Origin of *Spergularia ×kurkae*, a hybrid between the rare endemic *S. echinosperma* and its widespread congener *S. rubra*

Původ *Spergularia ×kurkae*, křížence mezi vzácným endemickým druhem *S. echinosperma* a široce rozšířeným *S. rubra*

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The origin of *Spergularia ×kurkae*, a presumed tetraploid hybrid between the diploid central-European endemic *S. echinosperma* and its widespread tetraploid congener *S. rubra*, was investigated by sequencing the nrDNA ITS region and cpDNA *rpo*C1 intron. *Spergularia echinosperma* and *S. rubra* differed markedly in their ITS sequences. The presence of both sequences within the genome of *S. ×kurkae* confirmed its hybrid origin and parentage; cpDNA sequences identified *S. echinosperma* as the sole maternal parent. Because both parental ITS homeologs were clearly visible in the sequences of almost all of the *S. ×kurkae* individuals, we conclude that this taxon is of a relatively young age. We hypothesize that *S. ×kurkae* might have evolved as a result of human-mediated introduction of *S. rubra* into fishponds. Cross-amplification of species-specific ITS primers revealed high levels of intra-individual ITS polymorphisms in *S. echinosperma* and *S. rubra*. Our results suggest ongoing gene flow from *S. ×kurkae* to *S. rubra*. In contrast, no evidence of gene flow from *S. ×kurkae* or *S. rubra* to *S. echinosperma* was found, providing, despite concerns, no support for the threat of the genetic assimilation of *S. echinosperma*. Our current data also support the view of *S. kurkae* as a stabilized, separate allopolyploid species.

K e y w o r d s: endemism, hybridization, introgression, repeat-specific amplification, *Spergularia*

Introduction

Interspecific hybridization is assumed to be a major force driving the evolution of vascular plants (Rieseberg et al. 1993, Ellstrand et al. 1996, Prentis et al. 2007, Soltis & Soltis 2009, Soltis 2013). It is estimated that as many as 50% of angiosperms may be of hybrid origin (Arnold 1997). The most significant trigger for hybridization has been the effect of humans on ecosystems. Creation of new habitats and the introduction of allochthonous species have led to the formation of an unprecedented number of hybrid zones (Levin et al. 1996, Rhymer & Simberloff 1996, Arnold 1997, Rieseberg 1997, Ellstrand & Schierenbeck 2000, Soltis & Soltis 2009). Whereas hybrid zones are of undeniable

importance for the study of evolutionary processes, they can also pose a threat to endangered species in the form of genetic assimilation by widespread congeners (Levin et al. 1996, Wolf et al. 2001, Prentis et al. 2007).

Gene flow is usually limited to species of the same ploidy level (Chapman & Abbott 2010). Heteroploid hybridization is hampered by the production of sterile odd-ploidy offspring and typically occurs only in higher-ploidy taxa (Schneider 1958, Brochmann et al. 1992, Kolář et al. 2009, Hülber et al. 2015). However, there are an increasing number of documented cases of gene flow between diploids and tetraploids (e.g. Neuffer et al. 1999, Bleeker & Matthies 2005, Thórsson et al. 2010, Jørgensen et al. 2011, Koutecký et al. 2011, Moraes et al. 2013). The most important mechanism of inter-ploidy gene flow appears to be the fusion of reduced (n) and unreduced (2n) gametes (Ramsey & Schemske 1998, Soltis et al. 2004).

Spergularia echinosperma (Čelak.) Asch. et Graebn. is one of the few species of vascular plants that is endemic to central Europe and does not occur in high mountains (Friedrich 1979, Dvořák 1990, Kúr et al. 2012). It is confined to the vegetation of annual wetland herbaceous plants (class *Isoëto-Nanojuncetea*) that are mainly recorded growing in the dried out bottoms of freshwater reservoirs that are periodically drained. The primary habitats of *S. echinosperma* are alluvial pools and sandy banks of rivers (Friedrich 1979, Dvořák 1990). Unfortunately, most of these habitats have been destroyed by the channelling of rivers. *Spergularia echinosperma* also occurs in secondary habitats, especially the bottoms of drained fishponds, where it has also been threatened by the intensification of fishpond management over the last century (Popiela 2005, Šumberová et al. 2005, 2006, Šumberová 2011).

Spergularia echinosperma is morphologically similar to *S. rubra* (L.) J. Presl et C. Presl, a nearly cosmopolitan weedy species that mainly occurs in disturbed habitats such as sandy fields, roadsides and waste ground (Dvořák 1979, Friedrich 1979, Monnier & Ratter 1993, Hartman & Rabeler 2005). *Spergularia rubra* also sometimes grows in the same habitats as *S. echinosperma*, e.g. river banks and drained bottoms of ponds, where the two species have the same ecological niche and mixed populations are occasionally found (P. Kúr et al., unpubl.). These species differ in their ploidy levels, with *S. echinosperma* diploid (2n = 2x = 18; Dvořák & Dadáková 1984) and *S. rubra* tetraploid (2n = 4x = 36; Dvořák 1990, Wisskirchen & Haeupler 1998); there are records of other ploidy levels in countries other than those in central Europe (Ratter 1964, Fernandes & Leitao 1971).

Morphological observations have led some authors to conclude that *S. echinosperma* and *S. rubra* hybridize (Jage 1974, Dvořák 1989, 1990), and the hybrid was formally described as *S.* ×*kurkae* F. Dvořák (Dvořák 1989). The formal description is supplemented with a chromosome count, which is tetraploid (2n = 36). A more detailed study by Kúr et al. (2012) reports tetraploid populations that match the description of *S.* ×*kurkae*. These populations are clearly morphologically intermediate between *S. echinosperma* and *S. rubra*, supporting their hybrid origin. However, their genome size deviates significantly from the genome size of a modelled allotetraploid hybrid *S. echinosperma* × *S. rubra*. Dvořák (1990) also assumes introgressive hybridization between *S.* ×*kurkae* and *S. echinosperma*. However, Kúr et al. (2012) reports no morphological indications of gene flow between these taxa. Rather, they detect possible hybridization at the tetraploid level between *S.* ×*kurkae* and *S. rubra*. Therefore, in the current study, we investigate the hybridization of the two *Spergularia* species using molecular methods.

