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Biosystematic revision of the *Spergularia echinosperma* complex

Ph.D. Thesis

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Annotation

This thesis is focused on the biosystematic study of the Central-European endemic *Spergularia echinosperma*. With the combined use of morphometric analyses, genome size measurements and molecular tools, the taxonomic issues associated with this species have been clarified. The existence of *S. kurkae*, a stable allotetraploid hybrid between diploid *S. echinosperma* and tetraploid *S. rubra*, has been proven. Based on several lines of evidence, including distinct morphological separation and frequent occurrence in the absence of the parental species, treating *S. kurkae* as a separate species is proposed. In addition, two infraspecific taxa within *S. echinosperma*—*S. echinosperma* subsp. *echinosperma* and *S. echinosperma* subsp. *albensis*—differing in distributions and ecology have been described. A complete revision of the localities of *S. echinosperma*, *S. kurkae* and *S. rubra* in the Czech Republic is also presented. Furthermore, the development of 16 polymorphic microsatellite loci for *S. echinosperma* is reported.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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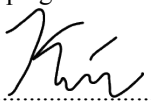

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Pavel Kúr

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My greatest thanks belong to my supervisor Milan Štech who in 2005 initiated the study of *Spergularia echinosperma* for my Bachelor's thesis, probably having no idea this topic was going to remain relevant till my PhD and result in 5 impacted articles. I must also thank Kateřina Šumberová, the foremost living Czech wetland botanist, for her valuable advice and cooperation, and to Michal Ducháček, the busiest living Czech herbarium curator, for supporting my research. Further thanks belong to Alžběta Manukjanová for her help with field sampling, Eliška Vicherová for her help with experiment preparation, and Jana Piherová for performing some of the morphological measurements.

Dedicated to my mom (1947–2016) whom a malignant disease prevented from witnessing this day...

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Kúr P.**, Štech M., Koutecký P. & Trávníček P. (2012): Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra*, and notes on potential hybridization of these two species. – Preslia 84: 905–924 (IF = 2.989).

Pavel Kúr participated in the field sampling and flow cytometry measurements, performed all the morphological measurements and statistical analyses, and wrote the draft of the manuscript.

II. **Kúr P.**, Košnar J. & Štech M. (2014): Characterization and cross-species amplification of 16 microsatellite loci in *Spergularia echinosperma* (Caryophyllales: Caryophyllaceae). – Conservation Genetics Resources 6: 571–573 (IF = 0.789).

Pavel Kúr participated in the search and testing of microsatellite markers from the SSR-enriched library, performed the statistical analyses and wrote the draft of the manuscript.

III. Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtek J., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., **Kúr P.**, Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – Preslia 88: 229–322 (IF = 2.989).

Pavel Kúr compiled the distributional data for two species (*Spergularia echinosperma*, *S. kurkae*) and participated in the revision of the records on the distributions of other four species (*S. rubra*, *S. marina*, *S. media*, *Cyperus fuscus*).

This participation is hereby confirmed by the signature of the first author:

Zdeněk Kaplan, Ph.D.

IV. **Kúr P.**, Košnar J., Koutecký P., Tremetsberger K. & Štech M. (2016): Origin of *Spergularia* ×*kurkae*, a hybrid between the rare endemic *S. echinosperma* and its widespread congener *S. rubra*. – Preslia 88: 391–407 (IF = 2.989).

Pavel Kúr performed major part of the laboratory analyses, contributed significantly to the specimen collection and wrote the draft of the manuscript.

V. **Kúr P.**, Amarell U., Jage H. & Štech M. (XXXX): Taxonomy and evolutionary diversification of the Central European endemic *Spergularia echinosperma* (Caryophyllaceae). *Manuscript*.

Pavel Kúr initiated the study, participated in the specimen collection, revised herbarium specimens, performed the statistical analyses and wrote the draft of the manuscript.

Contents

General introduction

Polyploidy

Polyploidy, defined as the possession of three or more complete sets of chromosomes, is nowadays recognized as the major force driving the evolution of plants (Ramsey & Schemske 1998, Adams & Wendel 2005, Soltis et al. 2014, 2015). All extant groups of angiosperms have undergone at least one, but in most cases multiple rounds of polyploidization (Soltis et al. 2014). Among other plant groups, polyploidy is also frequent in lycophytes and monilophytes (Husband et al. 2013). It is estimated that about 15% of speciation events in flowering plants and about 31% of speciation events in ferns directly involve polyploidy (Wood et al. 2009).

The reason why polyploidy is so important an evolutionary force is that it generates excessive genetic material, which opens up the door for evolutionary novelty. Higher gene dosage can protect the genome from the deleterious effects of recessive alleles and may lead to functional diversification of the homologs (Soltis et al. 2014). Therefore, polyploids often possess novel physiological and life-history characteristics, which may aid their adaptation to novel environments and help occupy new ecological niches (Ramsey & Schemske 1998).

Classification of polyploids

Based on the mode of origin, two main types of polyploids are recognized. Autopolyploids are formed by a polyploidization event within a single species. They are traditionally supposed to be characterized by the formation of multivalents during meiosis as a result of non-preferential pairing of three or more sets of homologous chromosomes. Hence, alleles at a given locus segregate at random which leads to polysomic (multisomic) inheritance. Allopolyploids, in contrast, arise in consequence of hybridization between different species. They are typically characterized by disomic inheritance and fixed heterozygosity (Soltis et al. 2014, 2016).

In reality, however, the clear discrimination of polyploids based on the mode of inheritance is not possible. In nature, there is a continuum between polyploids with strictly polysomic and strictly disomic inheritance, and different loci within a single polyploid frequently exhibit different modes of inheritance—this phenomenon is referred to as mixosomy and polyploids exhibiting such behavior are called segmental allopolyploids (Ramsey & Schemske 2002, Soltis et al. 2016). The mode of origin does not play a role. Autopolyploids are under selection pressure against polysomic inheritance (which leads to gametes with unbalanced chromosome numbers and reduced fertility) and towards rediploidization. In allopolyploids, on the other hand, the occurrence of multivalents is common as a result of partial homology of homeologous chromosomes (Ramsey & Schemske 2002).

There has been controversy on the relative frequency of auto- and allopolyploids in nature. Allopolyploids were traditionally considered to be more common than autopolyploids (Ramsey & Schemske 1998, Soltis et al. 2004). The reason was the abovementioned concerns about multivalent formation leading to reduced fertility in autopolyploids (Soltis et al. 2014). Empirical data, however, have disproved this traditional view. It is now believed that autopolyploids play a very significant role in nature (Soltis et al. 2007, 2014), and some studies indicate they might be even more frequent than allopolyploids (Barker et al. 2015).

Pathways of polyploid formation

There are three distinct mechanisms leading to the formation of new polyploids. The simplest pathway is crossing of two polyploids of differing ploidy levels that leads directly to the formation of a new cytotype. This way, triploids are commonly formed in the contact zones between diploid and tetraploid cytotypes (Ramsey & Schemske 1998).

Another pathway of polyploid formation is somatic doubling. It occurs when mitotic abnormalities result in polyploid cells within the somatic tissue of a plant, and these cells subsequently form reproductive organs. Gametes produced in such organs have double chromosome numbers than the maternal plant and a self-fertilisation event may result in the formation of a polyploid. This pathway is generally considered to be rare (Mason & Pires 2015), although it was responsible for the formation of the first described allopolyploid *Primula kewensis* (Soltis et al. 2014).

The most frequent mechanism of polyploid formation, however, is the production of unreduced gametes (i.e. gametes with the somatic chromosome number). Union of one reduced and one unreduced gamete or of two unreduced gametes leads to a one-step formation of a new polyploid (Ramsey & Schemske 1998). Unreduced gametes are a ubiquitous phenomenon in angiosperms and are produced by a plethora of different mechanisms across different taxa (Ramsey & Schemske 1998, Ramsey 2007, Mason & Pires 2015). Their production is currently viewed as an important mechanism for evolutionary speciation (Mason & Pires 2015). Although the overall frequency of unreduced gametes in nature is estimated to be very low—approximately 0.1–2.0% (Ramsey 2007)—it can vary substantially among different individuals and populations within a species. Only a small fraction of high-frequency 2n gamete producers within a population can affect the dynamics of polyploid generation significantly (Ramsey & Schemske 1998). Formation of unreduced gametes is also promoted by environmental stress (e.g. high or low temperatures, herbivory, wounding or water and nutrient deficit) and helps plants establish new polyploid lineages better adapted to harsh environments (Ramsey & Schemske 1998). In *Achillea borealis*, for example, individuals grown in a growth chamber under large temperature fluctuations produced nearly 3-times more unreduced pollen than individuals from the same population grown in the field (Ramsey 2007). The tendency of harsh environmental conditions to induce unreduced gametes is also probably related to the well-documented high incidence of polyploidy in high mountains and arctic regions (Ramsey & Schemske 1998, Brochmann et al. 2004).

