light microscope under 1000× magnification.

Flow cytometry

Flow cytometry was employed for estimating the relative DNA content and ploidy level of all sampled plants. The measurements were done using a Partec Ploidy Analyser II (Partec GmbH, Münster, Germany) at the Institute of Botany, Academy of Sciences in Průhonice in the season 2008 and at the Faculty of Sciences, University of South Bohemia in České

Budějovice in 2009. For the first analyses, we measured groups of 5 to 10 plants together. Due to problems with analyses quality and endopolyploidy in *S. echinosperma* samples, however, we decided to measure each individual separately. *Spergularia rubra* displayed no problems, so it was analyzed by 5 plants. Total of 225 samples (from the 515 analyzed plants) was measured.

We used the two-step procedure of nuclear isolation and staining (Otto 1990) slightly modified for plant tissues (Suda et al. 2007). Fresh leaves were used for the analyses. A few leaves (typically two or three) were finely chopped together with the appropriate amount of an internal DNA standard plant using a new razor blade in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). *Glycine max* cv. Polanka was used as the internal standard (2C = 2.50 pg, Doležel et al. 1994). The suspension was filtered through a nylon mesh (42 μm), and 1 ml of staining solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), fluorochrome (4 μg/ml DAPI) and β-mercaptoethanol (2 μl/ml) was added. The staining took 1–2 min. Fluorescence intensity of 5000 particles was recorded and the coefficient of variation (CV) and nuclei count for the analyzed plant and standard in each analysis were calculated. Only histograms with nuclei count for both the analyzed plant and the standard higher than 400 were considered reliable enough to be used for statistical comparisons of the relative genome sizes (150 samples with 237 plants). Samples of worse quality were used for assessing the ploidy level only. The one-way ANOVA in Statistica 8 (StatSoft 1998) was applied for the between-group comparisons of the relative genome sizes.

Since there are no records for the genus *Spergularia* in the plant DNA C-values database (Bennett & Leitch 2005), we decided to determine the species genome sizes in absolute units as well. The same method, but with fluorochrome propidium iodide (PI) together with RNase IIa (both in a final concentration of 50 μg/ml) replacing DAPI fluorochrome in the staining solution, was used for estimation of the absolute genome size. *Lycopersicon esculentum* cv. Stupické polní tyčkové rané (2C = 1.96 pg, Doležel et al. 1992) was used as the internal standard. Plants from nine populations (population *Driten* containing both diploids and tetraploids was split into two pseudo-populations according to the ploidy level), three populations for each group, grown from seeds in a climabox were used for absolute genome size measurements (see Appendix 1). Three plants from each population were analyzed, and each plant was repeatedly measured on three different days. If the variation between individuals exceeded 2%, additional measurements were performed and the most out-layered measurement excluded. Statistical evaluation of differences between the groups was performed by nested-design ANOVA in Statistica 8 (StatSoft 1998) with population nested in group.

Morphometry

In total, 18 quantitative and derived ratio characters, mainly from the seeds and vegetative parts of plants, were used for the analyses (Table 2). We chose some diagnostic characters reported by Dvořák (1979, 1990) and other important characters observed in the field. Unfortunately, it was not possible to record floral characters due to scarcity of flowering plants in the time of fruit ripeness. All characters with multiple occurrence on a plant were measured repeatedly, optimally three times (assuming the individual was big enough), and the mean values were used for analyses. Stipules were selected from the lower, middle and upper part of a stem, respectively. Since no significant differences between stipules from the particular parts were found, the final value was obtained by their averaging. Leaves were selected randomly from lower and middle parts of a plant (where the leaves were fully developed). For measurements of fruit pedicels and capsules, only those from the lowermost parts of a plant were used.

Seed dimensions and the length of the papillae 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 papilla upper part (usually broadened in *S. echinosperma* resembling to a "head") and the width of the lower part ("neck"). Papillae without the head wider than the neck were assigned the value of 1. Papillae density (*PapNum*) was expressed as the number of visible papillae to one quarter of the seed circumference. Three seeds, each from a different capsule, and three papillae per seed were measured.

The recorded data were processed by multivariate statistical analyses. CANOCO for Windows 4.5 (ter Braak & Šmilauer 2002) was used to conduct Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA). For the classificatory analysis, the statistical package R 2.8.1 (R Development Core Team 2008) was used as well as it was used for the classification trees. Statistica 8 (StatSoft 2008) was employed for the basic tests (normality testing, correlation matrix) and visualization of some results.

Prior to running multivariate analyses, a correlation matrix was computed (Spearman correlations) to detect groups of strongly correlated characters. Only one character from each of these groups was retained for further analyses. Subsequently, normality of the characters was tested (Shapiro-Wilk test). Characters most deviating from normality were log-transformed (see Results), which is a prerequisite for some of the multivariate methods used (PCA, discriminant analyses).

PCA was employed to get an insight into the overall pattern of variation and for visual inspection of morphological discontinuities among the groups. To find out which characters significantly separated the groups, LDA with forward selection (Monte-Carlo permutation test with 999 permutations) was applied. The linear discriminant functions were calculated from the selected characters and their predictive ability was tested in R by cross-validation using each population as a leave-out unit (user written function employing the lda function from the MASS package). Predictive ability was expressed as the percentage of correctly classified samples. For comparison, the classification test was repeated with all the characters recorded.

Besides the discriminant analysis, classification trees method was used for better visualization of the classification rules. Classification trees represent a non-parametric alternative to discriminant analysis. They work with a single character in each step finding the optimal rule for division into two subgroups. The result is a classificatory tree with classification rules in each branch, resembling to a determination key (Breiman et al. 1984). Similarly to LDA, classification tree algorithms use a cross-validation for estimating the optimal tree size where there is the smallest error rate. For the analysis of our data, we used the rpart function (rpart package) in R modified so that it used populations as the cross-validation units (original implementation divides the data set into several subsets in random).

Table 2. – Characters used in the morphometric analyses of *S. echinosperma* and *S. rubra*.

Acronym	Character [unit]
LeafLeng	length of leaves in the lower part of a stem [mm]
LeafWidt	width of leaves in the lower part of a stem [mm]
InterLen	length of the internodes adjacent to the measured leaves [mm]
Int-Leaf	ratio of the internode length and leaf length
FrPedLen	lower fruit pedicel length [mm]
CapsLeng	length of the adjacent capsule [mm]
Ped-Cap	ratio of the pedicel length and capsule length
StemsNum	number of stems (counted at the base)
StpWd	stipule width [mm]
StpLt	stipule length [mm]
StpRT	ratio of the stipule length and stipule width
PlHeight	height of the longest stem [cm]
LengSed	seed length [µm]
WidtSed	seed width [µm]
SedRat	ratio of the seed length and seed width
PapHei	papillae height [µm]
PapRat	ratio of the papilla upper part (head) width and papilla lower part (neck) width (papilla shape)
PapNum	number of papillae to one quarter of the seed circumference (papillae density)

Germination

Germination was tested on seeds collected from the nine populations used for the measurements of absolute genome sizes (three for each of the groups *S. echinosperma* diploid, tetraploid and *S. rubra*) in 2008 (see Appendix 1). Before conducting the experiment, the seeds were stored under dry conditions and room temperature for a half year.

Five treatments were tested. Two treatments involved wet chilling in sand-filled Petri dishes at 5°C in dark (100 seeds per dish). After chilling cessation, dishes were transferred to a climabox with a constant temperature (20°C) and 12/12 h light period, where they were permanently left (first treatment) or nightly transferred to a dark cool box at 5°C to simulate temperature fluctuation (second treatment). At the other two treatments, chilling was replaced by freezing in -15°C since we speculated that low temperatures could promote germination by breaking physical dormancy as it has been observed in many other species (Baskin & Baskin 1998), while subsequent temperature regimes were the same as in the previous case. The last treatment was control. Moreover, all treatments were combined with two different lengths of chilling/freezing, namely 6 and 12 weeks. Three replications were made for each treatment combination. Number of germinated seeds was recorded 6 times in 4-day intervals. A seed was considered germinated when a small seedling with two cotyledons emerged.

Each dish was characterized by three characteristics. Firstly, it was the final percentage of germinated seeds (germination percentage). Secondly, the germination rate was calculated as the time in days when half of the final number of germinated seeds had already germinated (T_{50}). Since germination was recorded in 4-day intervals, a more accurate estimate of T_{50} was obtained by linear interpolation in the interval where the half of germinated seeds

fitted into. Thirdly, germination delay was expressed as the number of the counting day (1 to 6) when first occurrence of germinated seeds was recorded.

Differences between treatments were evaluated using the appropriate ANOVA model with Statistica 8 (StatSoft 1998), for the final percentage of germinated seeds, germination rate T_{50}, and germination delay separately. The proportion of germinated seeds was subjected to angular transformation (arcsin√x), and the germination rate and delay were log-transformed. Cases with no germinated seeds were omitted from the T_{50} and germination delay analyses, resulting in unbalanced ANOVA (where some treatment combinations were completely missing due to zero germinability, see Results). Treatment and length were interacting main-plot factors, while population was a random effect factor nested in group and interacting with the main-plot factors too. If the main effects were significant, the Tukey HSD test was used for multiple comparisons.

Results

Chromosome counting

Chromosome counting of *Spergularia* samples turned out to be problematic. No mitosis of sufficient quality to ascertain the precise number of chromosomes was found, although various combinations of pretreatment durations and chemical agents (α-bromonaphthalene, colchicine, and 8-hydroxyquinoline) were tested. In the result, number of chromosomes could be estimated only roughly, which, however, was sufficient for distinguishing between diploids and tetraploids. So, tetraploid state for *S. rubra* and *S. echinosperma* tetraploids and diploid state for *S. echinosperma* diploids were confirmed.

Flow cytometry

The ploidy levels of all 515 plants were successfully determined. Samples from 9 populations of *S. echinosperma* turned out to be completely tetraploid; in 3 populations they were completely diploid, and those of the remaining 2 populations were mixed (i.e. they contained both tetraploid and diploid plants). The tetraploid/diploid ratios of the samples from the two mixed populations were 14:6 (*Driten*) and 19:1 (*Cky*). By contrast, all analyzed plants of *S rubra* were exclusively tetraploid (Fig. 1).

Tetraploids of *S. echinosperma* and *S. rubra* differed completely in their genome sizes with *S. echinosperma* possessing the larger genome (Table 3, Fig. 2). Comparison of the taxa based on absolute genome size showed 8.3% difference, while the difference in relative genome size was 7.8%. Monoploid genome size (hereafter referred to as absolute g. s.) differed significantly among all three groups ($F_{2, 17} = 1209.9$, $p < 0.001$). Tetraploids of *S echinosperma* had significantly lower monoploid genome size than diploids, with the average difference of 3.3%. Additionally, one plant of *S. rubra* (population *Luznice*) displayed distinctly lower genome size than the rest of the group, differing by 2.5%, which could possibly indicate an aneuploid plant (Table 4). Hence, this individual was excluded from the statistical analyses. Unfortunately, verification of the chromosome count was not possible due to difficulties with karyological samples preparation.