To accomplish our objective, we sequenced the biparentally inherited internal transcribed spacer (ITS) of nuclear ribosomal DNA and the maternally inherited *rpo*C1 intron of chloroplast DNA (cpDNA). The ITS region was chosen because it is a multicopy marker that often retains intra-individual polymorphism in hybrid taxa, thus allowing clear inferences about their parentage (Sang et al. 1995, Koch 2003). To evaluate interspecific gene flow, we also used repeat-specific amplification of the ITS region. This is a very sensitive method that enables the detection of minority sequence variants and is suitable for inferring hybridization and reconstructing phylogeny (Rauscher et al. 2002, 2004, Soltis et al. 2008, Laureto & Barkman 2011). The cpDNA was used to indicate the direction of hybridization (i.e. to identify the maternal species). The combined use of these two markers allowed us to answer the following two questions: (i) What is the parentage of tetraploid *S.* ×*kurkae*? (ii) Does introgression among *S.* ×*kurkae*, *S. echinosperma* and *S. rubra* occur?

Materials and methods

Plant sampling

A total of 516 plants from 91 populations of *Spergularia echinosperma*, *S. rubra* and *S.* ×*kurkae* (1–20 individuals per population and taxon) in the Czech Republic and Germany were sampled between the years 2008 and 2012 (Fig. 1; see Electronic Appendix 1 for the exact localities and acronyms of the populations used in the text). Voucher specimens were deposited in the herbarium CBFS. Additionally, the holotype of *S.* ×*kurkae* (deposited in the herbarium CB) was used for DNA sequencing.

Flow cytometry and chromosome counting

To confirm the determination of the plants analysed, flow cytometry was used to estimate the genome size and DNA ploidy level (sensu Suda et al. 2006) of all the plants collected. We followed the protocol presented in Kúr et al. (2012).

We calibrated the flow cytometric measurements using the chromosome counts of all three taxa. One plant from each of the populations Siglovec (*S. rubra*), Kojatín (*S. echinosperma*) and Nový Dářko (*S.* ×*kurkae*) were used. The apical root meristems of germinated seedlings were pre-treated with a saturated water solution of p-dichlorobenzene (3 h, room temperature) and fixed in a 3:1 mixture of 96% ethanol and glacial acetic acid overnight at 4 °C. Chromosome counts were made after digestion using enzymes and squashing as described by Schwarzacher & Heslop-Harrison (2000) and Schönswetter et al. (2007). The fixed material was maintained in citrate buffer (pH = 4.8; freshly prepared by mixing 4 ml of 0.1 M citric acid monohydrate $C_6H_8O_7 \cdot H_2O$ and 6 ml of 0.1 M trisodium citrate dihydrate $C_6H_5O_7Na_3 \cdot 2\ H_2O$, and diluting 10× with distilled water) for 20 min, transferred to an enzyme mixture containing 1% (w/v) cellulase Onozuka (Serva, Heidelberg, Germany), 0.4% (w/v) cytohelicase (Sigma-Aldrich, Vienna, Austria) and 0.4% (w/v) pectolyase (Sigma-Aldrich) in citrate buffer (pH = 4.8, pre-warmed at 37 °C) and incubated for 30 min at 37 °C. Next, the loose root material was washed in citrate buffer for a minimum of 30 min and transferred to a drop of 60% acetic acid on a microscopic slide. The material was then dissected using entomological needles under a stereomicroscope,

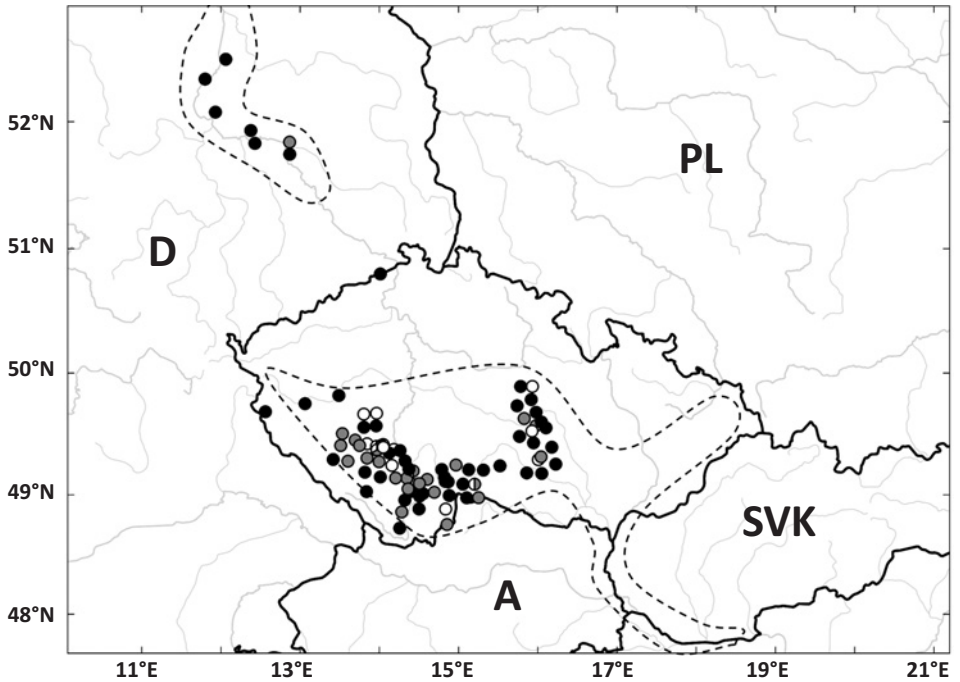

Fig. 1. – Distributions of the populations of *Spergularia echinosperma* (white), *S.* ×*kurkae* (grey) and *S. rubra* (black) studied. The dashed lines denote the distribution of *S. echinosperma* (compiled from the review of herbarium specimens; P. Kúr et al., unpubl.). The distribution of *S. rubra* is not mapped, as this species is widespread throughout the study area.