Hybridization

Hybridization, like polyploidy, also belongs to the major forces driving the evolution of plants (Harrison 1990, Arnold 1997, Mallet 2005). Hybridization, however, is not as ubiquitous as polyploidy. Estimates of the frequency of natural hybridization in plants differ depending on the author and methodology used. Arnold (1997) suggests that greater than 50% of angiosperms may be of hybrid origin. This estimate, however, relates to the number of ancient hybridization events and does not indicate that more than half of contemporary species are hybrids. Focusing on recent hybridization events, about 25% of plant species are known to hybridize with at least one other species (Mallet 2005). The estimates of the number of hybrid species vary around 10%, differing significantly between regions (Ellstrand et al. 1996, Whitney et al. 2010). There

has also been an increasing number of documented cases of interspecific hybridization in bryophytes (Wyatt et al. 1988, Natcheva & Cronberg 2004, Ricca & Shaw 2010).

It should be noted, however, that natural interspecific hybrids are distributed highly non-randomly among taxonomic groups. Only 40% of families and 16% of genera were found by Whitney et al. (2010) to contain hybrids. There seems to be a strong phylogenetic signal in the hybridization propensity, which is concentrated into a few groups of vascular plants only (Whitney et al. 2010).

Pathways of hybridization

There are two distinct types of hybridization. If the both hybridizing species are of the same ploidy, it is called homoploid hybridization (Yakimowski & Rieseberg 2014). Crossing of species of different ploidy levels is referred to as interploidy (interploid, interploidal, heteroploid) hybridization (Schatlowski & Köhler 2012).

The both types of hybridization have their own pitfalls. Homoploid hybridization often yields non-viable or sterile progeny due to gene and chromosome incompatibilities (Yakimowski & Rieseberg 2014). Even if fertile progeny is produced, they usually lack reproductive isolation from the parents, which leads to introgression and formation of hybrid swarms rather than hybrid speciation (Rieseberg 1997, Mallet 2007). However, there are two principal ways how a homoploid hybrid can escape backcrossing with parental species and become an independent entity. First, hybrids can colonize novel ecological niches with the absence of the parental species thereby reducing gene flow between them and parents (Mallet 2007). Second, polyploidization of a homoploid hybrid may create an instant reproductive barrier between the hybrid and its parents. It may also suppress complications associated with poor pairing of homologous chromosomes and gene incompatibilities between the two sub-genomes. Parental divergence has been indeed empirically proven to positively correlate with the likelihood of polyploidization in homoploid crosses (Chapman & Burke 2007, Paun et al. 2009).

Interploidy hybridization was traditionally believed to be rare in nature (Chapman & Abbott 2010). The reason was a theoretically predicted production of non-viable or sterile odd-ploidy progeny, in case of diploid-tetraploid crosses called "triploid block" (Köhler et al. 2010). Triploid block can have two different causes. First is the parental genomic imbalance. In angiosperm homoploid crosses, the ratios of maternal to paternal genomes are 1:1 in the embryo and 2:1 in the endosperm. Both these ratios are crucial for the correct development of the seed and their violations in triploid seeds lead to substantial developmental defects (Stoute et al. 2012, Sutherland & Galloway 2017). Second, even if a triploid hybrid is viable, it should suffer from reduced fertility as a result of production of aneuploid gametes (i.e. with unbalanced chromosome numbers) that cannot function normally (Ramsey & Schemske 1998).

However, there has been an increasing amount of evidence recently of the limited negative impact of triploid block on interploidy hybridization in nature. Parental genomic imbalance does not cause aberrations in endosperm development in all cases and viable odd-ploidy hybrids are commonly found in nature (Ramsey & Schemske 1998, Soltis et al. 2004, Schatlowski & Köhler 2012, Mason & Pires 2015). The interploidy barrier is even weaker in some higher-ploidy crosses where there is a more favorable maternal:paternal genome ratio in

the endosperm (Sutherland & Galloway 2017). It has been also demonstrated that odd-ploidy hybrids typically produce a fraction of euploid gametes (i.e. with balanced chromosome numbers) which function normally, and they may therefore be partially fertile (Chapman & Abbott 2010). Among the studies reviewed by Ramsey & Schemske (1998), for example, the average percentage of euploid pollen in triploids was around 10%. This pathway of interploidy hybridization is called "triploid bridge" (Paun et al. 2009, Ricca et al. 2011, Arnold et al. 2015).

The most important and widespread mechanism of interploidy hybridization, just like in the case of polyploid formation, is production of unreduced gametes. This pathway circumvents the obstacles associated with triploid formation and leads to a direct formation of even-ploidy progeny (Köhler et al. 2010). This pathway also effectively eliminates complications associated with parental genomic imbalance. Especially fusion of an unreduced gamete from a diploid with a reduced gamete from a tetraploid leads to embryo and endosperm having the maternal:paternal genome ratios close to the normal ratios in homoploid crosses (Sutherland & Galloway 2017).

Recurrent origins of hybrids

It is now well established, based on several lines of evidence, that recurrent formation of hybrids is the rule rather than the exception in nature (Levin 2001, Soltis & Soltis 2009). Interestingly, independently formed hybrids do not always follow the same evolutionary paths. In *Spartina*, for example, two independently formed homoploid hybrids between *S. alterniflora* and *S. maritima* had very different morphological and genomic consequences. Hybrid formed in England (*S. ×townsendii*) polyploidized and formed a very successful invasive allopolyploid species *S. anglica*. Independently formed hybrid from France (*S. ×neyrautii*) differed significantly in morphology and patterns of DNA methylation and gene expression from *S. ×townsendii* and did never undergo subsequent chromosome doubling (Ainouche et al. 2012, Soltis et al. 2016).

In contrast, evolution repeats itself in some systems of recurrently formed hybrids. A well-described example is the pair of species *Tragopogon mirus* and *T. miscellus*. Both the species are allopolyploids formed by hybridizations of *T. dubius* × *T. porrifolius* and *T. dubius* × *T. pratensis*, respectively (Lim et al. 2008). In these species, naturally occurring multiple origins, as well as multiple synthetic lines, were shown to replay the evolutionary scenario in much the same way, morphologically, genomically, and chromosomally (Soltis et al. 2016).

Hybridization as a thread to rare species

Although hybridization may have positive consequences in increasing the intraspecific genetic diversity thus promoting local adaptation, it may also pose a serious threat to rare species. Two main mechanisms by which hybridization may threat populations of rare species are demographic swamping and genetic assimilation.

Demographic swamping is a process when a hybrid ecologically outcompetes its parental species (Holderegger 1998). It should be noted that hybrids need not automatically have superior fitness than their parents and it largely depends on their genotypic constitution and the environment in which they occur (Arnold & Hodges 1995, Arnold & Martin 2010). However, there are some documented cases of hybrids locally outcompeting their parental

species, e.g. in *Typha* ×*glauca* (Huisman et al. 2012) or *Schoenoplectiella* ×*magrathii* (Smith & McKenzie 2013). Polyploidization of a primary hybrid may in some cases, like in *Spartina anglica* (Ainouche et al. 2012), significantly improve its competitive abilities (Rieseberg 1997).

Genetic assimilation is a phenomenon where genetic material from a more abundant species is transferred via hybridization into a rare species. The rare species consequently loses its genetic integrity and is absorbed by a hybrid swarm (Holderegger 1998). That genetic assimilation may pose a serious threat to endangered species have been documented in several cases. The most infamous one is that of *Cercocarpus traskiae*, species endemic to Catalina Island located off the SW coast of California. The species is represented by a single population, which consisted of only 11 adult trees and several tens of seedlings in the time when Rieseberg & Gerber (1995) carried out their genetic analysis. Using molecular markers, the authors detected that half of the adult trees and some of the seedlings were actually hybrids between *C. traskiae* and a more abundant congener *C. betuloides* var. *blancheae*. Only 6 (!) out of the 11 adult individuals were found to be pure *C. traskiae* (Rieseberg & Gerber 1995). Another example of an endemic species endangered by genetic assimilation from a widespread congener is that of *Argyranthemum coronopifolium*, an endemic of Canary Islands. Brochmann (1984) demonstrated that this species is being genetically eroded by *A. frutescens*, a weedy species that has migrated along road sites to the populations of *A. coronopifolium* as a result of the development of tourism. Although the above mentioned species represent extreme cases, local extinctions of populations of rare species may be a more common phenomenon (Rieseberg & Wendel 1993).