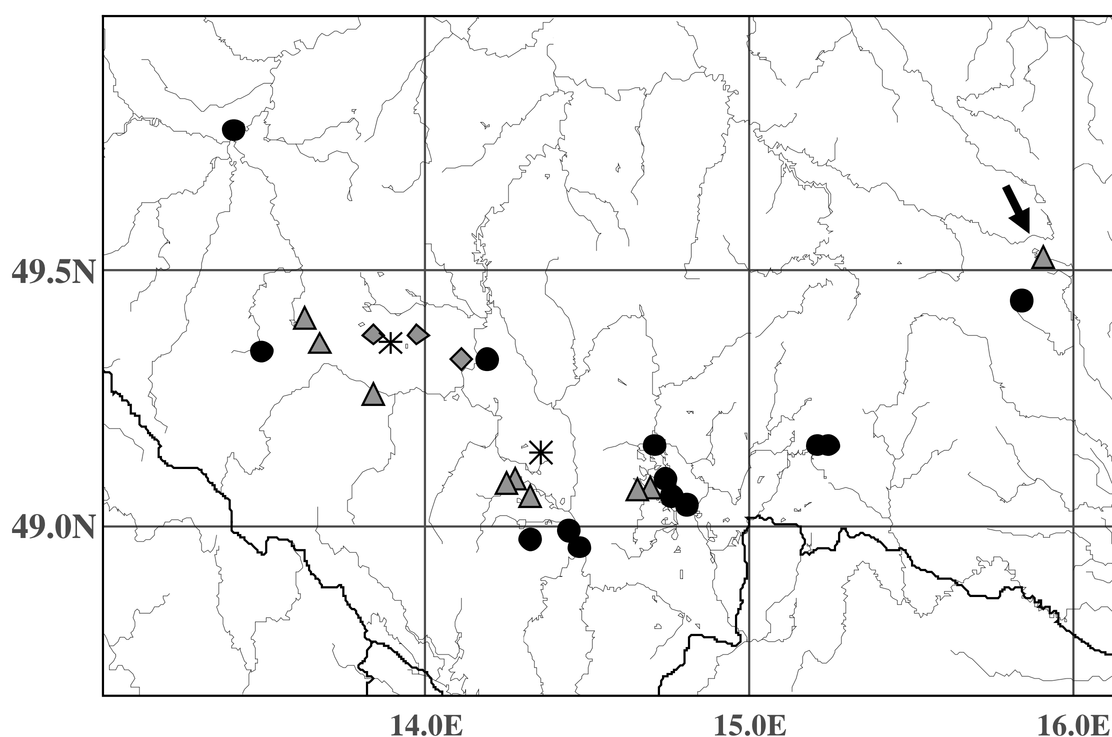

Fig. 1. – Distribution of the studied populations of *S. echinosperma* and *S. rubra*. ● *S rubra* tetraploids, ▲ *S. echinosperma* tetraploids only, ◆ *S. echinosperma* diploids only, ✱ *S echinosperma* diploids and tetraploids mixed. The arrow denotes the morphologically transitional population *Veselsky*.

Table 3. – Summary of the 2C relative genome sizes for *S. rubra*, *S. echinosperma* tetraploids and population *Veselsky* compared to the standard *Glycine max* (given as unit relative genome size). N – number of samples; SD – standard deviation; CV – range of coefficients of variance values for sample peaks. One-way ANOVA revealed significant differences between the groups ($F_{2,\ 126} = 376.3$, $p < 0.001$); different letters at mean values indicate groups that differ significantly at $p < 0.001$ (Tukey HSD test).

Group	N	Min	Max	Lower quartile	Upper quartile	Mean	Median	SD	CV (%)
S. rubra	16	0.395	0.415	0.406	0.411	0.408 a	0.408	0.005	2.6–4.9
S. echinosperma 4x	92	0.430	0.450	0.437	0.443	0.440 b	0.441	0.004	2.5–4.9
Veselsky	21	0.429	0.442	0.434	0.438	0.436 c	0.436	0.003	2.9–4.0

Table 4. – Summary of absolute genome sizes for *S. echinosperma* cytotypes and *S. rubra* (in picograms of DNA). *S. rubra* Outlier denotes the possibly aneuploid plant not included in the ANOVA. N – number of samples; SD – standard deviation; SE – standard error of mean; CV – range of coefficients of variance values for sample peaks. Different letters at mean Cx values indicate groups that differ significantly at $p < 0.001$ (Tukey HSD test).

Group	N	2C values							Cx value
		Min	Max	Mean	Median	SD	SE	CV (%)	Mean
S. echinosperma 2x	9	0.621	0.633	0.627	0.628	0.004	0.001	3.9–6.4	0.314 a
S. echinosperma 4x	9	1.210	1.224	1.217	1.218	0.004	0.001	3.1–6.3	0.304 b
S. rubra	8	1.120	1.127	1.124	1.125	0.002	0.001	2.9–4.9	0.281 c
S. rubra Outlier	1	-	-	1.097	-	-	-	3.4–5.2	0.274

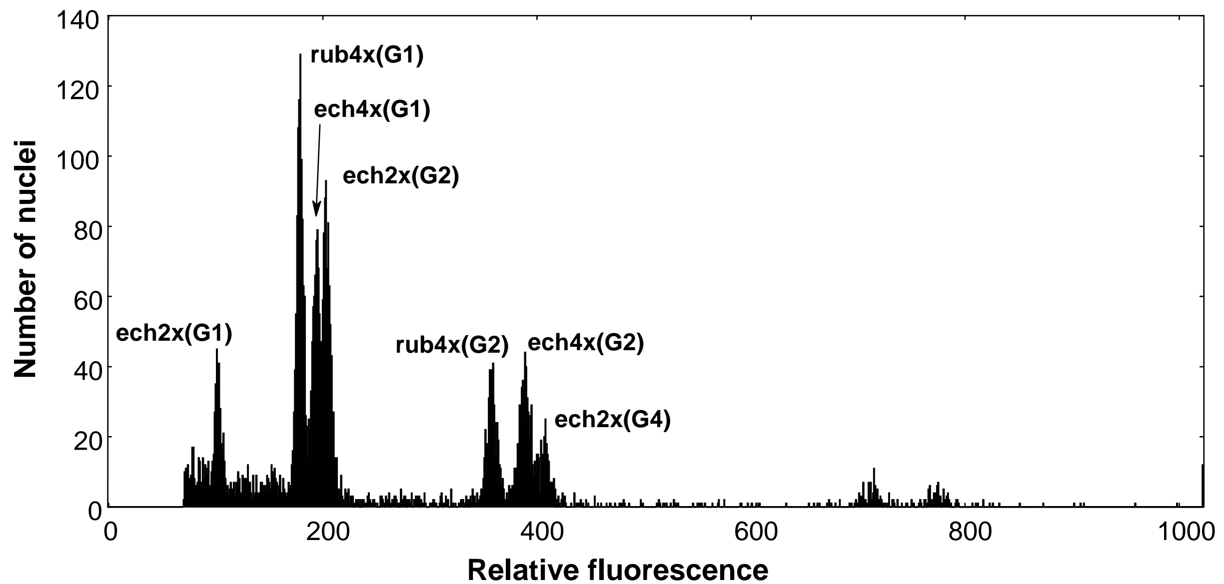

Fig. 2. – Histogram of the relative DNA content obtained during a simultaneous flow cytometry analysis of a *S. rubra* (rub4x), *S. echinosperma* tetraploid (ech4x) and *S. echinosperma* diploid (ech2x). Symbols in brackets indicate cell cycle phase of a particular peak.

Morphometry

Correlation matrix revealed three groups of strongly positively correlated characters (correlation coefficient exceeding 0.8). From the first group of two correlated characters, capsule pedicel length (*FrPedLen*) and pedicel-capsule length ratio (*Ped-Cap*), only *FrPedLen* was kept for further analyses because of its better predictive power. Other two strongly correlated characters were stipule length (*StpLt*) and stipule length-width ratio (*StpRT*). *StpRT* turned out to be a better predictor (and with more sensible applicability in filed determination), so it was retained. The last group of correlated characters comprised seed length (*LengSed*) and seed width (*WidtSed*), from which only *LengSed* was used.

Normality of the included characters was tested. The values of characters *LeafLeng*, *InterLen*, *Int-Leaf*, *LeafWidt*, *FrPedLen*, *CapsLeng*, *StemsNum*, *StpRT*, *PlHeight*, *LengSed*, *SedRat*, and *PapRat* were subjected to log-transformation for improving their fit to normal distribution.

The main pattern of variation in the data set and its relationship to the cytotype identity of the plants were examined by PCA (see Fig. 3). The ordination diagram shows a distinct separation of both the species (along the first and second ordination axes) and the cytotypes of *S. echinosperma* (along the third axis), although there is some overlap of the groups.