covered with a cover slip and squashed. Preparations were frozen on a cooling plate, air dried after cover slip removal, and stored at –20 °C until required. After application of 9 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 2 µg/ml DAPI to the dry preparations, the preparations were screened for well-spread mitotic metaphases under a Zeiss Axio Imager.M2 epifluorescence microscope equipped with an Axiocam HRm camera. Images were acquired using Zeiss AxioVision SE64 software (Carl Zeiss Meditec AG, Oberkochen, Germany).

DNA extraction

Parts of plants (typically a whole lateral branch) were silica-dried and processed using the rapid DNA extraction method of Werner et al. (2002). A small amount of plant material was ground in 30 µl of 0.5 M NaOH and centrifuged. The supernatant was diluted 1:10 with 100 mM Tris-HCl buffer (pH = 8.3). In order to obtain DNA isolates of high quality, DNA from plants that were only available as herbarium specimens was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Germany) following the manufacturer's protocol.

DNA sequencing and cloning

To amplify the ITS region, the ITS4i and ITS5i PCR primers (Roalson & Friar 2000) were used with the following cycling program: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min; and final elongation at 72 °C for 10 min. The *rpo*C1 intron was amplified using the ANU cp033-L and ANU cp034-R primers (Ebert & Peakall 2009) with the following cycling program: 94 °C for 2 min; 12 cycles of 94 °C for 30 s, 66–51 °C (gradually reduced by 3 °C every second cycle) for 30 s, 72 °C for 45 s; 33 cycles of 94 °C for 30 s, 47 °C for 30 s and 72 °C for 45 s; and final elongation at 72 °C for 10 min. The PCR reactions were carried out with 2.5 µl of Plain PP Master Mix (Top-Bio, Czech Republic), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, and 0.4 µl of the template DNA in a final reaction volume of 5 µl. The PCR products were sequenced using the ITS5i and ANU_cp033-L primers on an ABI PRISM 3130xl Genetic Analyzer (Laboratory of Genomics, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice). The resulting electropherograms were inspected in Finch TV 1.4 (Geospiza, USA).

All but seven samples of *S. ×kurkae* showed multiple additive peaks in the direct ITS sequences, indicating intra-individual variation in the sequences. To separate the particular ITS molecules, PCR products were cloned into competent *Escherichia coli* DH5 alpha cells using the pGEM-T Easy Vector System (Promega, USA) according to the manufacturer's instructions except that only one quarter of the recommended volumes for the reactions were used. Because the pattern of nucleotide additivity was the same for all of the samples, only one sample was chosen for cloning (population Cky). Five clones were sequenced to investigate the potential presence of different ITS copies.

Repeat-specific amplification

As the sequencing revealed two distinct ITS ribotypes, one specific to *S. echinosperma* and the other to *S. rubra* (see Results), we designed two sets of taxon-specific ITS primers using Primer3 (Koressaar & Remm 2007). Two primer pairs each consisted of a universal forward primer targeting both ribotypes (SIf, 5'-TCGTAACAAGGTTTCCGTAGGTG-3') in the 18S rRNA region and a reverse primer specific for *S. echinosperma* (EIr, 5'-CTCTAACGGGCGGGCG-3') or *S. rubra* (RIr, 5'-CTCTGGAAACGGGGCGG-3') ribotype, which targeted a variable site in the middle of the ITS1 region, producing a short partial fragment of the ITS1 region c. 100 bp long. The other two primer pairs each had a forward primer specific for *S. echinosperma* (EIf, 5'-TTGGTGCGTCCGCTCTAAC-3'; located 9 bp downstream of EIr) or *S. rubra* (RIf, 5'-CGCCCGCTCTGGAAAC-3'; located 7 bp downstream of RIr) and the universal reverse ITS4i primer, producing a long fragment ~ 500 bp long that included partial ITS1, complete 5.8S and complete ITS2 regions (Fig. 2). The *echinosperma*-specific amplification was done using 260 individuals of *S. rubra*, and the *rubra*-specific amplification was done using 58 individuals of *S. echinosperma*. In addition, the *rubra*-specific amplification was tested in the seven above-mentioned individuals of *S. ×kurkae* that had only the *S. echinosperma* homeolog visible on the direct sequences (see Electronic Appendix 1). The PCR program was 95 °C for 3 min; 35/45 cycles of 95 °C for 30 s, primer-pair specific annealing temperature for 60 s, and 72 °C for 60 s; and 72 °C for 10 min. The annealing temperatures were 69 °C for the EIf/ITS4i primer pair, 67 °C for the RIf/ITS4i primer pair, and 64 °C for the SIf/EIr