It has become clear that the most significant trigger for hybridization has been the effect of humans on ecosystems. It is processes like habitat disturbance, creation of new habitats, introduction of allochthonous species, and climatic changes caused by human activities that 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). It is not only the thread that hybrids present to individual rare species. Hybridization has also been proven to have the potential to increase the invasiveness of introduced plant species thus threatening whole plant communities (Ellstrand & Schierenbeck 2000, Schierenbeck & Ellstrand 2009).

Detecting hybridization

Proving hybridization may be a challenging task. Traditionally, the presence of hybridization has been inferred from morphological evidence. This approach is still widely-applied in floras and largely affects our current view on the frequency of hybridization in plants (cf. Whitney et al. 2010). Nevertheless, even if there are many examples of interspecific hybrids whose hybrid origin does reflect in their intermediate morphology, morphological character intermediacy in general is a poor predictor of hybrid ancestry (López-Caamal & Tovar-Sánchez 2014). In a revision of 46 studies exploring morphological character expression in hybrids, Rieseberg & Ellstrand (1993) pointed out that only less than half of the morphological characters investigated in F1 hybrids displayed intermediacy between the parental species.

Problems also arise in species which have a limited number of usable morphological characters. Many examples can be found among aquatic plants, which are infamous among

taxonomists for their reduced morphology and wide phenotypic plasticity (Les et al. 2009, 2010). In such cases, reliable hybrid recognition cannot be done without additional markers.

Besides phenotypic characters, hybrid recognition relies nowadays on the genetic data of individuals. Historically, many types of molecular methods have been employed for hybrid recognition. They can be divided based on multiple criteria, but the most practical one for our purposes is their division into single-locus and multi-locus methods.

Single-locus methods rely on the PCR amplification of a single DNA locus. Information from multiple such loci can be obtained, but it is combined post-hoc (Crawford & Mort 2004). Although for phylogeny reconstruction, low-copy or single-copy markers (i.e. those which are present in a limited number of copies in the genome) are generally preferred (Curto et al. 2012), hybridization hypotheses can be better tested with the use of multi-copy markers. Among the most popular ones is the nuclear ribosomal DNA (nrDNA), most notably the internal transcribed spacer (ITS) region (Feliner & Rosselló 2007). Its multi-locus nature and biparentall inheritance causes that it often retains intra-individual polymorphism in hybrid taxa, thus allowing clear inferences about their parentage (e.g. Sang et al. 1995, Koch 2003). In addition, there are a few more practical advantages, which make the use of the ITS popular. First, there are several sets of universal PCR primers available. Second, the multi-copy structure of the ITS facilitates PCR amplification from worse-quality material, e.g. herbarium specimens. However, the use of the ITS region is limited in some cases. Ribosomal DNA is subject to a process called "concerted evolution", which means homogenization of the divergent nrDNA copies within genome over time. This process can, in some cases very rapidly (Fuertes Aguilar et al. 1999, Kovarik 2005), obliterate the traces of one parental genome within a hybrid concealing its origin. This is particularly true in old hybrids (Feliner & Rosselló 2007).

In the cases where single-locus markers fail to reconstruct the hybrid ancestry, multi-locus markers can be tried instead. Multi-locus molecular methods utilize the information from a multitude of independent DNA loci, which is typically generated in a single run. The most popular examples of such methods include the Restriction Fragment Length Polymorphism (RFLP; Bernatzky 1989), Random Amplified Polymorphic DNA (RAPD; Hadrys et al. 1992), Amplified Fragment Length Polymorphism (AFLP; Vos et al. 1995), Inter Simple Sequence Repeats (ISSR; Godwin et al. 1997), and Simple Sequence Repeats (SSR or microsatellites; Zietkiewicz et al. 1994). Using statistical models such as Bayesian clustering (Porras-Hurtado et al. 2013), information from multiple loci can be used to cluster investigated individuals and to infer the probable hybrid ancestry. However, all of the above mentioned methods have some serious drawbacks. They are: (1) the requirement of large quantities of sample DNA (RFLP), (2) poor reproducibility (RAPD, to some extent also ISSR and AFLP), (3) dominant pattern of inheritance (impeding its usage for hybrid detection; AFLP, ISSR, RAPD), and (4) the necessity of prior knowledge of the genome of the target species (SSR) (López-Caamal & Tovar-Sánchez 2014). Thus, all of these methods (possibly except for SSR, see Hodel et al. 2016a, 2016b) are becoming obsolete (Edwards et al. 2015, Grover & Sharma 2016) and are being replaced by modern methods utilizing recent discoveries in the field of sequencing technologies (so called "next-generation sequencing" methods, Metzker 2010) that overcome most of the drawbacks associated with the traditional methods.

Genus *Spergularia*

Spergularia (Pers.) J. Presl & C. Presl is a nearly cosmopolitan genus of herbaceous plants from the family Caryophyllaceae (Friedrich 1979, Monnier & Ratter 1993). It is distributed from the temperate to the subtropical zones of the both hemispheres. However, its diversity is concentrated into two hotspots—South America and the Mediterranean region (Rossbach 1940, Meusel & Jäger 1992, Monnier & Ratter 1993).

Although the genus is currently of no economic importance, some representatives are used as model organisms in ecological studies (Telenius 1992, Gutterman 1997, Delesalle & Mazer 2002) and some studies point out possible use of some species as medical plants (Oliveira et al. 2013) or food source (Kubitzki et al. 2013, Wrigley et al. 2015). Despite its potential uses, genus *Spergularia* has been biosystematically very little investigated. This can be illustrated in the fact that the estimates of the total number of species differ 3-fold, ranging from 20 (Friedrich 1979, Dvořák 1990) to 60 (Bittrich 1993, Hartman & Rabeler 2005). The few studies addressing the biosystematics of *Spergularia* (Ratter 1964, 1965a, 1965b, 1969a, 1969b, 1972, 1973a, 1973b, 1976) suggested that hybridization and reticulate evolution have probably contributed significantly to the diversity of this genus. Another process that also seems to have played an important role in the evolution of the genus is polyploidy. This can be assumed from the very fact that genus *Spergularia* is cytotypically quite diverse. There is a series of known ploidy levels ranging from diploid through tetraploid and hexaploid to octoploid (Sanders 1983, Verlaque et al. 1992, Hartman et Rabeler 2005), and multiple ploidy levels can be often found within a single species (Monnier & Ratter 1993).

Genus *Spergularia* is ecologically very diverse. Most of the species are halophytes growing in either coastal or inland salt marshes. Two most notable examples are the species *S. marina* (L.) Besser and *S. media* (L.) C. Presl, which are a characteristic component of coastland vegetation communities thorough nearly the whole Northern hemisphere (Monnier & Ratter 1993, Hartman & Rabeler 2005). Some representatives have adapted to ruderal habitats and become widespread synanthropic species. It is especially the case of *S. rubra* (L.) J. Presl et C. Presl, which is the most widespread representative of the genus that has been introduced to all continents except Antarctica due to human activities (Hultén & Fries 1986, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). Among all of this, the species *Spergularia echinosperma* (Čelak.) A. et Gr., the only European representative growing in exposed margins of freshwater bodies (Monnier & Ratter 1993), represents a sheer rarity.

Spergularia echinosperma

Spergularia echinosperma is a rare species confined to the vegetation of annual wetland herbaceous plants (class Isoëto-Nano-Juncetea) that occurs in the dried out bottoms of freshwater reservoirs that are periodically drained. It has two main centers of distribution—Germany and the Czech Republic (Friedrich 1979, Dvořák 1990). In Central Europe, the species is also marginally distributed in Austria (Fischer et al. 2008) and Slovakia (Dvořák 1979, Goliašová 2012). There are also reports from Poland (Szafer et al. 1967, Krämer & Fartmann 2007), which, however, are probably incorrect (L. Rutkowski, pers. comm.).

Spergularia echinosperma is considered to be a Central European endemic by Central European authors (Friedrich 1979, Dvořák 1990). However, there also exist reports of its

occurrence in other countries out of Central Europe, namely France (Jalas 1988, Monnier & Ratter 1993, Chagneau 2013), Spain (Jalas 1988, Ratter 1990, Monnier & Ratter 1993), Morocco (Monnier 1968), and USA (Hartman & Rabeler 2005). The affiliation of these occurrences to the species *S. echinosperma* is problematic, and they require further investigation.