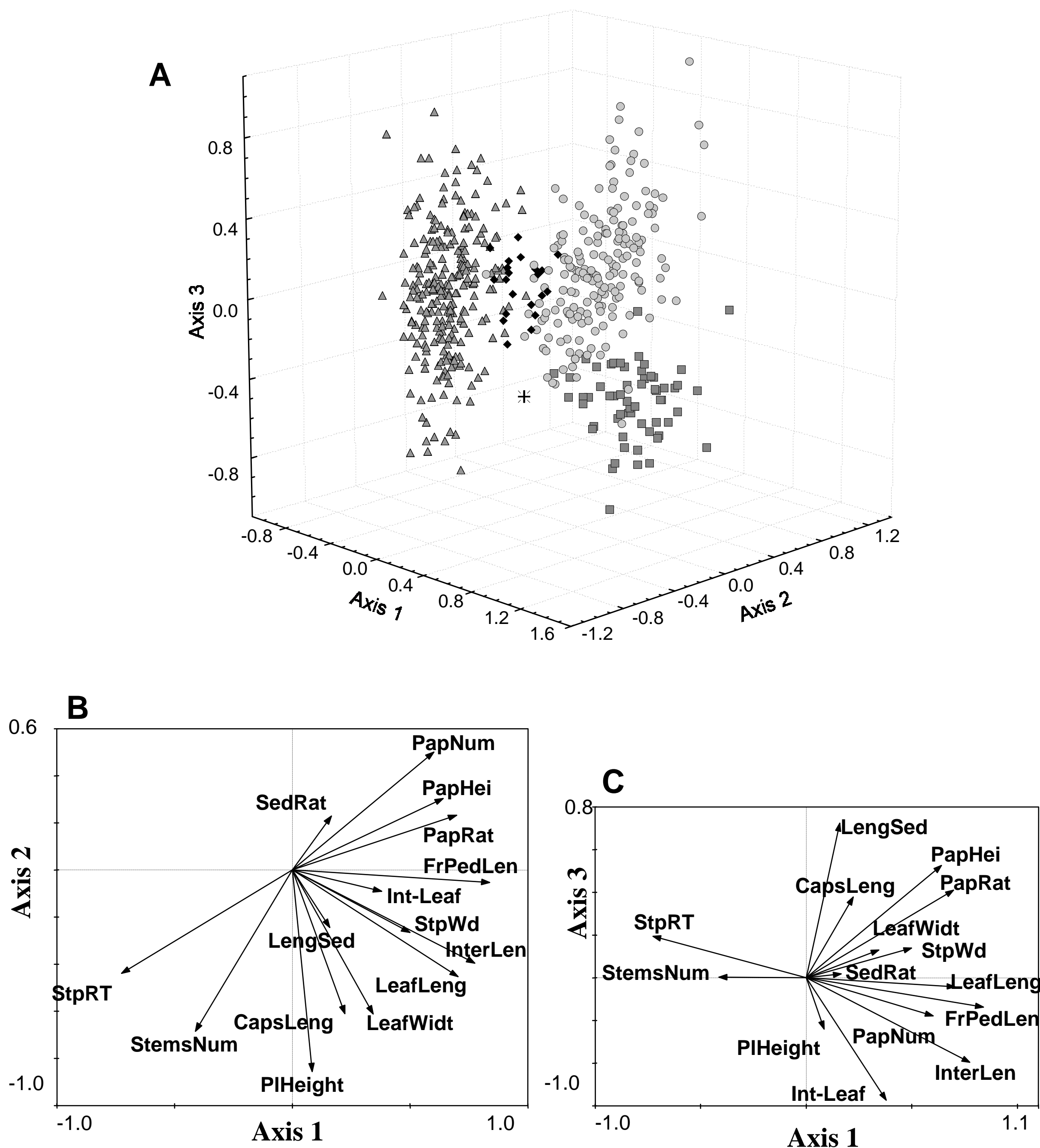

Fig. 3. – PCA ordination diagram of specimens (A) and characters (B, C) of *S. rubra* (▲), *S echinosperma* tetraploids (●), *S. echinosperma* diploids (■), population *Veselsky* (◆), and *S.* ×*kurkae* holotype (+). The first, second and third axes account for 29.1%, 20.6% and 11.9% of the variability, respectively.

Remarkably, one population assigned to tetraploid *S. echinosperma* (*Veselsky*) deviated from the pattern and was morphologically transitional between *S. echinosperma* and *S. rubra*, indicating possible hybrid origin. Therefore we used this population as a separate group in the comparisons of relative genome sizes (Table 3). Although it displayed significantly lower genome size than the group of tetraploid *S. echinosperma*, this difference was minute (mean difference of 0.9%) and is probably just an artifact caused by unbalanced numbers of samples within the compared populations (due to restricting criteria on the histogram quality). Practically all genome size values of the population *Veselsky* fitted into

the range of the values for tetraploid *S. echinosperma*. Because of its possible hybrid origin, population *Veselsky* was excluded from the discriminant and classification analyses.

Similarly, the holotype of *S. ×kurkae* also displayed transitional values of the morphological characters.

LDA was employed to test the significance of the discriminatory power of the 15 selected characters. Marginal effects (i.e. effects of each character as if it was the only predictor in the analysis) of all the characters were highly significant ($p < 0.001$). The forward selection identified 12 characters that contributed most to the prediction of group membership (Table 5, Fig. 4). Both standardized canonical coefficients (denoting the partial contribution of each variable to the discriminant functions) and factor structure coefficients (denoting the correlations between variables and the functions) are presented (Table 5). The best predictors were primarily stipule length/width ratio (*StpRT*), differing considerably between all 3 groups, papillae height (*PapHei*) for discrimination between the species, and papillae density (*PapNum*) and seed length (*LengSed*) discriminating between diploids and tetraploids of *S. echinosperma*. See Table 6 for summary of all measured characters.

The predictive ability of the 12 selected characters was tested by a classificatory analysis. Posterior probabilities for all samples were computed using a cross-validation, and the percentage of misclassified samples in each group served as the predictive ability measure. Only samples of *S. rubra* were classified correctly at 100%. *Spergularia echinosperma* was erroneously classified in 3.7% of cases. 4.9% of diploids were incorrectly classified as tetraploids while tetraploids displayed 1.1% of samples erroneously assigned to *S. rubra* and 2.2% of samples misclassified as diploids. A comparative analysis using all 15 morphological characters resulted in slightly worse classification due to emergence of one sample of *S. rubra* misclassified as *S. echinosperma* tetraploid (misclassification pattern in *S. echinosperma* remained the same).

Classification tree confirmed the prime role of stipule length/width ratio. It can be roughly stated that stipules shorter than wide led to *S. echinosperma* diploids while longer stipules belonged to *S. echinosperma* tetraploids or *S. rubra*. The boundary between *S. echinosperma* and *S. rubra* was about the ratio of 1.7. Inside *S. echinosperma*, diploids also displayed smaller seeds with more densely papillose testa. Moreover, *S. rubra* differed from tetraploid *S. echinosperma* by lower papillae and higher number of stems (Fig. 5). The overall predictive power of this model was slightly higher than that of the classificatory analysis. 1.2% of samples of *S. rubra* were misclassified as *S. echinosperma* tetraploids while there were 3.2% of misclassified samples of *S. echinosperma* diploids (i.e. two samples, one classified as tetraploid and the other one as *S. rubra*) and 1.5% of samples of *S. echinosperma* tetraploids erroneously classified as *S. rubra*.

Table 5. – Characters selected in the forward selection and the respective standardized coefficients of the discriminant functions (LD1, 2) and factor structure coefficients. LambdaA – eigenvalue representing the effect of each character when added to already selected characters.

Forward selection				Stand. Coeff.		Structure Coeff.	
Character	LambdaA	F	P	LD1	LD2	LD1	LD2
StpRT	0.783	315.088	0.001	-1.6351	0.6011	-0.8833	0.0495
PapHei	0.396	235.400	0.001	0.3194	0.6376	0.5348	0.5193
PapNum	0.098	65.898	0.001	0.8802	-0.6357	0.8046	-0.1167
LengSed	0.072	53.464	0.001	-0.4566	0.494	-0.2007	0.5354
StemsNum	0.050	40.074	0.001	-0.4992	-0.1299	-0.6658	-0.1316
FrPedLen	0.042	36.492	0.001	0.4281	0.7164	0.6044	0.2521
Int-Leaf	0.020	17.526	0.001	0.021	-0.2154	0.2502	-0.1796
PlHeight	0.014	12.390	0.001	-0.0489	-0.5087	-0.3734	-0.1121
PapRat	0.014	12.986	0.001	0.3841	0.3293	0.5577	0.4278
StpWd	0.011	10.514	0.001	-0.3205	0.156	0.1924	0.1553
SedRat	0.011	10.638	0.001	0.3116	-0.1156	0.2567	-0.007
CapsLeng	0.005	5.371	0.017	-0.074	0.1845	-0.2114	0.3423

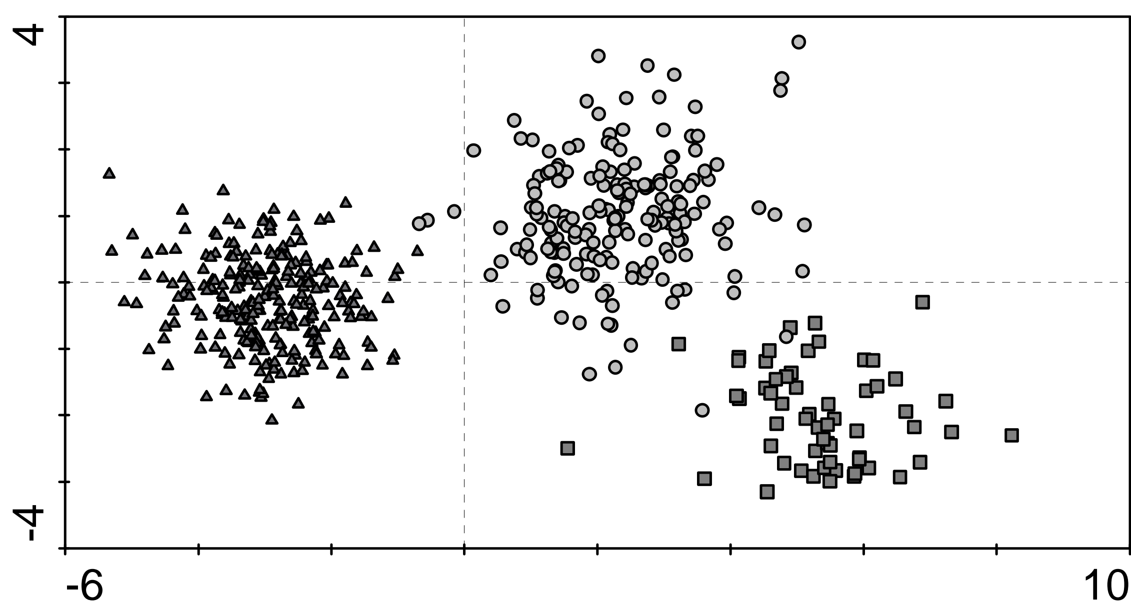

Fig. 4. – Linear discriminant analysis of individuals of *S. rubra* (▲), *S. echinosperma* tetraploids (●), and *S. echinosperma* diploids (■). The two canonical axes extract 45.8% and 30.0% of variation among groups.

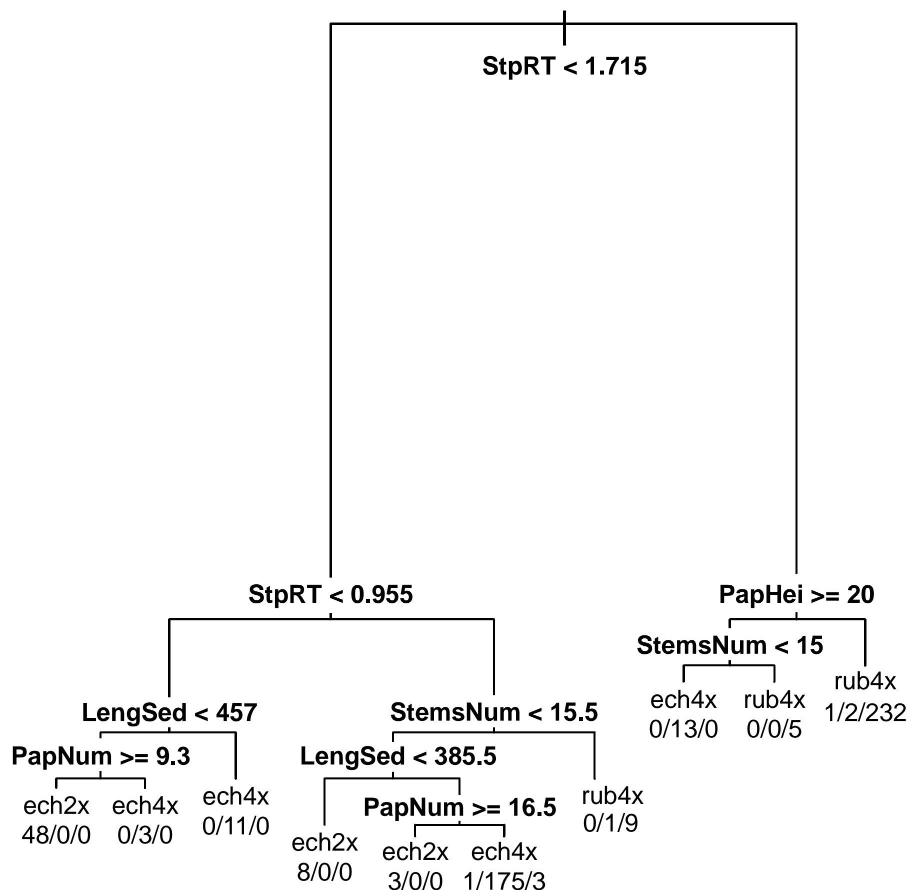

Fig. 5. – Classification tree of samples of *S. rubra* and *S. echinosperma* cytotypes (rub4x – *S. rubra*, ech4x – *S. echinosperma* tetraploids, ech2x – *S. echinosperma* diploids). If a classification rule is fulfilled, the determination continues to the left branch. Length of branches resembles the relative discriminatory power of the adjacent rule. Numbers separated by the slashes signify numbers of samples classified to a particular group (ech2x/ech4x/rub4x).