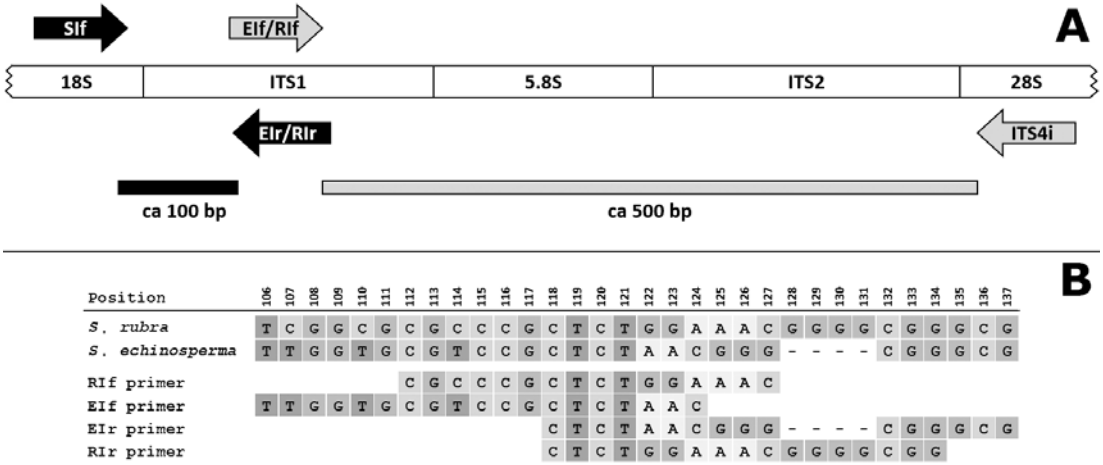

Fig. 2. – (A) Scheme of the primers used in the repeat-specific amplification of *Spergularia echinosperma* and *S. rubra*, with approximate lengths of the PCR products. (B) Variable site in the ITS sequences of *S. echinosperma* and *S. rubra* targeted by the repeat-specific PCR primers.

and SIf/RIr primer pairs. Each PCR was run in two replicates consisting of 35 and 45 cycles. The PCR products were visualized on a 1.5% (w/v) agarose gel.

The percentages of individuals with positive amplification were calculated for each population of *S. echinosperma* and *S. rubra* tested, with at least 3 individuals analysed for each primer pair. Populations with significantly higher amplification rates were detected using Grubbs' test for outlier detection in R 3.2.0 (R Development Core Team 2015; grubbs.test function from the outliers package, ver. 0.14; Komsta 2011).

In addition, the products of the repeat-specific amplification were sequenced in a subset of the samples (17 in EIf/ITS4i, 19 in SIf/EIr, 6 in RIf/ITS4i and 13 in SIf/RIr; Table 1). PCR products showing multiple peaks in the direct sequences were cloned. The resulting sequences were aligned manually in BioEdit 7.2.0 (Hall 1999) and compared with the sequences obtained by direct sequencing. A ribotype network was constructed in TCS 1.21 (Clement et al. 2000) with a 90% connection limit and gaps included as the 5th state. Because of the problems with the RIf/ITS4i sequencing of the longer fragment in *S. echinosperma*, the network was computed only for the sequences of the short fragments (i.e. SIf/EIr and SIf/RIr amplifications).

PCR-RFLP

Only two distinct *rpo*C1 haplotypes were found among all of the samples sequenced, one being specific to *S. echinosperma* and the other to *S. rubra* (see Results). The *rubra*-haplotype possessed a restriction site for the enzyme PdmI (XmnI) at the 569th position of the alignment where the *echinosperma*-haplotype had a one-base substitution preventing this enzyme from cutting. Therefore, a PCR-RFLP protocol for fast identification of particular *rpo*C1 haplotypes was developed. The reactions were as follows: 1.8 µl of the PCR

Table 1. – Ribotypes recorded using repeat-specific PCR amplification in *Spergularia echinosperma* (E) and *S. rubra* (R), and percentages of individuals with positive amplification per population (only populations with more than two individuals tested are considered). Ribotypes EIr0 and RIr0 match the sequences obtained from the direct sequencing of *S. echinosperma* and *S. rubra*, respectively. Values in bold indicate populations with a significantly higher amplification rate for a particular PCR replicate (Grubbs' outlier test; $\alpha = 0.05$).

Population	Individual	Taxon	Haplotypes	Percentage of positive amplification of alternative ribotypes [%]			
				Long fragment (EIf/RIf)		Short fragment (EIr/RIr)	
				35 cycles	45 cycles	35 cycles	45 cycles
Babák	1	E	RIr0, 1	–	–	–	–
	2		RIr1, 4, 8				
Dříteň	1	E	–	0	50	0	0
Hůrka	1	E	–	0	20	0	0
Chvalovec	3	E	RIr1	0	20	0	14
	4		RIr1				
Hoděmyšl	1	E	RIr3	**17**	33	0	40
Jenšov	1	E	RIr0, 9	–	–	–	–
	2		RIr1, 6, 7				
Malobor	1	E	RIr1, 4, 5	0	0	0	0
	2		RIr0, 1				
Mlýnhor	1	E	–	0	0	0	0
Pařezný	1	E	RIr1, 2	0	0	0	0
	2		RIr1				
Podhůrský	1	E	–	0	50	0	17
Skopec	1	E	RIr1	0	33	0	33
Švihov	1	E	–	0	17	0	50
Vosecký	1	E	RIr3	0	0	0	25
Beranov	1	R	EIf0	0	**67**	33	67
	2		EIf0				
	3		EIf0				
	4		EIr0				
Beranov-road	1	R	EIr0, 1	22	11	0	29
Bleddin-road	1	R	–	17	50	0	17
Bohdalov	1	R	–	0	17	0	40
Březejc	1	R	–	0	33	0	0
Černá	1	R	–	0	0	0	40
Dvořák	1	R	–	0	0	0	25
Hoděmysl-road	1	R	–	17	0	0	50
HorMez	1	R	–	0	0	20	0
Chvalovec	1	R	EIf0	**71**	71	67	83
	2		EIr0, EIf0				
Chvalovec-road	1	R	EIf0	**94**	94	35	31
	2		EIr0, EIf0				
Dessau	1	R	EIf0	17	17	17	33
	2		EIr0				
Dobev	1	R	EIr0	17	0	33	17
	2		EIr0, EIf0				
Domburg	1	R	EIf0	0	25	0	0
Grieben	1	R	EIr0	0	17	33	17
Heinrichsberg	1	R	EIf0	**33**	17	0	40
Januš	1	R	EIf1	–	–	–	–
Klec	1	R	–	0	0	0	0
Klieken	1	R	EIf0	**33**	17	17	40
	2		EIr0				
Konračský-road	1	R	–	0	0	0	17