The current primary habitats of *S. echinosperma* are alluvial pools and river banks. At present, the species grows in this type of habitats in Germany. Here, it is found in periodically exposed substrates in the alluvium of the Elbe River (Friedrich 1979). In the Czech Republic, *S. echinosperma* occurs exclusively in secondary habitats, which is the bottoms of drained fishponds (Dvořák 1979, 1990). The species is endangered in both the primary and secondary habitats. Alluvial pools have been vastly destroyed by the channeling of rivers. In fishponds, the species is threatened by the intensification of fishpond management over the last century. It is especially abandoning the traditional summer drying of fishponds, excessive fish stock, duck farming, manuring, and liming, which destroys the populations of sensitive annual species including *S. echinosperma* (Šumberová et al. 2005, 2006, Šumberová 2011).

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

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

Jage (1974) and Dvořák (1979) reported the occurrence of more distinct morphotypes within *S. echinosperma*. Later, results of a more detailed study were published as a part of the *Spergularia* treatment for the Flora of the Czech Republic (Dvořák 1990). This author revealed the existence of *S. echinosperma* populations with morphological characters typical of *S. rubra* (especially seed color and length of stipules and fruit pedicels), which he ultimately explained by interspecific hybridization. He supposed that hybridization leads to the formation of a primary tetraploid hybrid, which he described as *S. ×kurkae* F. Dvořák (Dvořák 1989), accompanied by further gene introgression from *S. rubra* to *S. echinosperma*. However, the assumed tetraploid state of *S. ×kurkae* was documented only by a single chromosome count (Dvořák 1989), as in the case of *S. echinosperma*. With such limited data, Dvořák (1990) could

not credibly infer the cytotype structure of the populations and morphotypes of the plants he studied.

The situation is further complicated by the fact that there is a conflict in the descriptions of *S. echinosperma* between Czech and German authors. There has been contradictory information as for the actual values of some seed characters. Specifically, it is the difference in the indicated seed color. Czech authors describe *S. echinosperma* as possessing black seeds (Dostál 1989, Dvořák 1990), including the description of this taxon by Čelakovský (1881). German plants, on the other hand, are characterized by (dark) brown seed color (Jage 1974, Friedrich 1979). This discrepancy in the reported seed color between the Czech and German plants could be correlated with the differences in other morphological characters and could be taxonomically important.

Aims of the thesis

The current state of knowledge of the Central-European endemic *Spergularia echinosperma* is fairly fragmentary. The morphological and ploidy differences between *S. echinosperma* and *S. rubra* and their putative hybrid *S. ×kurkae* are only poorly described, and the evidence of the presence of interspecific hybridization is dubious. It is obvious that *S. rubra* and *S. echinosperma* need to be revised based on an extensive screening of their morphological and cytotype variation in all parts of the disjunct distribution range of *S. echinosperma*.

The aim of Paper I was to assess the morphological and ploidy variation of *S. echinosperma*, *S. rubra* and their putative hybrid *S. ×kurkae* in the Czech Republic. The data on the morphology and genome size were also used to elucidate the existence of hybrids between *S. echinosperma* and *S. rubra*.

In Paper II, the hybridization between *S. echinosperma* and *S. rubra* was investigated using molecular tools. Specifically, questions regarding the parentage of *S. ×kurkae* and gene introgression between *S. echinosperma* and *S. rubra* were addressed.

In Paper III, the distributions of the species of genus *Spergularia* in the Czech Republic were mapped. The paper is a part of a large series of articles dealing with the distributions of selected species of vascular plant in the Czech Republic within the framework of the PLADIAS (Plant Diversity Analysis and Synthesis Centre) project.

Paper IV dealt with the taxonomy of the species *S. echinosperma*. Morphological variation of the species in the both parts of its disjunct distribution range was investigated and correlated with ecology and geographical distribution.

In Paper V, 16 polymorphic microsatellite markers were developed for the species *S. echinosperma* and their cross-amplification in *S. rubra* was tested.

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Paper I

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

Kúr P., Štech M., Koutecký P. & Trávníček P. (2012): *Preslia 84: 905–924*.

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

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

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Kúr P., Štech M., Koutecký P. & Trávníček P. (2012): Morphological and cytological variation in *Spergularia echinosperma* and *S. rubra*, and notes on potential hybridization of these two species. – Preslia 84: 905–924.

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

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

Introduction

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

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

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

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

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

Materials and methods

Plants

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

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

Cytological analyses

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

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

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

Morphometry

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

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

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

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

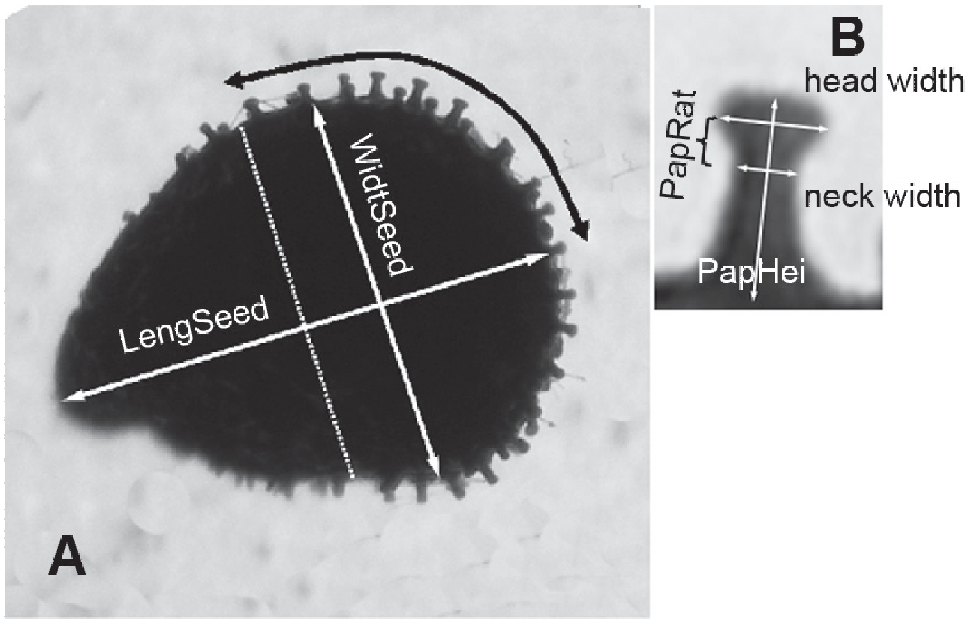

Fig. 1. – Characters measured on the seeds (A) and surface papillae (B). The black curved line specifies the part of the seed circumference where the density of papillae was determined. The longitudinal border of this part is a plane halving the vector of maximal seed length and perpendicular to it (indicated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

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

We also reanalysed the data by classification trees that represent a non-parametric alternative to the classificatory discriminant analysis. The essential difference between these two methods is that classification trees, instead of using all characters together, create

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

Results

Cytological analysis

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

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

Morphometry

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

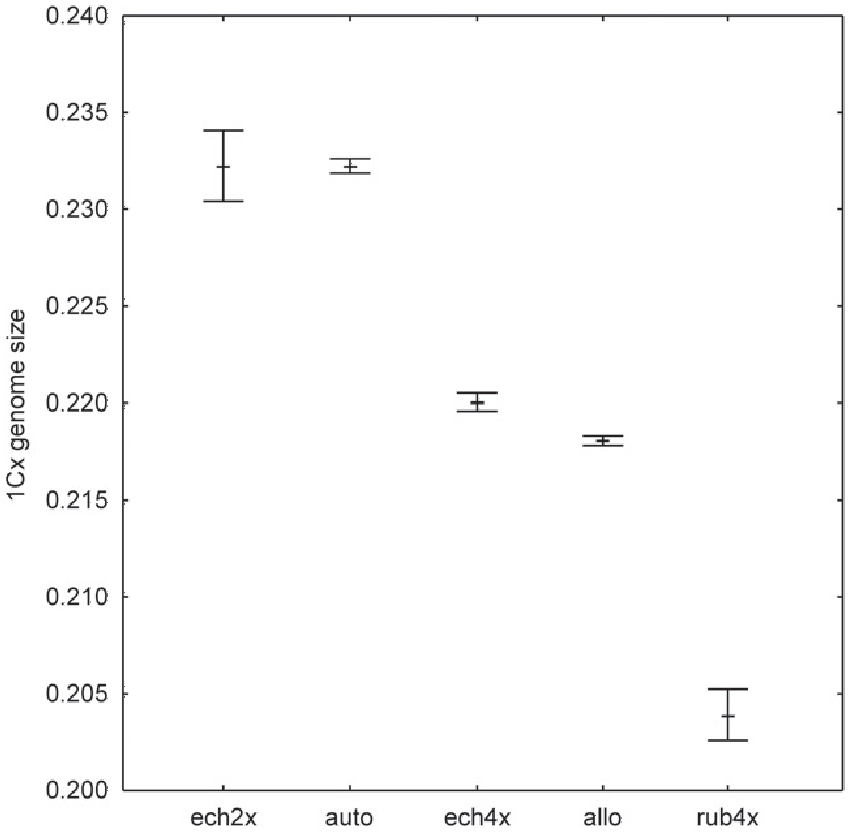

Fig. 2. – Box-and-whisker plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x), *S. rubra* (rub4x), a hypothetical *S. echinosperma*-*S. rubra* allopolyploid (allo) and hypothetical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