Germination

There were significant differences in germination, between both the species and the *S. echinosperma* cytotypes (Table 7). Germination percentages ranged from 100% to 0%. No germination was recorded in 76 out of 270 dishes, sometimes occurring in a whole group-treatment combination (see Fig. 6).

Spergularia rubra displayed the highest mean germination percentage (72%). Tetraploid and diploid *S. echinosperma* reached mean germination percentages of only 10% and 6% respectively. Moreover, there were considerable differences between the treatments. The lowest germination was generally observed in the two treatments involving freeze. *Spergularia rubra* had the best germination in the control treatment while both cytotypes of *S. echinosperma* germinated noticeably in the treatment with fluctuating temperature only. In the case of *S. echinosperma* diploids and tetraploids, there were highly significant differences in their reactions to the two lengths of chilling. Diploids displayed higher germination percentages at 12-week chilling compared to 6-week chilling (48% versus 11%), whereas the difference in tetraploids was less pronounced (33% versus 20%). There were also some differences among populations within groups, especially in the reactions of *S. rubra* populations to freezing (see Fig. 7). Besides, population *Smrzov* of tetraploid *S. echinosperma* demonstrated generally increased germination percentages compared to the other two populations.

Table 6. – Summary of all measured morphological characters for *S. rubra* (249 individuals), *S. echinosperma* tetraploids (184 individuals), *S. echinosperma* diploids (61 individuals), and population *Veselsky* (21 individuals). Mean ± standard deviation and a range between lower and upper quartiles (with minimum and maximum in brackets) are shown.

	S. rubra	*S. echinosperma* tetraploid	*S. echinosperma* diploid	Population *Veselsky*
LeafLeng	7.7±3.9 (3.0–)5.4–8.2(–24.8)	11.0±4.8 (2.5–)6.9–13.5(–24.7)	9.4±3.5 (4.1–)6.9–11.1(–18.8)	10.8±2.2 (6.2–)9.0–11.8(–15.2)
InterLen	7.7±5.4 (1.3–)4.3–8.8(–28.4)	11.1±5.9 (2.2–)6.6–14.3(–33.5)	12.0±3.6 (5.9–)9.6–13.9(–26.8)	8.6±2.0 (5.1–)6.8–10.2(–12.2)
Int-Leaf	0.98±0.38 (0.24–)0.73–1.17(–2.86)	1.03±0.37 (0.37–)0.79–1.21(–2.77)	1.35±0.36 (0.78–)1.10–1.56(–2.49)	0.83±0.17 (0.54–)0.76–0.91(–1.37)
LeafWidt	0.6±0.2 (0.2–)0.5–0.7(–1.2)	0.6±0.1 (0.3–)0.5–0.7(–1.1)	0.5±0.1 (0.3–)0.4–0.6(–0.7)	0.7±0.1 (0.5–)0.6–0.7(–0.8)
FrPedLen	3.6±1.4 (1.5–)2.7–4.2(–8.4)	6.9±2.8 (2.0–)5.1–8.1(–23.7)	6.0±1.7 (1.7–)5.1–6.8(–12.5)	4.8±1.2 (3.1–)3.9–5.4(–8.4)
CapsLeng	3.5±0.4 (2.3–)3.3–3.8(–4.6)	3.6±0.5 (2.6–)3.3–3.9(–5.5)	3.0±0.4 (1.9–)2.8–3.2(–4.1)	3.6±0.3 (3.2–)3.4–3.7(–4.4)
Ped-Cap	1.02±0.35 (0.40–)0.78–1.16(–2.37)	1.90±0.71 (0.70–)1.42–2.19(–5.39)	2.02±0.56 (0.68–)1.69–2.31(–3.68)	1.34±0.33 (0.97–)1.11–1.42(–2.39)
StemsNum	18±12 (2–)8–25(–63)	5±4 (1–)2–8(–19)	4±3 (1–)1–7(–19)	9±1 (6–)8–10(–12)
StpWd	1.6±0.2 (1.0–)1.4–1.7(–2.4)	1.7±0.3 (0.8–)1.6–2.0(–2.5)	1.7±0.3 (0.7–)1.5–1.9(–2.3)	1.9±0.3 (1.4–)1.7–2.1(–2.4)
StpLt	3.5±0.5 (2.1–)3.2–3.9(–4.9)	2.2±0.4 (1.3–)1.9–2.4(–4)	1.4±0.2 (1.0–)1.3–1.5(–1.8)	2.2±0.3 (1.7–)2.0–2.4(–3.2)
StpRT	2.34±0.41 (1.43–)2.04–2.60(–4.04)	1.31±0.27 (0.75–)1.12–1.48(–2.09)	0.86±0.24 (0.48–)0.71–0.93(–2.00)	1.28±0.34 (0.64–)1.07–1.56(–2.05)
PlHeight	10±5 (3–)7–11(–31)	7±4 (1–)4–10(–23)	6±2 (3–)4–8(–12)	7±1 (4–)6–7(–9)
LengSed	458±34 (368–)437–480(–564)	475±37 (389–)449–499(–569)	400±25 (349–)381–420(–456)	452±19 (414–)437–458(–495)
WidtSed	355±31 (279–)335–372(–444)	359±30 (251–)339–377(–435)	299±23 (237–)282–310(–359)	345±18 (306–)337–352(–383)
SedRat	1.30±0.07 (1.10–)1.25–1.34(–1.54)	1.33±0.07 (1.17–)1.28–1.37(–1.57)	1.35±0.07 (1.22–)1.30–1.39(–1.55)	1.32±0.05 (1.18–)1.28–1.36(–1.37)
PapHei	16±2 (11–)15–18(–23)	22±3 (15–)20–23(–31)	18±2 (14–)17–19(–23)	19±1 (16–)19–20(–21)
PapRat	1.15±0.10 (1.00–)1.07–1.21(–1.44)	1.49±0.24 (1.09–)1.30–1.64(–2.25)	1.29±0.15 (1.04–)1.17–1.38(–1.78)	1.25±0.15 (1.03–)1.16–1.36(–1.63)
PapNum	7±2 (3–)6–8(–12)	11±2 (7–)10–13(–16)	15±2 (10–)13–17(–20)	7±2 (4–)6–8(–10)

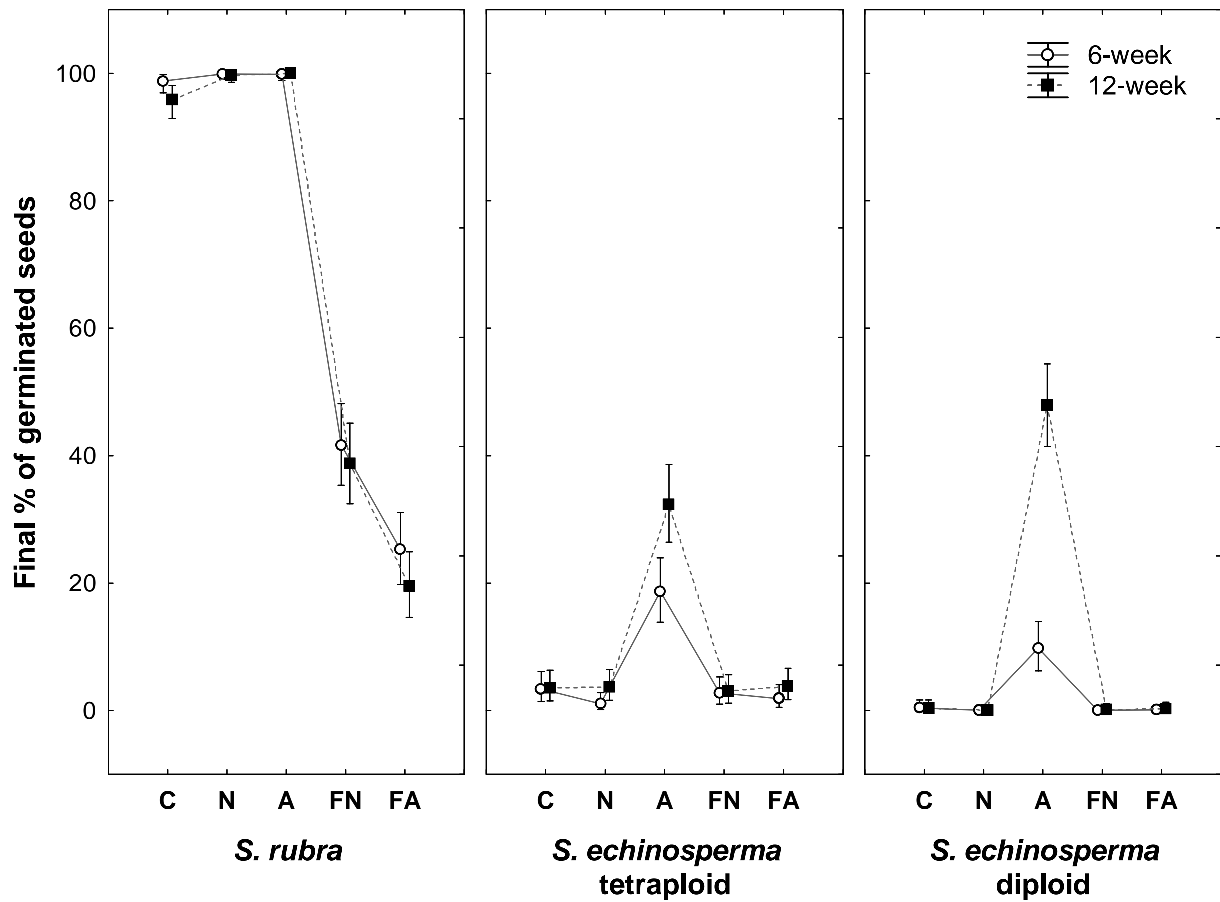

Fig. 6. – Final percentages of germinated seeds for combinations of group, treatment and chilling/freezing length (6-week versus 12-week). Treatments: C – control; N – chilling with subsequent non-fluctuating temperature; A – chilling with subsequent fluctuating temperature; FN – freezing with subsequent non-fluctuating temperature; FA – freezing with subsequent fluctuating temperature. Symbols indicate least squares means; vertical bars denote 0.95 confidence intervals.