Population	Individual	Taxon	Haplotypes	Percentage of positive amplification of alternative ribotypes [%]			
				Long fragment (EIf/RIf)		Short fragment (EIr/RIr)	
				35 cycles	45 cycles	35 cycles	45 cycles
KrLes	1	R	EIf0	14	29	0	29
Lužnice	1	R	–	0	0	0	0
Máj	1	R	–	0	0	0	0
Mlýnhor-road	1	R	–	0	0	0	33
Mříč	1	R	EIr0	0	0	17	67
Mýto	1	R	–	0	0	0	0
Nový Dářko-road	1	R	–	0	0	0	33
Pecihrádek	1	R	–	0	0	20	0
Písek	1	R	–	0	0	0	40
Pláňava	1	R	–	0	0	0	60
Pobočenský	1	R	EIr0	0	0	25	0
Polom	1	R	EIr0	0	33	33	60
Ptáčov	1	R	EIr0	0	17	17	33
Rožmitál	1	R	–	0	0	0	0
Siglovec	1	R	EIf0	17	50	0	20
	2		EIf2				
Skopec-road	1	R	EIr0	0	50	33	67
Slavkovický	1	R	EIr0, EIf0	20	20	20	20
St Hlína	1	R	–	0	20	0	0
Strmilov	1	R	–	0	0	25	0
Švihov-road	1	R	EIr0	0	33	17	33
Telč Štěpnice	1	R	EIr0	0	17	17	50
Vlkov	1	R	–	0	0	0	0
Vosecký-road	1	R	–	0	11	0	11
Vrbinec	1	R	EIr0	0	0	17	50
Waidhaus	1	R	–	0	20	0	0
Zavlekov	1	R	EIr0	0	0	40	20

product was added to a mixture containing 1.35 U of PdmI enzyme (Fermentas, Lithuania), 0.27 µl of 10× Tango buffer (Fermentas) and 1.98 µl of sterile H_2O. The mixture was incubated at 37 °C for 6 h and the entire reaction volume was analysed electrophoretically on a 1.5% (w/v) agarose gel. The specificity of this method was tested using 27 samples of *S. echinosperma* and 60 samples of *S. rubra*, the identities of which were confirmed by direct sequencing.

Results

Chromosome counts and ploidy levels

Three different cytotypes were found, corresponding to diploid *S. echinosperma*, tetraploid *S.* ×*kurkae* and tetraploid *S. rubra*. All three taxa also differed in monoploid genome size (Fig. 3). An exception was one individual of *S.* ×*kurkae* (population Gbelinek), which had a monoploid genome size significantly larger than that of the other *S.* ×*kurkae* individuals. Chromosome counts confirmed 2n = 36 for *S. rubra* and *S.* ×*kurkae* and 2n = 18 for *S. echinosperma* (Fig. 4).

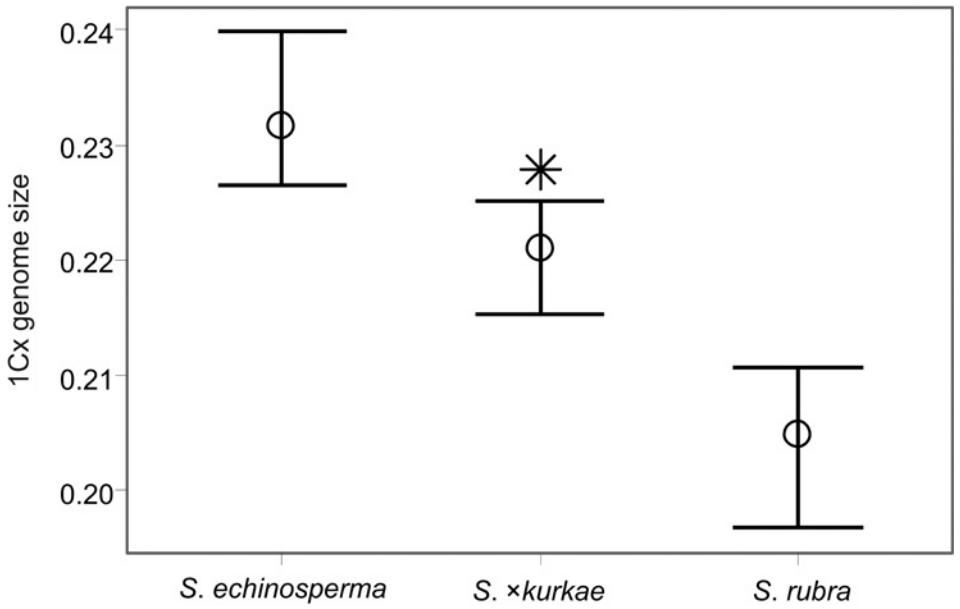

Fig. 3. – Range plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for *Spergularia echinosperma*, *S.* ×*kurkae*, and *S. rubra* expressed in terms of a ratio with the 1C value of the internal standard *Glycine max*. Midpoint = median; error bar = min–max. The *S.* ×*kurkae* outlier is marked with an asterisk.