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

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

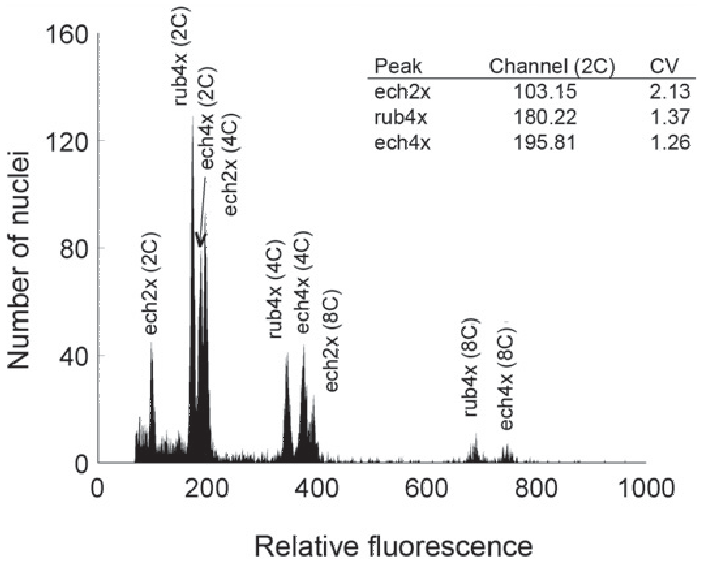

Fig. 3. – Histogram of relative fluorescence of DAPI-stained nuclei of the diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and tetraploid *S. rubra* (rub4x) corroborating the differences in the genome sizes of these three taxa. The genus *Spergularia* displays considerable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

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

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

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

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

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

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

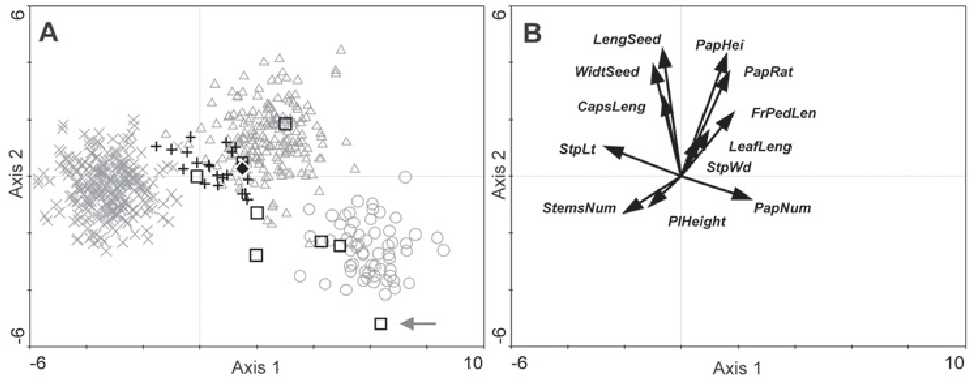

Fig. 4. – Results of CDA of individuals (A) and characters selected by forward selection (B). *Spergularia echinosperma* diploids: grey circles; *S. echinosperma* tetraploids: grey triangles; *S. rubra* tetraploids: grey X-crosses; population Veselsky: black crosses; *S. echinosperma* syntypes: black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

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

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

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

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

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

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

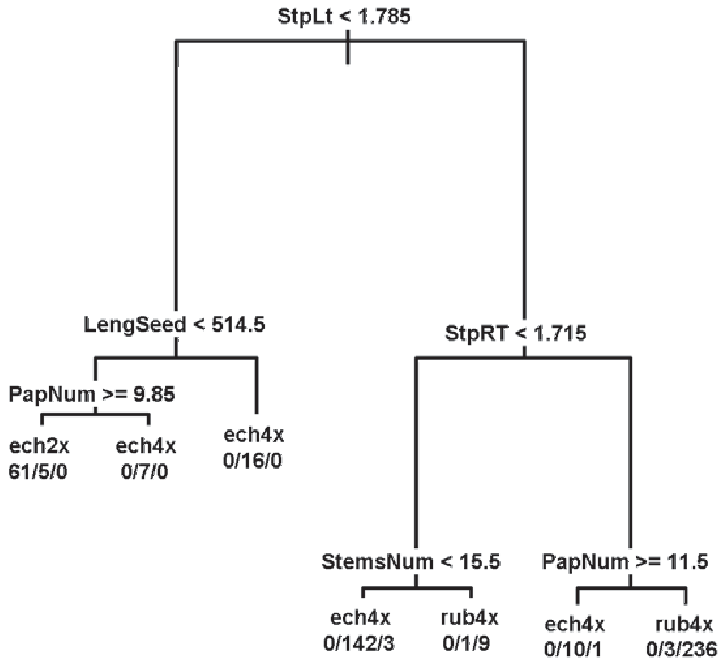

Fig. 5. – Classification tree of individuals of diploid *Spergularia echinosperma* (ech2x), tetraploid *S. echinosperma* (ech4x) and *S. rubra* (rub4x). If a character value matches the classification rule, the determination continues to the left branch, otherwise to the right branch. Lengths of the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

Discussion

Ploidy levels and morphology

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

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

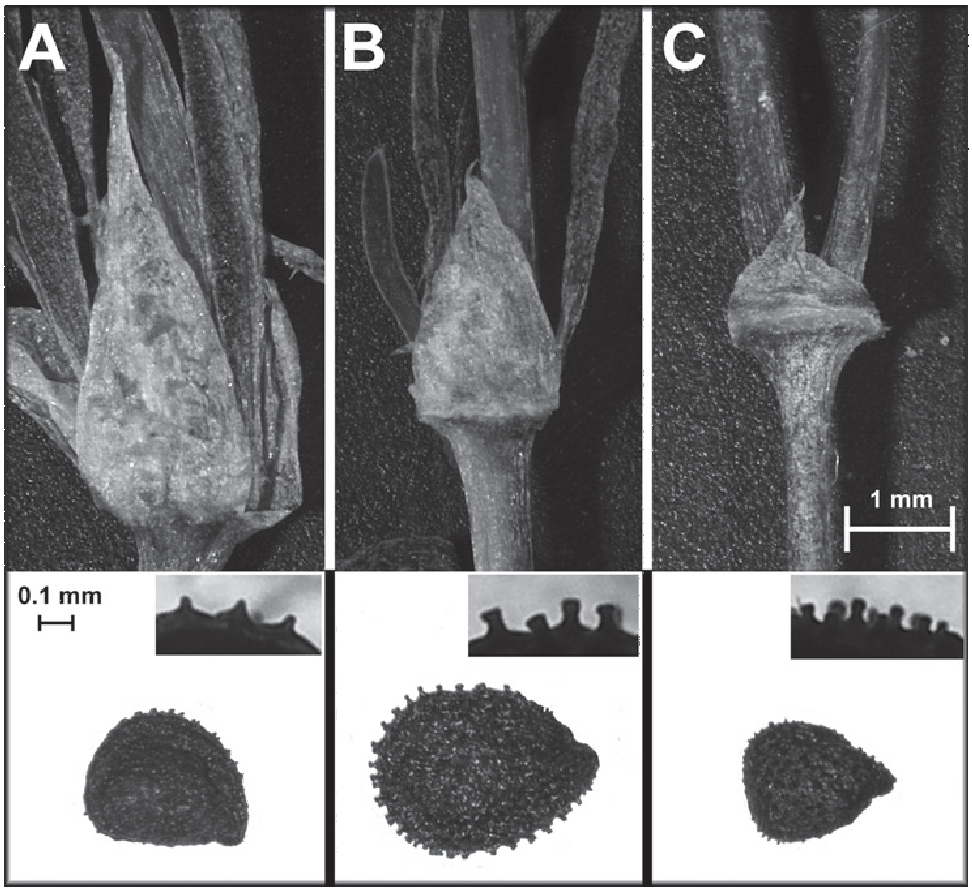

Fig. 6. – Typical stipules and seeds of *Spergularia rubra* (A), tetraploid *S. echinosperma* (B) and diploid *S. echinosperma* (C).