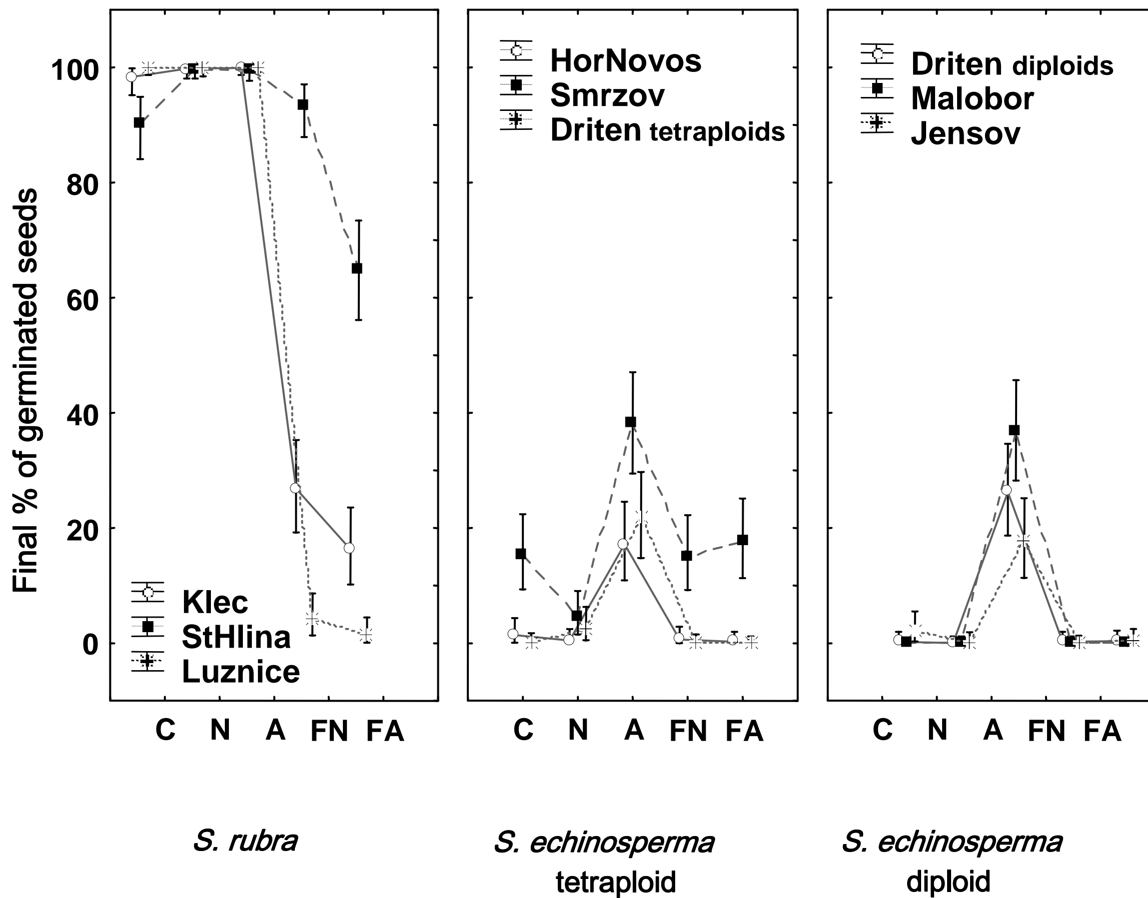

Fig. 7. – Final percentages of germinated seeds for population-treatment combinations. Treatments: C – control; N – chilling with subsequent non-fluctuating temperature; A – chilling with subsequent fluctuating temperature; FN – freezing with subsequent non-fluctuating temperature; FA – freezing with subsequent fluctuating temperature. Symbols indicate least squares means; vertical bars denote 0.95 confidence intervals.

The groups also showed significant differences in the germination rate T_{50} and germination delay (Table 8). *Spergularia echinosperma* displayed significantly slower germination than *S. rubra* did, with mean T_{50} 9.0 and 4.8 days respectively. There was no significant difference in germination rate between the *S. echinosperma* cytotypes. However, there was a distinct effect of the extended length of chilling. The both cytotypes germinated considerably faster after 12-week chilling (mean T_{50} was 8.2 days) compared to 6-week chilling (with mean T_{50} 10.0 days) although this difference was not significant for diploids due to small number of observations. The groups also differed significantly in the germination start. 73% of all germinated seeds of *S. rubra* had germinated (except the freeze treatments) as early as on the first counting day. On the contrary, *S. echinosperma* displayed delayed germination with diploids more delayed than tetraploids. 46% and 40% of tetraploids germinated during the first and second counting interval, respectively. On the contrary, only 5% of diploids germinated during the first interval, and the major proportion (53%) germinated as late as during the second interval. Again, slight differences between individual populations were detected.

Table 7. – Summary of ANOVA of final germination percentage. Brackets after a factor signify that this factor is nested in the factor named in the brackets.

	DF	MS	F	p
Group	2	26.838	53.573	<0.001
Treatment	4	3.472	16.121	<0.001
Length	1	0.109	8.845	0.025
Group*Treatment	8	1.227	5.696	<0.001
Group*Length	2	0.109	8.848	0.016
Treatment*Length	4	0.126	9.876	<0.001
Group*Treatment*Length	8	0.037	2.933	0.020
Population(Group)	6	0.501	2.331	0.065
Population(Group)*Treatment	24	0.215	16.862	<0.001
Population(Group)*Length	6	0.012	0.966	0.469
Population(Group)*Treatment*Length	24	0.013	1.305	0.165
Error	180	0.010		

Table 8. – Summary of ANOVAs of germination rate T_{50} and germination delay. Brackets after a factor signify that this factor is nested in the factor named in the brackets.

		Germination rate T_{50}			Germination delay		
	DF	MS	F	p	MS	F	p
Group	2	10.705	19.479	0.002	6.307	21.354	<0.001
Treatment	4	2.896	9.349	<0.001	2.128	14.221	<0.001
Length	1	0.958	6.406	0.038	0.158	1.723	0.220
Group*Treatment	8	0.746	2.241	0.063	0.255	1.629	0.170
Group*Length	2	0.311	1.940	0.217	0.079	0.862	0.460
Treatment*Length	4	0.131	1.715	0.186	0.159	1.419	0.265
Group*Treatment*Length	8	0.085	1.099	0.405	0.124	1.095	0.409
Population(Group)	6	0.620	1.393	0.274	0.328	2.433	0.182
Population(Group)*Treatment	22	0.360	4.509	<0.001	0.165	1.407	0.236
Population(Group)*Length	6	0.186	2.221	0.097	0.100	0.804	0.581
Population(Group)*Treatment*Length	15	0.085	1.770	0.047	0.126	1.990	0.022
Error	115	0.048			0.063		

Discussion

Cytotype distribution

The majority of *S. echinosperma* individuals analyzed in this study turned out to be tetraploid (205 out of 266), which is in concordance with our preliminary findings (Kúr 2007). Unfortunately, owing to substantially limited number of populations discovered, no conclusions about cytotype distribution on the regional scale can be made.

The discovery of two mixed-ploidy populations of *S. echinosperma* poses questions about possible origin of the tetraploids and their coexistence with the diploids. Generally, co-occurrence of polyploids and their diploid ancestors in common populations is regarded as not permanently sustainable because the rare cytotype (initially the polyploid) is put into a selective disadvantage (Felber 1991, Fowler & Levin 1984). The reason is that the rare cytotype experiences gamete wastage since the mating between diploids and tetraploids (or any other corresponding polyploid combination) either completely fails (triploid block, Marks 1966) or generates triploid offspring with usually lowered viability (Felber 1991, Fowler &

Levin 1984). However, there are several factors that can potentially overcome this barrier and facilitate polyploid establishment in common populations. They are formation of prezygotic mating barriers, increased autogamy rates, and increased relative fitness of the rare cytotype (Burton & Husband 2001, Rausch & Morgan 2005, Halverson et al. 2008). As for these factors, high *S. echinosperma* potential for autogamy was really observed (P. Kúr, unpublished results), which could be one of the mechanisms enabling tetraploid survival.

Additionally, different cytotypes within a polyploid aggregate are sometimes found to occupy different ecological niches, which may lead to an effective elimination of mutual matings (Baack 2005, Kim et al. 2006, Burton & Husband 2001). Since no *in situ* examination of the distribution of the particular *S. echinosperma* cytotypes was conducted, the existence of a microgeographic separation of the cytotypes cannot be disproved.

Moreover, tetraploids can be replenished by recurrent formation via unreduced gametes formed by the diploids (Soltis & Soltis 1999). Since rates of unreduced gametes production in angiosperms are generally low (typically below 1%; Ramsey & Schemske 1998), direct tetraploid formation via two unreduced gametes is considered to be a rare phenomenon. More frequently, tetraploid establishment is accomplished via triploids generated by fusion of reduced and unreduced gametes. If these are viable, they typically produce some proportion of euploid gametes, whose union can be subsequently responsible for tetraploid formation (triploid bridge) (Felber & Bever 1997, Ramsey & Schemske 1998, Husband 2004, Yamauchi et al. 2004). However, this does not apply universally, and there are some taxa where no or very scarce incidence of triploids was observed (Baack 2005, Kolář et al. 2009, Koutecký 2007). This also seems to be the case of mixed-ploidy populations of *S. echinosperma* since no triploid plants were detected during the cytologic screening. Nonetheless, without a more intensive research, the possibility of recurrent formation of *S. echinosperma* tetraploids cannot be dismissed.

Furthermore, *S. rubra* was found exclusively tetraploid in the studied populations. Ratter (1964), however, reported some occurrences of hexaploid plants from the Mediterranean area and speculated that this type could be more widespread through the region. Similarly, Fernandes & Leitao (1971) reported a single discovery of diploid plants in Western Portugal. Since our research was limited to only a few populations from human affected habitats, the presence of other than tetraploid cytotypes in Central Europe cannot be disproved. Suitable sites to focus potential further cytotype screening on could possibly be relict forestless areas with possible autochthonous occurrence of *S. rubra*. Most promising localities are sand dunes, sandy river banks, gravelly glades or suitable undisturbed habitats above the timberline, where the species has also been found to occur (Friedrich 1979, Hartman & Rabeler 2005).