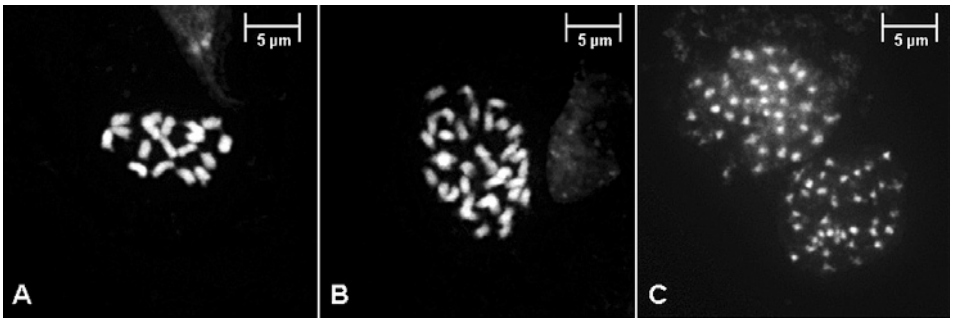

Fig. 4. – Mitotic chromosome spreads of *Spergularia echinosperma* (A; 2n = 18), *S.* ×*kurkae* (B; 2n = 36) and *S. rubra* (C; 2n = 36).

Direct sequencing and cloning

Direct sequencing of the ITS region of 112 plants resulted in two distinct ribotypes that were specific for *S. echinosperma* and *S. rubra* (GenBank acc. no. KU662347–KU662348). The alignment had a length of 674 bp and contained 16 substitutions and 4 indels (Electronic Appendix 2). Cloning of *S.* ×*kurkae* resulted in two ITS sequences that were identical with the ribotypes of *S. echinosperma* and *S. rubra*. The pattern of nucleotide

additivity of these two ribotypes was recorded in the direct sequences of 39 individuals of *S.* ×*kurkae*, including the holotype. Seven individuals were exceptions, in which only the peaks of *S. echinosperma* were observable (populations: Špitálský – 3 individuals, Chvalovec, Bleddin, Kozcin, Veselský – 1 individual each).

Only two different *rpo*C1 haplotypes were found among all of the 95 plants sequenced: one unique for *S. rubra* and the other unique for *S. echinosperma* and *S.* ×*kurkae* (GenBank acc. no. KU671397–KU671398). The alignment had a length of 802 bp and contained two substitutions and one indel (Electronic Appendix 3). The PdmI assay consistently produced two fragments for *S. rubra* (200 and 600 bp), whereas no digestion was detectable for *S. echinosperma*. We confirmed the presence of the *S. echinosperma* haplotype in all 164 individuals of *S.* ×*kurkae* (Electronic Appendix 1). The attempts to amplify the *rpo*C1 intron in the holotype of *S.* ×*kurkae* failed.

Repeat-specific amplification

Both sets of the *rubra*-specific ITS primers amplified positively in five out of the seven individuals of *S.* ×*kurkae* for which the *S. rubra* ribotype was not visible in the direct sequences (populations Bleddin, Chvalovec, Kozcin, Špitálský, and Veselský). The other two samples (population Špitálský) did not show any positive amplification.

The *echinosperma*-specific ITS primers amplified positively in 18% of the 267 individuals of *S. rubra*, averaged over both PCR replicates. The rate of positive amplification was distributed unequally among the *S. rubra* populations, with only a few highly amplifying populations (Beranov, Chvalovec, Chvalovec-road, Heinrichsberg, and Klieken; Table 1). The *rubra*-specific primers amplified positively in 10% of the 64 individuals of *S. echinosperma*, averaged over both PCR replicates. The rates of positive amplification were distributed randomly among the populations of *S. echinosperma*, and there were no populations with consistently higher amplification rates (Table 1).

A subset of the PCR products of the *echinosperma*-specific amplification in *S. rubra* (32 individuals) and the *rubra*-specific amplification in *S. echinosperma* (13 individuals) was sequenced. The longer *echinosperma*-specific EIf/ITS4i products from *S. rubra* were found in all but two of the individuals to be identical with the ITS sequence of *S. echinosperma* (ribotype EIf0). The two *S. rubra* individuals produced sequences differing from this ribotype by a single substitution (ribotypes EIf1–EIf2; Tables 1 and 2). Sequencing of the longer *rubra*-specific RIf/ITS4i products from *S. echinosperma* was unsuccessful due to a very weak signal.

The shorter *echinosperma*-specific SIf/EIr products from *S. rubra* were in nearly all of the individuals identical with the ITS ribotype of *S. echinosperma* (ribotype EIr0). Additionally, one individual displayed intra-individual sequence variation and contained another unique sequence differing from the EIr0 ribotype in one substitution (ribotype EIr1; Tables 1 and 3). The shorter *rubra*-specific SIf/RIr products from *S. echinosperma* resulted in 10 different *rubra*-like ribotypes (RIr0–RIr9), which were clearly separated from the *echinosperma*-like ribotypes (EIr) in the TCS network (Fig. 5). The separation between the RIr and EIr groups of ribotypes was distinct, with at least three hypothetical missing haplotypes. Importantly, the ribotype matching the ITS sequence of *S. rubra* (RIr0) was very rare in *S. echinosperma*, being detected in only three individuals (Tables 1 and 3). The remaining *rubra*-like ribotypes (RIr1–9) found in 13 individuals of *S. echinosperma*

were derived from the RIr0 ribotype of *S. rubra* and differed by 1–4 substitutions. There was no clear pattern in the geographic distribution of the different ribotypes (Electronic Appendix 4).

Table 2. – Ribotypes recorded using repeat-specific amplification by EIf/ITS4i primers in *Spergularia rubra* and their comparison with the ITS sequence of *S. rubra*. Only variable sites are shown. The position numbers correspond to the alignment of the whole ITS region (Electronic Appendix 2).