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

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

Other relatively reliable characters, which were less useful in the field, were seed size and testa structure. In accordance with the original description (Čelakovský 1881) and other authors (Friedrich 1979, Dvořák 1990) the seeds of *S. rubra* differ from those of *S. echinosperma* in having a low density of surface papillae, which are also considerably smaller. In addition, the *S. echinosperma* cytotypes strongly differed from each other in seed morphology. The diploids displayed significantly smaller and more densely verrucose

seeds with a lower density of papillae and less pronounced papilla heads than the tetraploids (Fig. 6). Based on the results of the morphometric analyses, we compiled the following determination key for the taxa/cytotypes:

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

Genome size

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

Origin of the tetraploid cytotype of Spergularia echinosperma

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

We are aware that one-step hybridization through unreduced gametes of the diploid is not the only possibility. However, we think it is the most likely scenario. Angiosperms commonly produce unreduced gametes and this is viewed as the primary source of neopolyploid formation, especially in diploid-tetraploid crosses (Ramsey & Schemske 1998). For *Spergularia* it is reported that a few tetraploid seeds were produced by a cross between *S. maritima* (All.) Chiov. (♀, diploid) and *S. rupicola* Lebel ex Le Jolis (♂, tetraploid) (Ratter, 1976). The alternative pathway of allotetraploid formation involves an intermediate stage of (at least partly) fertile triploid progeny formed by fusion of normally developed gametes of the parental species ("triploid bridge"). These triploids can produce tetraploid offspring by selfing or backcrossing to one of the parental taxa (Bretagnolle & Thompson 1995). Though rare, this pathway of polyploid formation can be significant in

diploid-tetraploid hybridization (e.g. Vardi & Zohary 1967, Anamthawat-Jónsson & Thorsson 2003, Aagaard et al. 2005, Lo et al. 2010). In *Spergularia*, nearly all triploid offspring of various diploid-tetraploid crosses are sterile and the fertility of seeds from triploid plants is very low (0.1–0.2%) (Ratter 1976). This together with the absence of triploids in wild populations (both our data and in the literature) makes the triploid bridge pathway highly improbable.

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

Taxonomy and nomenclature

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

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

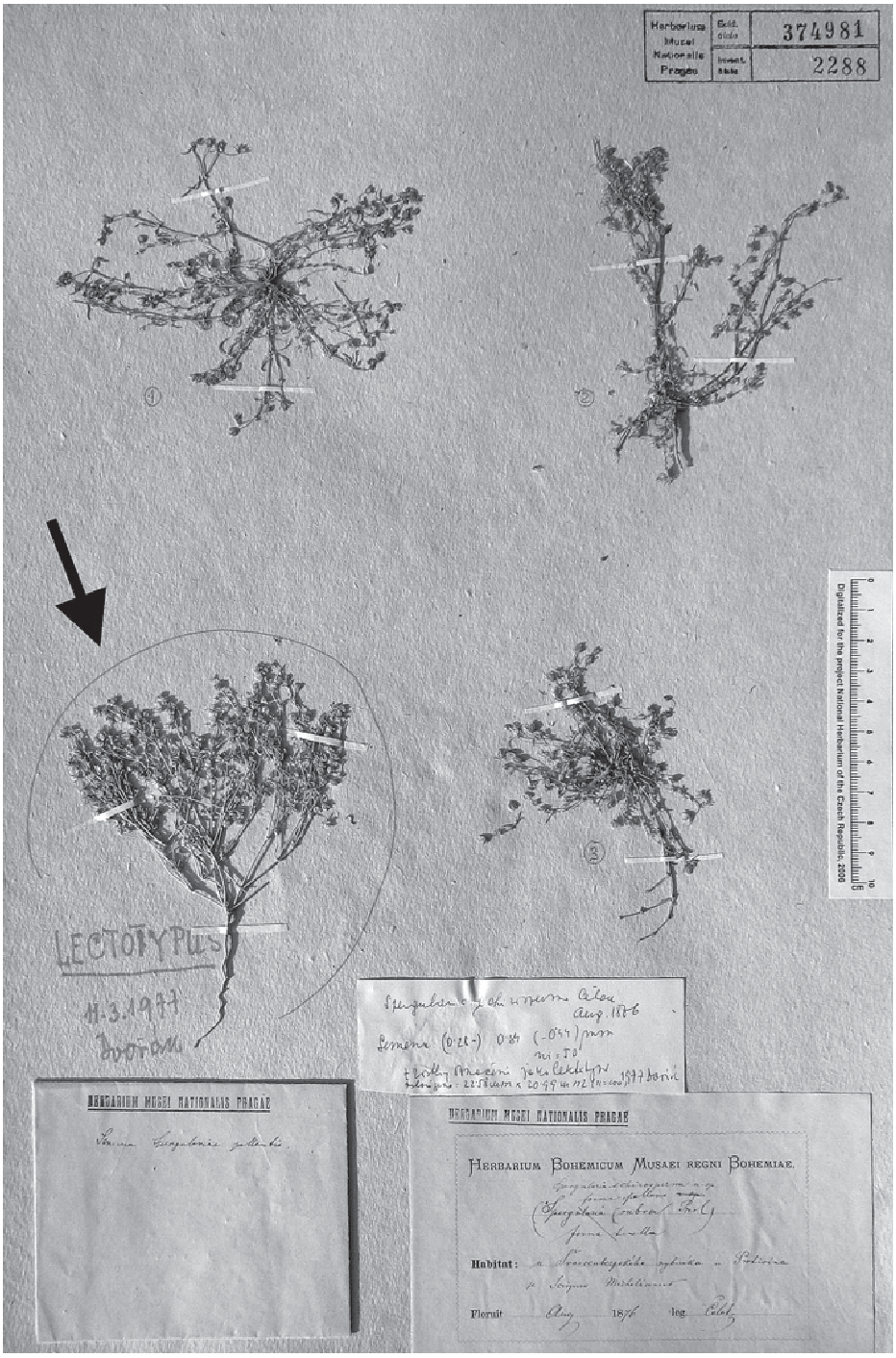

Fig. 7. – The proposed lectotype for the name *Spergularia echinosperma* (Čelak.) Asch. et Graebn., PR 374981, marked by the arrow. The text on the label reads: "*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.".

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

Nomenclature of *S. echinosperma*:

Spergularia echinosperma (Čelak.) Asch. et Graebn. in Ber. Deutsch. Bot. Ges. 11: 516, 1893.
≡ *Spergularia rubra* [subsp.] b. *echinosperma* Čelak. in Prodr. Fl. Böhmen 4: 867, 1881.
Lectotype (**designated here**): "*Spergularia echinosperma* n. sp. forma *pallens*, u Švarcenberského rybníka u Protivína se *Scirpus Michelianus*, Aug 1876 leg. Čelak.", PR 374981, left bottom individual (marked by the arrow in Fig. 7); the lectotype belongs to the diploid cytotype.

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Souhrn

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

1a Semena hnědá, řídce bradavčitá (hustota 5–9 papil na 1/4 obvodu semene); palisty alespoň 1,7× delší než široké, alespoň 2,9 mm dlouhé; rostliny obvykle s více než 5 lodyhami .....***S. rubra***
1b Semena černá, hustěji bradavčitá (hustota 8–17 papil na 1/4 obvodu semene); palisty méně než 1,7× delší než široké, kratší než 2,8 mm; rostliny obvykle s méně než 9 lodyhami 2
2a Palisty kratší než široké, kratší než 1,6 mm; semena kratší než 0,48 mm, hustota povrchových papil 12–17 na 1/4 obvodu semene .....***S. echinosperma*, diploidní cytotyp**
2b Palisty delší než široké, delší než 1,7 mm; semena delší než 0,48 mm, hustota povrchových papil 8–14 na 1/4 obvodu semene .....***S. echinosperma*, tetraploidní cytotyp**

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

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

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

Paper II

Characterization and cross-species amplification of 16 microsatellite loci in *Spergularia echinosperma* (Caryophyllales: Caryophyllaceae).