Genome size analyses

Unfortunately, attempts to verify precise chromosome numbers failed, which is a situation often occurring in plants possessing extremely small chromosomes (Hancock 1942, Sinkovič & Bohanec 1988, Nathewet et al. 2009). This is obviously the case of the genus *Spergularia*, so more effective procedures will have to be used in the future.

Genome size turned out to be a reliable marker for distinguishing *S. rubra* and *S. echinosperma* tetraploids. *Spergularia echinosperma* genome was larger by approximately 8%.

Moreover, the genome size of tetraploid *S. echinosperma* was 3.3% lower than double of the genome size of diploids. Presence of this discrepancy can indicate the tetraploids to be of allopolyploid origin with the other parent possessing genome size smaller than diploid *S. echinosperma*. The fact that *S. echinosperma* tetraploids turned out to be somewhat morphologically transitional between the diploids and *S. rubra* would put tetraploid *S. rubra* into the position of a probable candidate. In some cases, viable allotetraploids have been

documented to arise from diploids and tetraploids, either via union of unreduced gametes of the diploid and normal gametes of the tetraploid (Hanneman & Peloquin 1968, Kron & Husband 2009, Borrill & Lindner 1971), or via triploid bridge (Jones & Bamford 1942, Thompson 1930). In this case, recurrent formation is also possible as in the case of autopolyploids (Ramsey & Schemske 1998). However, the genome size of *S. rubra* was revealed to be too low to attain the genome size detected in tetraploid *S. echinosperma* when crossed with unreduced gametes of the diploids, so the possibility of recurrent tetraploid formation directly via unreduced gametes does not seem to be probable. However, recurrent formation via triploid bridge could be theoretically conceivable. During experimental crosses between several diploid and tetraploid *Spergularia* species, Ratter (1972, 1973) obtained viable, and even slightly fertile, triploids in a few cases. Nonetheless, the absence of triploids in the investigated populations makes this scenario improbable in the case of *S. echinosperma* and *S. rubra*.

Alternatively, current tetraploids of *S. echinosperma* need not be arising repeatedly, but they could be a result of an ancient polyploidization event, speculatively involving a presumed diploid ancestor of *S. rubra*.

However, lowered genome size does not mean the tetraploids cannot be of autopolyploid origin. Polyploidization has been often found to produce progeny possessing genome significantly smaller than double of the parental genomes (genome downsizing; Leitch & Bennet 2004). Observed 3.3% difference between actual and predicted values does in no way deviate from differences commonly observed in other species (Mahelka et al. 2005, Eilam et al. 2008, Mráz et al. 2009, Chrtek et al. 2009). Hence, genome size itself is not a sufficient marker for elucidating the origin of the tetraploids, and genetic analyses will be necessary to employ.

Interestingly, one individual of *S. rubra* displayed the genome size smaller by 2.5% than the other analyzed individuals of the species. The most probable explanation is that the investigated plant was aneuploid, although the precise chromosome count was not possible to verify karyologically. Aneuploidy is a very widespread phenomenon in angiosperms, especially in the case of polyploid taxa (Ramsey & Schemske 2002). Despite scarcity of studies on *Spergularia*, some cases of aneuploidy in this genus are also known. Ratter (1965a,b, 1969a,b, 1976) frequently observed aneuploid plants in the progeny descended from artificial interspecific crosses of several *Spergularia* species, sometimes accompanied by polyploidization. However, occurrence of aneuploidy in the progeny of newly formed polyploids, especially allopolyploids, is more frequent since they often experience difficulties with proper chromosome segregation and therefore produce increased numbers of aneuploid gametes (Ramsey & Schemske 2002). Besides, one possible known example of naturally occurring aneuploidy was detected in *S. australis*. In this species, chromosome count of $2n = 40$ was reported, although the base chromosome number for other *Spergularia* species is $x = 9$ (Moore 1982). Similarly, aneuploid plants were found in other genera of *Caryophyllaceae*, for example *Cerastium* (Brysting 2000) and *Arenaria* (Valcarcel et al. 2006).

In addition, the absolute genome sizes recorded fill a gap in the DNA C-values database (Bennett & Leitch 2005), which so far does not contain any data for the whole genus *Spergularia*. Mean somatic genome sizes (2C-values) for the groups investigated were: 0.63 pg for *S. echinosperma* diploids, 1.22 pg for *S. echinosperma* tetraploids, and 1.12 pg for *S. rubra*. All these values lies at the lower limit of genome sizes reported for the family *Caryophyllaceae* in the database (median 2C genome size for the family is 2.90 pg, minimum–maximum with 10% and 90% percentiles are 1.05(1.38)–(6.29)6.59 pg, number of cases is 30). The genome size of the diploids is even lower than the lowest genome size reported in the family (for *Herniaria glabra*, Bennett & Leitch 2005). In addition, genome sizes of the *Spergularia* taxa are also very low in the context of all angiosperms, in which the

lowest so far recorded 2C genome size is 0.20 pg for *Fragaria viridis* (Antonius & Ahokas 1996, Bennett & Leitch 2005).

Morphological variation and possible hybridization

We found significant morphological differences between both the species and the cytotypes within *S. echinosperma*. However, the only character that fully differentiated the species was seed color, on the basis of which the groups were originally delimited and which corresponded with the group partition based on genome sizes. No plants with both brown and black seeds together, as reported by Dvořák (1989, 1990), were found, with the exception of scarce, probably underdeveloped seeds in some *S. echinosperma* capsules. No fully discriminating character for *S. echinosperma* cytotypes was found. Still, the predictive ability of all selected characters together was good enough for largely reliable distinguishing between both the species and cytotypes. Stipule length/width ratio turned out to be the best quantitative predictor for the delimitation of not only the species, as it was reported by previous authors (Dvořák 1979, Čelakovský 1881, Ascherson & Graebner 1893), but also the *S. echinosperma* cytotypes. Furthermore, tetraploids of *S. echinosperma* displayed generally bigger organs, especially seeds and capsules. This situation is in accordance with the general trend of polyploids to possess bigger cells and organs than their diploid ancestors (Briggs & Walters 1997, Kondorosi et al. 2000).

The description of *S. echinosperma* by Dvořák (1990) with its short stipules resembles more likely the diploid cytotype, which corresponds with the chromosome counts detected by Dvořák & Dadáková (1984). On the other hand, some of the *S. echinosperma* morphotypes reported by the author, namely those with elongated fruit pedicels and those with relatively long stipules, allegedly affected by gene introgression from *S. rubra*, fits into the morphological variability of the tetraploids. So, it seems probable that some of the hybrid morphotypes reported by the author were, in fact, just tetraploid *S. echinosperma*.

Yet, the analyses revealed a morphologically intermediate population *Veselsky*, whose genome size, however, was practically indistinguishable from that of other *S. echinosperma* tetraploids. Individuals from this population displayed transitional values of characters distinguishing the two species, which could indicate its origin via hybridization with *S. rubra*. Moreover, seeds of plants from this population were not clearly black but had slightly brown tinge. This morphotype may resemble one group of the hybrids described by Dvořák (1990) (with elongated stipules and dark brown seeds), which the author reported just from the area of Českomoravská vrchovina highlands and close surrounding. Moreover, the holotype specimen of *S. ×kurkae*, collected in the Třeboňská pánev basin, also displayed values of the morphological characters slightly deviating from normal *S. echinosperma* tetraploids but outlaying from the population *Veselsky* too. The hybrid origin, however, cannot be reliably confirmed without a more detailed research, including genetic analyses. Another possible explanation is that these morphotypes only represent extremes within the normal variability of *S. echinosperma*.

With the exception of the population *Veselsky*, no substantially isolated morphotype within both *S. rubra* and *S. echinosperma* cytotypes was detected. However, the biotopically unusual population of *S. rubra Cakov* from a bare pond bottom comprised somewhat more robust plants with elongated capsules and fruit pedicels (up to 8.4 mm long). Although this kind of habitat is somewhat unusual for this species, its occurrence here may be explained by its spread during previous mud removal activities. Similar morphology was found in plants from the population *Provazce*, whose habitat was a sand heap at a quarrel. These morphotypes could resemble *S. rubra* f. *longipes* (Lange) Gürke, which, according to Dvořák (1990), frequently inhabits pond margins, especially in South Bohemia, and similar stands with sparse vegetation. Its fruit pedicels, however, are to be up to 19 mm long. It seems probable that

these types are just ecomorphoses conditioned by environment without competition of other plants.

Morphological distinguishability of the *S. echinosperma* cytotypes makes it possible to describe them formally as separate taxa. However, we hold the view that before any taxonomic treatment of the cytotypes, more information about their distribution, ecology and origin should be gathered.

Based on the results, it is possible to construct the following key to the identification of the species and cytotypes. Although the use of separate characters is not sufficient for reliable distinguishing of the groups, especially as for the *S. echinosperma* cytotypes, the key may be, with caution, used for determining typical individuals at least.

1a Seeds brown; stipules more than 1.7× longer than wide – papillae on seed testa lower than 19 µm; plants richly branched (usually more than 10 stems)..***S. rubra***

1b Seeds black; stipules less than 1.7× longer than wide – papillae on seed testa higher than 19 µm; plants typically with fewer than 10 stems.. **[*S. echinosperma*] 2**

2a Stipules longer than wide; seeds longer than 430 µm, sparsely verrucose (number of papillae to one quarter of seed circumference lower than 14) – capsules longer than 3.5 mm; leaves typically longer than internodes... ***S. echinosperma* tetraploids**

2b Stipules shorter than wide; seeds shorter than 430 µm, densely verrucose (number of papillae to one quarter of seed circumference lower than 14) – capsules shorter than 3.5 mm; leaves typically shorter than internodes ....***S. echinosperma* diploids**

Germination behavior

Spergularia echinosperma and *S. rubra* displayed significant differences in the germination ecology. Virtually no primary dormancy was observed in *S. rubra* since its seeds germinated to high percentages immediately after sowing, and no special dormancy-breaking treatment was required. The lack of primary dormancy in this species is in concert with the fact that *S. rubra* inhabits biotopes not experiencing predictable destructive events during the growing season, like flooding or intensive drought. Hence, dormancy would be nowise effective in helping the plants to avoid unfavorable conditions for their survival (Fenner & Thompson 2005). Similarly, absence of predictable disruptions during the growing season probably allows the species to germinate rapidly and almost instantly, so that it can swiftly occupy initial succession stages before the site is dominated by perennial species (Baker 1974, Sutherland 2004, Roberts & Feast 1973).

Spergularia echinosperma, on the contrary, displayed strong primary dormancy. The only treatment that was able to distinctly break the dormancy was chilling with subsequent temperature fluctuation. A longer period of chilling significantly promoted the germination. The need for cold stratification followed by alternating temperatures was revealed in many other species of flooded sites (Poschlod et al. 1999, Pietsch 1999, Lampe 1996). This mechanism signals upcoming spring and prevents the seeds from coming up in the precipitately unfavorable summer period of the year (Šumberová 2005). In addition, the seeds demonstrated significantly delayed germination onset and slow germination course, which seems to be an adaptation in case of unexpected flooding of the locality. This way, there is always a part of the seed bank that stays dormant, so an untimely flowage does not destroy the whole population.