Haplotype/position	131	171	190	202	205	208	235	306	488
S. rubra	G	A	C	C	T	T	C	A	C
EIf0 (= *S. echinosperma*)	–	A	T	T	C	C	T	A	T
EIf1	G	G	T	T	C	C	T	A	T
EIf2	G	A	T	T	C	C	T	C	T

Table 3. – Ribotypes recorded using repeat-specific amplification by SIf/EIr primers in *Spergularia rubra* (haplotypes EIr0-EIr1) and SIf/RIr primers in *S. echinosperma* (haplotypes RIr0-RIr9). Only variable sites are shown. The position numbers correspond to the alignment of the whole ITS region (Electronic Appendix 2).

Haplotype/position	73	74	77	78	79	80	87	95	107	110	112	114	116
EIr0 (= *S. echinosperma*)	G	G	C	G	C	C	–	T	T	T	C	T	C
EIr1	A	G	C	G	C	C	–	T	T	T	C	T	C
RIr0 (= *S. rubra*)	G	G	C	G	C	C	C	C	C	C	C	C	C
RIr1	G	G	A	G	C	C	T	C	T	T	C	C	C
RIr2	G	G	C	G	C	C	C	C	C	C	A	C	C
RIr3	G	G	C	G	C	C	C	C	C	T	C	C	C
RIr4	G	G	C	G	C	T	C	C	C	C	C	C	C
RIr5	G	G	C	A	C	C	C	C	T	T	C	C	C
RIr6	G	G	C	G	T	C	C	C	C	C	C	C	C
RIr7	G	T	C	G	C	C	C	C	C	C	C	C	C
RIr8	G	G	C	G	C	C	C	C	C	C	C	T	C
RIr9	G	G	C	G	C	C	C	C	C	C	C	C	A

Discussion

Origin of Spergularia ×kurkae

The chromosome counts and flow cytometric measurements confirmed previous reports of the ploidy levels for all the taxa studied. Only one individual of *S. ×kurkae* displayed an exceptionally high genome size and may be an aneuploid. The concurrent presence of the ITS ribotypes from *S. echinosperma* and *S. rubra* in nearly all of the individuals of *S. ×kurkae* (including the holotype) convincingly demonstrates the hybrid origin of *S. ×kurkae*. This finding is in accordance with the morphological evidence of Kúr et al. (2012).

The chloroplast DNA revealed that *S. echinosperma* was the maternal progenitor of *S. ×kurkae* in all cases. No triploids were found among the populations of *S. echinosperma* and *S. rubra* in either this study or in that of Kúr et al. (2012), indicating that triploids do not play a role in the evolution of this group. It is therefore likely that the formation of *S. ×kurkae* was a one-step process that involved unreduced gametes of *S. echinosperma*.

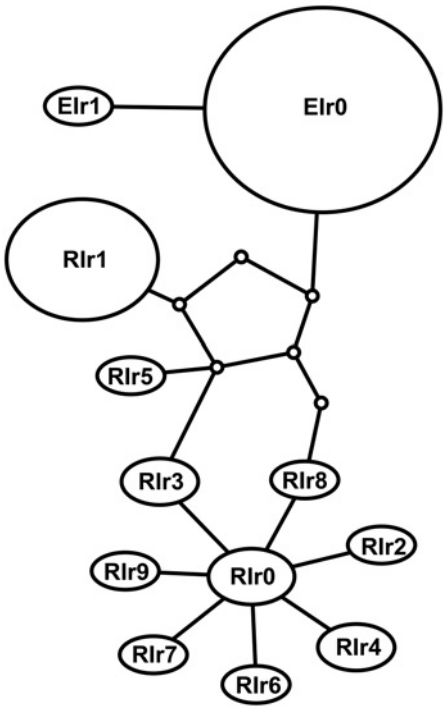

Fig. 5. – Ribotype network based on the sequences obtained from repeat-specific amplification with SIf/EIr and SIf/RIr primers. The size of the ovals indicate the relative frequencies of particular ribotypes in our data. The empty circles represent hypothetical missing haplotypes.

The incomplete concerted evolution in *S.* ×*kurkae* (i.e. lacking homogenization of divergent rDNA copies; Zimmer et al. 1980, Hillis et al. 1991, Elder & Turner 1995), an annual species with a short generation time, indicates its young age (cf. Sang et al. 1995, O'Kane et al. 1996, Koch 2003). We hypothesize that *S.* ×*kurkae* formed after *S. echinosperma* and *S. rubra* came into contact as a result of human-mediated introduction of *S. rubra* into fishponds (e.g. possibly due to grazing of summer-drained fishponds and the sowing of cereals and other culture plants; Šumberová 2003). In Bohemia, such introductions might be associated with fish farming, which began in the 11th century and was most intensive in the 15th and 16th centuries (Šumberová et al. 2006). Before the advent of fish farming, it was likely that the contact between *S. echinosperma* and *S. rubra* was limited as these species were likely ecologically separated at that time based on their contemporary primary habitats along the river banks of the Elbe in Germany (U. Amarell, pers. comm.). Additional insight into the origin of *S.* ×*kurkae* is expected to be provided by an ongoing study based on microsatellite markers (Kúr et al. 2014).

Interspecific gene flow

As the origin of the tetraploid *S. rubra* is unknown, the high incidence of the *S. echinosperma* ITS ribotype in some populations of *S. rubra* might be explained in two

ways. First, *S. rubra* might be of allopolyploid origin, with one parental genome from *S. echinosperma* or a species closely related to *S. echinosperma*. In this scenario, the observed intra-individual ITS variation within *S. rubra* would represent the remains of the *S. echinosperma*-like ancestor retained within *S. rubra*. This pathway could also explain the discrepancy between the recorded genome size of *S. ×kurkae* and that predicted by combining the genome sizes of its parents (Kúr et al. 2012). If *S. rubra* acted as a segmental allopolyploid, it might produce gametes with a higher genome size than half of that of the *S. rubra* somatic genome size, leading to the apparent genome upsizing in *S. ×kurkae*.