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MICROSATELLITE LETTERS

Characterization and cross-species amplification of 16 microsatellite loci in *Spergularia echinosperma* (Caryophyllales: Caryophyllaceae)

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Abstract Sixteen polymorphic microsatellite loci are described for *Spergularia echinosperma*, an endangered plant endemic to Central Europe. Based on 51 individuals, the number of alleles per locus ranged from 2 to 5, and the observed and expected heterozygosities were both 0.00–0.17. The markers can be valuable tool to conservation genetics of this species across its distributional range.

Keywords Conservation genetics · Endemic plant · 454-Pyrosequencing · SSR markers

Spergularia echinosperma (Čelak.) Asch. et Graebn. is a representative of the Europe-wide threatened flora of annual herbs of ephemeral wetlands. It is also one of the few Central-European plant endemics (Kur et al. 2012). Despite being rare and red-listed in all the Central-European countries, it has not been given much attention by conservation scientists. This study reports 16 microsatellite loci in this diploid species and their cross-amplification in related tetraploid *S. rubra* (L.) J. et C. Presl.

The microsatellites were developed using SSR-enrichment and 454-pyrosequencing as described in Drag et al. (2013). The final dataset comprised 11,349 reads, and yielded 42 putative SSR loci, for 19 of which PCR primers were designed. The markers were tested using M13-tailed assay (Schuelke 2000) on 51 individuals of *S. echinosperma* from six Czech and German populations. PCRs contained 0.5 µl of DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.08 µM of M13-tailed forward primer, 0.33 µM of fluorescently labeled M13 primer and reverse primer, 0.25 U of Taq polymerase (Top-Bio), and water to final volume of 5 µl. Cycling parameters were: 30 cycles of 94 °C (30 s), locus-specific annealing temperature (30 s), 72 °C (30 s); 8 cycles of 94 °C (30 s), 46 °C (30 s), 72 °C (30 s); and final elongation of 72 °C (10 min). The PCR products were analyzed on ABI 3730xl DNA Analyser (ABI). Finally, 16 loci were selected that had consistent amplification and sufficient polymorphism (Table 1); the sequences are deposited in GenBank (see Supplementary materials). Based on the results from GenAlEx 6.5 (Peakall and Smouse 2012), the number of alleles per locus ranged from 2 to 5, and the average observed (H_O) and expected (H_E) heterozygosities over all loci were extremely small (0.043 and 0.063, respectively). Genepop 4.2.1 (Rousset 2008) detected 7 of the loci to significantly differ from the Hardy–Weinberg equilibrium, but no evidence of linkage disequilibrium between all the loci.

The amplification in *S. rubra* (16 individuals from 4 populations) yielded products only for 9 of the loci, of which 3 demonstrated some level of polymorphism (Table 1). In conclusion, all the 16 markers proved to be a suitable tool for population studies in *S. echinosperma*, and some of them might also be helpful in genetic studies of the related species *S. rubra*.

Electronic supplementary material The online version of this article (doi:10.1007/s12686-014-0141-8) contains supplementary material, which is available to authorized users.

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Table 1 Characterization of the 16 microsatellite loci designed for *Spergularia echinosperma*

Locus	Primer sequences (5′ → 3′)	Motif	Ta	*S. echinosperma*					*S. rubra*	
				Size range (bp)	N_A	H_O	H_E	GenBank accession no.	Size range (bp)	N_A
SE_1*	F: AATGTGGTGGTTTTATCTGTG R: AGCATAGAATGGTCTTGTTGGA	$(GT)_{11}$	57	115–121	3	0.167	0.167	KF953667	107–115	2
SE_4*	F: CTGCCCTTGAATCTTTGACC R: TCTGCACTAACACGCATA	$(TTG)_{13}$	57	422–446	5	0.000	0.158	KF953668	×	
SE_8*	F: CGTCGTCGGAAACTGAAC R: CATCTGCCTGTAAGAAGAAACAAA	$(CT)_{14}$	57	248–254	4	0.000	0.110	KF953669	242	1
SE_37	F: CCCCTTATTCTTCACTGCTAT R: GAATGTTGAATGCGTGATGT	$(AC)_{11}$	57	346–348	2	0.167	0.083	KF953670	346	1
SE_69	F: CAAAGAGTGGCTTAGTGA R: TTATGGAAACCTGGGAGT	$(CA)_{11}$	53	308–310	2	0.000	0.000	KF953671	×	
SE_82	F: TGATTAGTAGGAATGCTTGTTC R: TTGTACCAAGTTCTCTTTTCT	$(CA)_{13}$	55	298–300	2	0.000	0.000	KF953672	288–290	2
SE_91*	F: CGGGAAAATGGACAAACC R: CTTGGCTACACGCACAATCA	$(CAA)_{20}$	57	260–269	4	0.000	0.083	KF953673	223	1
SE_108	F: TCTAATATCGCTTGAACTGCT R: GCTTCAGACCCATTCATC	$(TTG)_{20}$	53	278–290	3	0.017	0.046	KF953674	×	
SE_151	F: TGATTTGGTTTATGGTATTTGGA R: CCTATTGTTCGTCATCTTCATCT	$(CA)_{15}$	57	405–407	2	0.000	0.030	KF953675	387	1
SE_255	F: TGATTTGATTATTGAGTATTATTTGAC R: GGCAAGAGCGTGTAAGTG	$(TTG)_{11}$	53	370–376	3	0.000	0.030	KF953676	×	
SE_261	F: TGTATTGGACCCGCTTT R: TATRTTTGGTTAGTTATTTTTCTGC	$(CAA)_{20}$	53	270–276	2	0.000	0.000	KF953677	×	
SE_451*	F: CAGGTGAGGAAACAGCAGAA R: CGAATGTGGTGGAGACGA	$(AC)_{11}$	57	249–253	2	0.167	0.083	KF953678	249–253	2
SE_495*	F: AAACTGAAATTAACTCCTTCATTCAAC R: ATGGGGAACCTACCTTTTGG	$(TTG)_{24}$	53	209–212	2	0.000	0.053	KF953679	×	
SE_548	F: CTCTGCGTTCTGCTGGTTT R: CTGATTATGAGGTCGTGAAAGA	$(GTT)_{18}$	55	171–180	2	0.000	0.000	KF953680	136	1
SE_677*	F: TTCAAGGCTCAACAACGACA R: CACAAGTCCATAGTCAACGAAA	$(TGT)_{14}$	57	515–527	4	0.000	0.088	KF953681	488	1
SE_906	F: CTAATAAATAGCCCAATGTCCTT R: GCATCAGACCCCTTCTAACA	$(CAA)_{11}$	53	333–345	2	0.167	0.083	KF953682	×	

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, *Ta* annealing temperature [°C], * Significant deviation from Hardy–Weinberg equilibrium, × failed amplification

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Paper III

Distributions of vascular plants in the Czech Republic. Part 2.

Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtek J., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): *Preslia 88: 229–322*.

Considering the extreme length of this article, only sections contributed by Pavel Kúr are included in the thesis. Full text of the article is available at http://www.preslia.cz.

Distributions of vascular plants in the Czech Republic. Part 2

Rozšíření cévnatých rostlin v České republice. Část 2

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Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtek J. Jr., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – Preslia 88: 229–322.

The second part of the publication series on the distributions of vascular plants in the Czech Republic includes grid maps of 87 taxa of the genera *Antennaria*, *Aposeris*, *Astragalus*, *Avenula*, *Bidens*, *Carex*, *Cenchrus*, *Centunculus*, *Convallaria*, *Crocus*, *Cryptogramma*, *Cyperus*, *Dryopteris*, *Gladiolus*, *Gratiola*, *Helictochloa*, *Hierochloë*, *Lindernia*, *Maianthemum*, *Myriophyllum*, *Nothoalaena*, *Nymphoides*, *Radiola*, *Schoenoplectus*, *Sisyrinchium*, *Spergularia*, *Tillaea*, *Veratrum* and *Veronica*. The maps were produced by taxonomic experts based on all available herbarium, literature and field records. The plants studied include 56 taxa registered in the Red List of vascular plants of the Czech Republic, some of which showed remarkable declines. *Astragalus arenarius*, *Hierochloë odorata* and *H. repens*, as representatives of vegetation of inland sand dunes, are critically threatened due to conversion of their habitats to arable land, local sand mining, afforestation, changes in landscape management and eutrophication followed by succession. Each of them survives at a few localities and their populations are poor. Competitively weak wetland annuals, confined to open habitats such as exposed fishpond littorals and river beds, abandoned sand-pits and wet arable fields, have considerably declined and disappeared from large areas as a result of agriculture and fish-farming intensification, in particular fertilization and restriction of summer drainage of fishponds, and other changes in land-use. These include *Centunculus minimus*, *Cyperus flavescens*, *C. michelianus*, *Lindernia procumbens*, *Radiola linoides* and *Tillaea aquatica*. Observed recently at a few sites only, they are all classified as critically threatened. A map is for the first time provided also for *Spergularia kurkae*, a newly recognized species and a central-European endemic. *Astragalus asper*, *Schoenoplectus supinus* and *Veronica pumila* are now extirpated from the country's flora. In contrast, *Spergularia marina*, until recently confined to natural saline habitats and very rare, has been spreading along roads that are treated by de-icing salts. Examination of an old herbarium voucher showed that the only record of *Astragalus alopecuroides* in the Czech flora actually refers to the species whose correct name is *A. alopecurus*.