Moreover, some differences in germination between populations within the species were detected. Generally, germination differences between populations of a single species can be caused by either different environmental conditions of the maternal populations or interpopulation genetic differences (Pérez-García et al. 2006, Andersson & Milberg 1998, Fenner 1991). To filter out the effect of maternal population, germination experiments ought to be done with seeds harvested from plants grown for at least one generation under the same conditions. Although our experiment was not designed in this way, some speculations about

the nature of interpopulation differences in germination behavior can be made. In fragmented landscapes (like the Central European one), individual populations are often found in relatively discrete habitats, which causes the gene flow to other populations is usually restricted, enabling their separate evolution (Koutecká & Lepš 2009). While *S. rubra* is probably primarily spread by human activities to ruderal habitats over long distances; pond bottoms, a habitat of *S. echinosperma*, form relatively isolated entities within the landscape. Hence, we speculate that the differences between *S. rubra* populations most probably reflect local conditions, while reproductive isolation of populations of *S. echinosperma* may really have resulted in their individual adaptations.

In the search for causes of the relative scarcity of *S. echinosperma* diploids, the detected differences in cytotypes germination ecology could provide one possible explanation. Tetraploids displayed lower total germination percentages, but their germination onset was less delayed than in the case of diploids (and they also showed faster subsequent growth). In the light of constantly increasing intensification of fishpond management in the last years (Šumberová et al. 2005, Šumberová 2003), the period of summer pond drying has been significantly shortened. Most of the fishponds are refilled as early as at the beginning of May, which makes considerable demands on completion of the plants life cycles. This extremely short growing period probably creates a selection pressure on quick plant maturing and seed shedding. So, it seems plausible that the accelerated germination and faster growth of the tetraploids is an adaptation to these stress conditions, while the diploids are evolutionarily handicapped. Moreover, increased germination percentages of the diploids may indicate a reduced ability of creating a persistent seed bank, which would put the diploid cytotypes into a disadvantageous situation again. For that matter, better polyploids adaptability to unfavorable environmental conditions is a frequently observed phenomenon in flowering plants (Briggs & Walters 1997, Levin 1983, Wendel 2000).

In addition, freezing significantly reduced germinability of seeds of the both species. One possible explanation is that it triggered their transition into the state of conditioned dormancy. Many plant species, especially weedy ones, are known to undergo annual cycles of dormancy and non-dormancy (Baskin & Baskin 1985, 1998). Seeds of such species, no matter whether they possess primary innate dormancy or not, may re-become dormant if environmental conditions are unfavorable for germination. As seeds reenter dormancy, they first become conditionally dormant, during which the range of conditions under which they can germinate is narrowing. In the end, the seeds become fully dormant, which is then called secondary dormancy. This may be the case of *S. rubra* and *S. echinosperma*, when the species, after being exposed to frost, must undergo an additional dormancy-breaking impulse to be able to fully germinate. An alternative explanation, however, may be that constant deep freeze impaired the seeds and reduced their viability.

References

Andersson L. & Milberg P. (1998): Variation in seed dormancy among mother plants, populations and years of seed collection. – Seed Sci. Res. 8: 29–38.

Antonius K, Ahokas H. (1996): Flow cytometric determination of polyploidy level in spontaneous clones of strawberries. – Hereditas 124: 285.

Ascherson P. & Graebner P. (1893): Beiträge zur Kenntniss der norddeutschen Flora. – Ber. Deutsch. Bot. Ges. 11: 516–530.

Baack E. J. (2005): Ecological factors influencing tetraploid establishment in snow buttercups (*Ranunculus adoneus, Ranunculaceae*): minority cytotype exclusion and barriers to triploid formation. – Amer. J. Bot. 92(11): 1827–1835.

Baker H. G. (1974): The evolution of weeds. – Annu Rev Ecol Syst 5:1–24.

Baskin C. C. & Baskin J. M. (1998): Seeds: ecology, biogeography, and evolution of dormancy and germination. – Academic Press, San Diego.

Baskin J. M. & Baskin C. C. (1985): The Annual Dormancy Cycle in Buried Weed Seeds: A Continuum. – BioScience 35(8): 492–498.

Bennett M. D. & Leitch I. J. (2005): Angiosperm DNA C-values database (release 6.0). – URL: [http://www.kew.org/cvalues].

Bittrich V. (1993): *Caryophyllaceae*. – In: Kubitzki K., Rohwer J. & Bittrich V. (eds), The families and genera of vascular plants 2: 206–236, Springer Verlag, Berlin.

Borrill M. & Lindner R. (1971): Diploid-tetraploid sympatry in *Dactylis* (*Gramineae*). – New Phytol. 70: 1111–1124.

Breiman L., Friedman J., Stone C. J. & Olshen R. A. (1984): Classification and regression trees. – Chapman & Hall, Pacific Grove.

Briggs D.& Walters S. M. (1997): Plant Variation and Evolution. Ed. 3. – Cambridge University Press, Cambridge.

Brysting A. K. (2000): Chromosome number variation in the polyploid *Cerastium alpinum*-*C. arcticum* complex (*Caryophyllaceae*). – Nordic J. Bot. 20(2): 149–156.

Burton T. L. & Husband B. C. (2001): Fecundity and offspring ploidy in matings among diploid, triploid and tetraploid *Chamerion angustifolium* (*Onagraceae*): consequences for tetraploid establishment. – Heredity 87: 573–582.

Čelakovský L. (1881): Prodromus der Flora von Böhmen 4. – Praha.

Chrtek J., Zahradníček J., Krak K. & Fehrer J. (2009): Genome size in *Hieracium* subgenus *Hieracium* (*Asteraceae*) is strongly correlated with major phylogenetic groups. – Ann. Bot. 104(1):161–178.

Doležel J., Doleželová M. & Novák F. (1994): Flow cytometric estimation of nuclear DNA amount in diploid bananas (Musa acuminata and M. balbisiana). – Biol. Pl. 36: 351–631.

Doležel J., Sgorbati S. & Lucretti S. (1992): Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. – Physiol. Pl. 85: 625–631.

Dvořák F. & Dadáková B. (1984): Chromosome counts and chromosome morphology of some selected species. – Scr. Fac. Sci. Natur. Univ. Purkyn. Brun.-Biol. 14: 463–468.

Dvořák F. (1979): Některé výsledky studia druhu *Spergularia* echinosperma Čelak. [Some study results of the species *Spergularia* echinosperma Čelak.]. – Zpr. Čs. Bot. Společ. 14: 109–116.

Dvořák F. (1989): Chromosome counts and chromosome morphology of some selected species. – Scr. Fac. Sci. Natur. Univ. Purkyn. Brun.-Biol. 19: 301–322.

Dvořák F. (1990): *Spergularia* – kuřinka. – In: Hejný S. & Slavík B. (eds.), Květena České republiky [Flora of the Czech Republic] 2: 81–86, Academia, Praha.

Eilam T., Anikster Y., Millet E., Manisterski J. & Feldman M. (2008): Nuclear DNA amount and genome downsizing in natural and synthetic allopolyploids of the genera *Aegilops* and *Triticum*. – Genome 51: 616–627.

Felber F. & Bever J. D. (1997): Effect of triploid fitness on the coexistence of diploids and tetraploids. – Biol. J. Linn. Soc. 60: 95–106.

Felber F. (1991): Establishment of a tetraploid cytotype in a diploid population: effect of relative fitness of the cytotypes. – J. Evol. Biol. 4: 195–207.

Fenner M. (1991): The effects of the parent environment on seed germinability. – Seed Sci. Res. 1: 75-84.

Fenner M.& Thompson K. (2005): The Ecology of Seeds. – Cambridge University Press, New York.

Fernandes A. & Leitao M. T. (1971): Contribution à la connaissance cytotaxonomique des Spermatophyta du Portugal III, Caryophyllaceae. – Bolm. Soc. Broteriana 45: 143–176.

Fowler N. L. & Levin D. A. (1984): Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. – Am. Nat. 124: 703–711.

Friedrich H. C. (1979): Familie *Caryophyllaceae.* – In: Reichinger K.H. (ed.), Illustrierte Flora von Mitteleuropa III/2: 763–1182, Verlag, Berlin.

Hadač E. (1977): Poznámky o endemických rostlinách České socialistické republiky [Notes about endemic plants of the Czech Socialistic Republic]. – Zpr. Čs. Bot. Společ. 12: 1–15.
Halverson K., Heard S. B., Nason J. D. & Stireman J. O. (2008): Origins, distribution, and local co-occurrence of polyploid cytotypes in *Solidago altissima* (Asteraceae). – Amer. J. Bot. 95(1): 50–58.
Hancock B. L. (1942): A Schedule for Chromosome Counts M Some Plants with Small Chromosomes. – Biotech. Histochem. 17(2): 79–83.
Hanneman R. E. & Peloquin S. J. (1968): Ploidy levels of progeny from diploid-tetraploid crosses in the potato. – Am. J. Potato Res. 45(7): 255–261.
Hartman R. L. & Rabeler R. K. (2005): *Spergularia*. – In: Flora Of North America Editorial Committee (eds.), Flora of North America 5: 16–23, Oxford University Press, New York and Oxford.
Husband B. C. (2004): The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. – Biol. J. Linn. Soc. 82: 537–546.
Jones R. E. & Bamford R. (1942): Chromosome number in the progeny of triploid *Gladiolus* with special reference to the contribution of the triploid. – Am. J. Bot. 29: 807–813.
Kim J. H., Kyung H. Y., Choi Y. S., Lee J. K., Hiramatsu M. & Okubo H. (2006): Geographic distribution and habitat differentiation in diploid and triploid *Lilium lancifolium* of South Korea. – J. Fac. Agr., Kyushu Univ. 51(2): 239–243.
Kolář F., Štech M., Trávníček P., Rauchová J., Urfus T., Vít P., Kubešová M. & Suda J. (2009): Towards resolving the *Knautia arvensis* agg. (*Dipsacaceae*) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. – Ann. Bot. 103(6): 963–974.
Kondorosi E., Roudier F. & Gendreau E. (2000): Plant cell-size control: growing by ploidy? – Curr. Opin. Plant Biol. 3: 488–492.
Koutecká E. & Lepš. J. (2009): Effect of Light and Moisture Conditions and Seed Age on Germination of Three Closely Related Myosotis Species. – Folia Geobot. 44: 109–130.
Koutecký P. (2007): Morphological and ploidy level variation of *Centaurea phrygia* agg. (*Asteraceae*) in the Czech Republic, Slovakia and Ukraine. – Folia Geobot. 42: 77–102.
Kron P. & Husband B. C. (2009): Hybridization and the reproductive pathways mediating gene flow between native *Malus coronaria* and domestic apple, *M-domestica*. – Botany-Botanique 87(9): 864–874.
Kúr P. (2007): Cytologická variabilita a potenciální hybridizace druhů *Spergularia* echinosperma a *S. rubra* – rešerše problematiky v celém rodě a pilotní studie [Cytological variability and possible hybridization of the species *Spergularia* echinosperma and *S. rubra* – a review of the issue in the whole genus and a pilot study. Bc. Thesis, in Czech]. – Faculty of Biological Sciences, University of South Bohemia, České Budějovice.
Lampe M. (1996): Wuchsform, Wuchsrhythmus und Verbreitung der Arten der Zwergbinsengesellschaften. – Diss. Bot. 266: 1–353.
Lavorel S. & Garnier E. (2002): Predicting changes in community composition and ecosystem functioning from plant traits: revisiting the Holy Grail. – Funct Ecol 16:545–556.
Leitch I. J. & Bennet M. D. (2004): Genome downsizing in polyploid plants. – Biol. J. Linn. Soc. 82: 651–663.
Levin D. A. (1983): Polyploidy and novelty in flowering plants. – Am. Nat. 122: 1–25.
Mahelka V., Suda J., Jarolímová V., Trávníček P. & Krahulec F. (2005): Genome size discriminates between closely related taxa *Elytrigia repens* and *E. intermedia* (*Poaceae*: *Triticeae*) and their hybrid. – Folia Geobot. 40: 367–384.
Marks G. E. (1966): The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. – Evolution 20: 552–557.