Alternatively, the *S. echinosperma* ITS variants within *S. rubra* might be the result of ongoing gene flow between *S. ×kurkae* and *S. rubra*. Gene introgression between these two taxa was previously suggested by the existence of morphologically intermediate plants (Kúr et al. 2012). Importantly, in the present study, all five populations of *S. rubra* that had significantly high rates of amplification of the *echinosperma*-specific ITS primers (Table 1) were located near present (Beranov, Chvalovec, Chvalovec-road) or historical (Heinrichsberg, Klieken) localities of *S. echinosperma* or *S. ×kurkae*. We consider these findings as a good indicator of interspecific gene flow. However, the two hypotheses are not mutually exclusive, and both processes might be involved.

In contrast, we found no reliable evidence of gene flow from *S. ×kurkae* to *S. echinosperma* as the *S. rubra* ITS ribotype (RIr0) was almost never present within *S. echinosperma*. The several *rubra*-like ribotypes found in *S. echinosperma* (Table 1) were more divergent and are likely a result of an ancestral polymorphism retained within *S. echinosperma*. If there was recent gene flow from *S. rubra* to *S. echinosperma*, we would expect the frequent occurrence of the RIr0 ribotype in *S. echinosperma.*

Therefore, our results conflict with Dvořák (1990), who argues that there is a constant gene flow from *S. rubra* to *S. echinosperma*. We conclude that *S. echinosperma* is not currently threatened by genetic assimilation. However, even if the ploidy barrier protects this species from assimilation, it might still be threatened by ecological competition from *S. ×kurkae* (demographic swamping; Levin et al. 1996), as the latter has a higher fitness than *S. echinosperma* in terms of higher seed set and more rapid growth (P. Kúr, unpubl.). For example, in *Typha ×glauca* (Huisman et al. 2012) and *Spartina anglica* (Begon et al. 1991, Ennos & Sheffield 2009), hybrids successfully outcompeted the parental species. Further studies are needed to reliably assess the risks that *S. ×kurkae* poses to the rare endemic *S. echinosperma*.

Status of Spergularia ×kurkae

Since the description of *Spergularia ×kurkae* by Dvořák (1989), this taxon has not been listed in any of the central-European floras or checklists (Fischer et al. 2008, Jäger 2011, Danihelka et al. 2012, Goliašová 2012) except for the Flora of the Czech Republic (Dvořák 1990). Our current data, however, support *S. ×kurkae* as an independent taxon that mostly occurs in the absence of the parental species, which is consistent with its distinct morphological separation (Kúr et al. 2012). Although there are some indications of ongoing hybridization between *S. ×kurkae* and *S. rubra*, it appears to be rare, with little effect on the boundary between the taxa. In addition, according to preliminary data on germination ecology (P. Kúr, unpublished), the percentage germination recorded for

S. ×*kurkae* is similar to that of *S. echinosperma* and it does not suffer from reduced fertility. We therefore propose that *S. kurkae*, originally described as a primary hybrid, be treated as a separate allopolyploid species, in a similar way to *Bolboschoenus laticarpus* Marhold et al. (Marhold et al. 2004), *Galeopsis tetrahit* L. (Bendiksby et al. 2011) and *Veronica hederifolia* L. (Albach et al. 2008).

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Souhrn

Cílem předkládané práce bylo studium původu taxonu *Spergularia* ×*kurkae*, předpokládaného křížence diploidního středoevropského endemita *S. echinosperma* a široce rozšířeného tetraploidního druhu *S. rubra*. Celkem bylo analyzováno 516 rostlin z 91 populací z území České republiky a Německa, a to včetně typové položky jména *S.* ×*kurkae*. Použitými metodami bylo sekvenování jaderného ITS regionu a chloroplastového *rpo*C1 genu. *Spergularia echinosperma* a *S. rubra* se výrazně lišily ve svých ITS sekvencích. Oba ITS ribotypy se rovněž vyskytovaly pohromadě v genomu téměř všech jedinců *S.* ×*kurkae*, což přesvědčivě dokazuje hybridní původ tohoto taxonu. Chloroplastová DNA rovněž prokázala, že ve všech případech byl mateřským rodičem druh *S. echinosperma*. U téměř všech jedinců *S.* ×*kurkae* byly oba ITS ribotypy zřetelně patrné na přímých sekvencích, což ukazuje na neúplnou homogenizaci ribozomální DNA (incomplete concerted evolution) a naznačuje, že *S.* ×*kurkae* je pravděpodobně relativně mladým taxonem. Je možné, že se taxon *S.* ×*kurkae* vyvinul následkem člověkem zapříčiněné introdukce druhu *S. rubra* na obnažená dna rybníků, pravděpodobně ve spojitosti s rozvojem rybníkářství v Čechách. Reciproká amplifikace druhově specifických ITS primerů rovněž naznačila možnost genového toku na tetraploidní úrovni mezi *S.* ×*kurkae* a *S. rubra*, který je ale poměrně vzácný. Naproti tomu nebyly nalezeny důkazy o ohrožení *S. echinosperma* genetickou erozí a genovým tokem od *S. rubra*. Na základě spojení těchto výsledků a předchozích studií (morfologické rozdíly, samostatný výskyt nezávislý na rodičovských druzích) doporučujeme klasifikovat *S. kurkae* jako samostatný allopolyploidní druh.

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