Monnier P. & Ratter J. A. (1993): *Spergularia*. – In: Tutin T. G., Burges N. A., Chater A. O., Edmondson J. R., Heywood V. H., Moore D. M., Valentine D. H.,Walters S. M. & Webb D. A. (eds), Flora Europaea. Ed. 2, 1: 186–188, Cambridge University Press, Cambridge.

Moore D. M. (1982): Flora Europaea Check-list and Chromosome Index. – Cambridge University Press, Cambridge.

Mráz P., Chrtek J. & Šingliarová B. (2009): Geographical parthenogenesis, genome size variation and pollen production in the arctic-alpine species *Hieracium alpinum*. – Bot. Helv. 119(1): 41–51.

Nathewet P., Yanagi T., Iwastubo Y., Sone K., Takamura T. & Okuda N. (2009): Improvement of staining method for observation of mitotic chromosomes in octoploid strawberry plants. – Sci. Hort. 120: 431–435.

Otto F. (1990): DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. – In: Crissman H. A. & Darzynkiewicz Z. (eds), Methods in cell biology, Vol. 33, p. 105–110, Academic Press, New York.

Pérez-García F., Huertas M., Mora E., Pena B., Varela F. & González-Benito M. E. (2006): *Hypericum perforatum* L. seed germination: interpopulation variation and effect of light, temperature, presowing treatments and seed desiccation. – Genet. Resources Crop Evol. 53: 1187–1198.

Pietsch W. (1999): Zum Keimverhalten ausgewählter Arten mitteleuropäischer Zwergbinsengesellschaften. – Mitt. bad. Landesver. Naturkunde u. Naturschutz, ser. nov., 17(2): 261–274.

Poschlod P., Böhringer J., Fennel S., Prume C. & Tiekötter A. (1999): Aspekte der Biologie und Ökologie von Arten der Zwergbinsenfluren. – Mitt. bad. Landesver. Naturkunde u. Naturschutz 17(2): 219–260.

R Development Core Team (2008): R: A language and environment for statistical computing. – R Foundation for Statistical Computing, Vienna, Austria, URL: [http://cran.r-project.org].

Ramsey J. & Schemske D. W. (1998): Pathways, mechanisms, and rates of polyploid formation in flowering plants. – Annu. Rev. Ecol. Syst. 29: 467–501.

Ramsey J. & Schemske D. W. (2002): Neopolyploidy in flowering plants. – Annu. Rev. Ecol. Syst. 33: 589–639.

Ratter J. A. (1964): Cytogenetic studies in *Spergularia* I. Cytology of some Old World species. – Not. Roy. Bot. Gar. Edinburgh 25: 293–302.

Ratter J. A. (1965a): Cytogenetic studies in *Spergularia* II. An attempt to discover cytogenetic relationships of some species. – Not. Roy. Bot. Gar. Edinburgh 26: 203–223.

Ratter J. A. (1965b): Cytogenetic studies in *Spergularia* III. Some interspecific hybrids involving S. marina (L.). – Not. Roy. Bot. Gar. Edinburgh 26: 224–236.

Ratter J. A. (1969a): Cytogenetic studies in *Spergularia* IV. Some further interspecific hybrids. – Not. Roy. Bot. Gar. Edinburgh 29: 213–223.

Ratter J. A. (1969b): Cytogenetic studies in *Spergularia* V. Some interspecific hybrids involving S. media (L.). – Not. Roy. Bot. Gar. Edinburgh 29: 225–232.

Ratter J. A. (1972): Cytogenetic studies in *Spergularia* VI. The evolution of true breeding, fertile tetraploids from a triploid interspecific hybrid. – Not. Roy. Bot. Gar. Edinburgh 32: 117–125.

Ratter J. A. (1973): Cytogenetic studies in *Spergularia* VIII. Barriers to the production of viable interspecific hybrids. – Not. Roy. Bot. Gar. Edinburgh 32: 297–301.

Ratter J. A. (1976): Cytogenetic studies in *Spergularia* IX. Summary and conclusions. – Not. Roy. Bot. Gar. Edinburgh 34: 411–428.

Rausch J. H. & Morgan M. T. (2005): The effect of self-fertilization, inbreeding depression, and population size on autopolyploid establishment. – Evolution 59(9): 1867–1875.

Roberts H. A. & Feast P. M. (1973): Emergence and longevity of seeds of annual weeds in cultivated and undisturbed soil. – J. Appl. Ecol. 10(1): 133–143.
Rohlf F. J. (2008): TpsDig 2.12. – Ecology & Evolution, SUNY at Stony Brook, URL: [http://life.bio.sunysb.edu/morph].
Rossbach R. P. (1940): *Spergularia* in North and South America. – Rhodora 42: 57–83, 105–143, 158–193, 203–213.
Salisbury E. J. (1970): The pioneer vegetation of exposed muds and its biological features. – Phil. Transact. Roy. Soc. London, ser. B, 259: 207–255.
Sinkovič T. & Bohanec B. (1988): Chromosome counts and karyotype analysis in buckwheat (Fagopyrum esculentum Moench.). – Fagopyrum 8: 20-22.
Soltis D. E. & Soltis P. S. (1999): Polyploidy: recurrent formation and genome evolution. – Trends Ecol. Evol. 14(9): 348–352.
StatSoft (1998): Statistica for Windows. Ed. 2. – StatSoft Inc., Tulsa.
Suda J., Weiss-Schneeweiss H., Tribsch A., Schneeweiss G. M., Trávníček P. & Schönsweiter P. (2007): Complex distribution patterns of di-, tetra-, and hexaploid cytotypes in the European high mountain plant Senecio carniolicus (Asteraceae). – Amer. J. Bot. 94(8): 1391–1401.
Šumberová K. (2003): Veränderungen in der Teichwirtschaft und ihr Einfluss auf die Vegetation in der Tschechischen Republik. – Mitt. bad. Landesver. Naturkunde u. Naturschutz 18: 7–24.
Šumberová K. (2005): Co víme o vegetaci tříd Isoëto-Nanojuncetea a Bidentetea v České republice? [What do we know about the vegetation of the classes Isoëto-Nanojuncetea and Bidentetea in the Czech Republic?]. – Zpr. Čes. Bot. Spol. 40: 195–220.
Šumberová K., Horáková V. & Lososová Z. (2005): Vegetation dynamics on exposed pond bottoms in the Českobudějovická basin (Czech Republic). – Phytocoenologia 35: 421–448.
Šumberová K., Lososová Z., Fabšicová M. & Horáková V. (2006): Variability of vegetation of exposed pond bottoms in relation to management and environmental factors. – Preslia 78: 235–252.
Sutherland S. (2004): What makes a weed a weed: life history traits of native and exotic plants in the USA. – Oecologia 141: 24–39.
ter Braak C. J. F. & Šmilauer P. (2002): CANOCO Reference manual and CanoDraw for Winows User's guide: Software for Canonical Community Ordination (version 4.5). – Microcomputer Power, Ithaca, NY.
Thompson W. P. (1930): Cytology and genetics of crosses between fourteen- and seven-chromosome species of wheat. – Genetics 16: 309–324.
Valcarcel V., Vargas P., Feliner G. N. (2006): Phylogenetic and phylogeographic analysis of the western Mediterranean *Arenaria* section *Plinthine* (*Caryophyllaceae*) based on nuclear, plastid, and morphological markers. – Taxon 55(2): 297–312.
Wendel J. F. (2000): Genome evolution in polyploids. – Plant Mol. Biol. 42: 225–249.
Yamauchi A., Hosokawa A., Nagata H. & Shimoda M. (2004): Triploid bridge and role of parthenogenesis in the evolution of autopolyploidy. – Amer. Naturalist 164(1): 101–112.

Appendix 1. – List of localities of *S. echinosperma* and *S. rubra* populations used in the study together with their cytotype composition detected by flow cytometry. Populations marked by an asterisk are those from which plants used for the measurements of the absolute genome size and for the germination experiments originated.

No.	Label	Locality	Latitute	Longitude	Altitude (m)	Number of plants	Species and cytotype
1	*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
2	*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
3	*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
4	*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
5	*Driten**	S Bohemia, Dříteň: bare pond bottom of the Kočínský rybník pond	49°08'56.1"N	14°21'15.0"E	460	20	*S. echinosperma* 4x + 2x
6	*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
7	*HorMez*	Českomoravská vrchovina highlands, Horní Meziříčko: lowny playground in the village	49°09'19.0"N	15°14'29.7"E	580	19	*S. rubra* 4x
8	*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
9	*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
10	*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
11	*Klec**	S Bohemia, Klec: lawn in the village	49°05'49.5"N	14°44'56.6"E	420	20	*S. rubra* 4x
12	*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
13	*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
14	*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
15	*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
16	*Luznice**	S Bohemia, Lužnice: road margin in the village	49°03'46.0"N	14°45'37.5"E	420	24	*S. rubra* 4x
17	*Maj*	S Bohemia, České Budějovice, Máj: sandy playground	48°59'20.2"N	14°26'08.5"E	400	20	*S. rubra* 4x
18	*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
19	*Pecihradek*	W Bohemia, Plzeň, Pecihrádek: field margin	49°46'06.5"N	13°24'57.0"E	330	22	*S. rubra* 4x
20	*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
21	*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
22	*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
23	*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
24	*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
25	*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
26	*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
27	*Zavlekov*	W Bohemia, Zavlekov: lawn in the village	49°20'20.5"N	13°29'36.2"E	570	20	*S. rubra* 4x