Further introduced casuals mapped in this paper include *Bidens pilosus*, *Cenchrus echinatus*, *Gratiola neglecta* and *Lindernia dubia*, each introduced to only a few sites. *Bidens connatus* was recorded at two dozen sites and appears to have spread as a consequence of the great floods in 2002. Typical examples of naturalized neophytes are *Veronica filiformis* and *V. peregrina*, both currently known from many parts of the country. Invasive aliens are represented by *Bidens frondosus*, which began to spread in the 1930s and now is frequent throughout the country. Spatial and temporal dynamics of individual species are shown in maps and documented by records included in the Pladias database and available in Electronic appendices. The maps are accompanied by comments, which include additional information on distribution, habitats, taxonomy and biology of the species.

K e y w o r d s: alien species, central Europe, chorology, Czech Republic, distribution atlas, distribution patterns, endangered species, endemic, flora, grid maps, herbaria, phytogeography, plant record, vascular plants

Introduction

A recent review on the Czech flora (Kaplan 2012) emphasized that no comprehensive piece of work with distribution maps in this country is available in spite of a long history of botanical research. The project of mapping plant distributions in the Czech Republic was launched two years ago with the aim to establish a modern plant record database and to prepare the first sets of distribution maps as a basis for a future complete atlas of the distribution of vascular plants in the Czech Republic. The first results of our effort were published a half year ago (Kaplan et al. 2015) within the PLADIAS project (www.pladias.org). The paper contained 75 grid distribution maps produced by taxonomic experts and based on critically evaluated and sorted records.

From September 2015 to February 2016 the plant record database has increased by ca 154,000 new records. Of these about 45,000 records resulted from critical examination of herbarium specimens by taxonomic experts. Maps of further 87 taxa, both native and alien, were finished at the beginning of February 2016 and these are published in this paper.

Current revisions of national plant diversity have brought several species new to the flora of the Czech Republic, which include both newly recognized native endemics (Kolář et al. 2015, Lepší et al. 2015) as well as recently introduced alien species (Kocián 2014, Hadinec & Lustyk 2015). Two changes in identification and nomenclature involve genera dealt with in this paper, which require an update of the checklist of vascular plants of the Czech Republic (Danihelka et al. 2012). Examination of an old herbarium voucher and the nomenclatural history of the respective plant group showed that the only record on the casual occurrence of *Astragalus alopecuroides* in the Czech flora actually refers to the species whose correct name is *A. alopecurus*. The name *Crocus albiflorus* has to be replaced by *C. vernus*, which was shown to be the correct name for the species largely known as *C. albiflorus* (Peruzzi et al. 2013).

Materials and methods

Taxonomic scope

The following groups of vascular plants are mapped: native taxa, naturalized aliens and most casuals, and selected hybrids. Distribution maps are produced for species and sub-

species, in exceptional cases also for varieties or infrageneric taxa (e.g. sections). Plants of species groups that are difficult to assign to species may be mapped as species aggregates. Field crops and plants deliberately cultivated in gardens and parks are not included in the mapping project. Nomenclature, taxonomic concepts and delimitation of species aggregates mostly follow Danihelka et al. (2012), with differences indicated where necessary. For taxa not included in that checklist, a taxonomic reference is given. Publication of maps does not follow any alphabetical or systematic order but those maps that have resulted from recent revisions are preferably printed.

Data sources

All relevant floristic data sources are used. Major national herbaria and some local and foreign collections were consulted, incl. BRA, BRNL, BRNM, BRNU, CB, CBFS, CESK, CHOM, FMM, GM, HOMP, HR, KHMS, LIM, LIT, MJ, MMI, MP, MZ, NJM, OH, OL, OLM, OMJ, OP, OSM, OSTR, OVMB, PL, PR, PRA, PRC, ROZ, SAV, SLO, SOB, SOKO, SUM, VM, VYM, W, WU and ZMT (acronyms follow Thiers 2016), as the main source of taxonomically revised records. Most records for maps of common and easy-to-identify taxa come from the recently developed Pladias database (hosted at the Institute of Botany, Průhonice; previously tentatively named CzechDistrib database), which has integrated all available records on the distribution of vascular plants in the Czech Republic. Among the most important incorporated databases are the Database of the Distribution of Vascular Plants in the Czech Republic (FLDOK), the Czech National Phytosociological Database (CNPD), plant records from the Floristic Summer Schools and other activities of the Czech Botanical Society, the Species Occurrence Database of the Nature Conservation Agency of the Czech Republic (NDOP) and the Database of Forest Typology of the Forest Management Institute of the Czech Republic (DLT). Unpublished field records previously entered into the Pladias database by the authors of maps or regional contributors were also considered.

Procedure of mapping

All records used for mapping are entered into the Pladias database and geographically sorted according to the traditionally used CEBA (Central European Basic Area) grid template (Niklfeld 1999) divided into quadrants of 5 × 3 arc minutes (corresponding to approximately 5.5 × 5.9 km). The territory of the Czech Republic is covered by 2551 quadrants, of which 2181 are completely within the border of the country. Individual records as well as the whole distribution pattern of each taxon are checked and evaluated by the author of a particular map in a web-based mapping interface of the Pladias database. Because maps of taxonomically critical groups are often highly inaccurate in distribution atlases (Gregor 2009), maps of such taxa are based solely or mainly on herbarium records revised by taxonomic experts; these cases are indicated in the text accompanying the particular map. Maps of all other taxa are based on records from databases, literature and herbaria, which were scrutinized by the authors of the respective maps. Records used for producing maps are listed in Electronic Appendices 1–87. In selected maps, native versus introduced occurrences are distinguished and corresponding records in the database classified accordingly. Draft distribution maps and the background records are released in a web-based review process for scrutiny to field botanists, regional collabora-

tors and members of the Czech Botanical Society. Their comments and additional records are collected in the database and returned to the responsible specialists for consideration before producing final distribution maps.

Final maps and comments

The treatment of each taxon consists of a grid distribution map and of an accompanying text; authors of maps are indicated in the figure captions, and they also took major part in preparing the first drafts of the respective texts. Maps are displayed using spherical Mercator projection (EPSG:3857) where meridians and parallels are shown perpendicular, and the mapping CEBA grids are thus nicely displayed. The background relief was derived from the SRTM data (http://www2.jpl.nasa.gov/srtm/, the version provided by http://srtm.csi.cgiar.org), and the river network was adapted from data provided by CENIA (www.cenia.cz). When appropriate, different symbols are used in the maps in order to distinguish one of the following attributes of the plant distribution records: (1) recent versus old records, (2) native occurrences versus introductions, or (3) records based on revised herbarium specimens versus all other records. These classifications of records are used only for those taxa where such distinction provides important information and, in addition, the amount and quality of records are sufficient. The mapping symbols used to indicate the different attributes of the records in the particular grid cell are shown in Table 1. Symbols specific to individual maps are explained in their captions. In the caption to each map, counts of occupied quadrants are indicated according to the symbols used in the map; uncertain occurrences are not included in the counts. The accompanying text includes the accepted scientific name, a brief outline of the total distribution, information on habitats occupied by the species and a description of its distribution in the Czech Republic. Where appropriate, comments on the taxonomy, biology and details of the spatial and temporal dynamics of the distribution are given.

Table 1. – The mapping symbols used in the distribution maps to indicate the different attributes of the occurrence in a particular grid cell.

Attribute distinguished	Symbol	Attribute state
None	●	all records
Time	●	recent occurrence (at least one record since 2000)
	◍	old occurrence (all records before 2000, or demonstrably being extirpated from all localities after 2000, or all records undated)
Origin	●	native (at least one record)
	×	alien
Source data	●	a revised herbarium specimen (at least one record)
	▲	all other
All	?	only record(s) uncertain regarding identification and/or locality

Distribution maps and comments

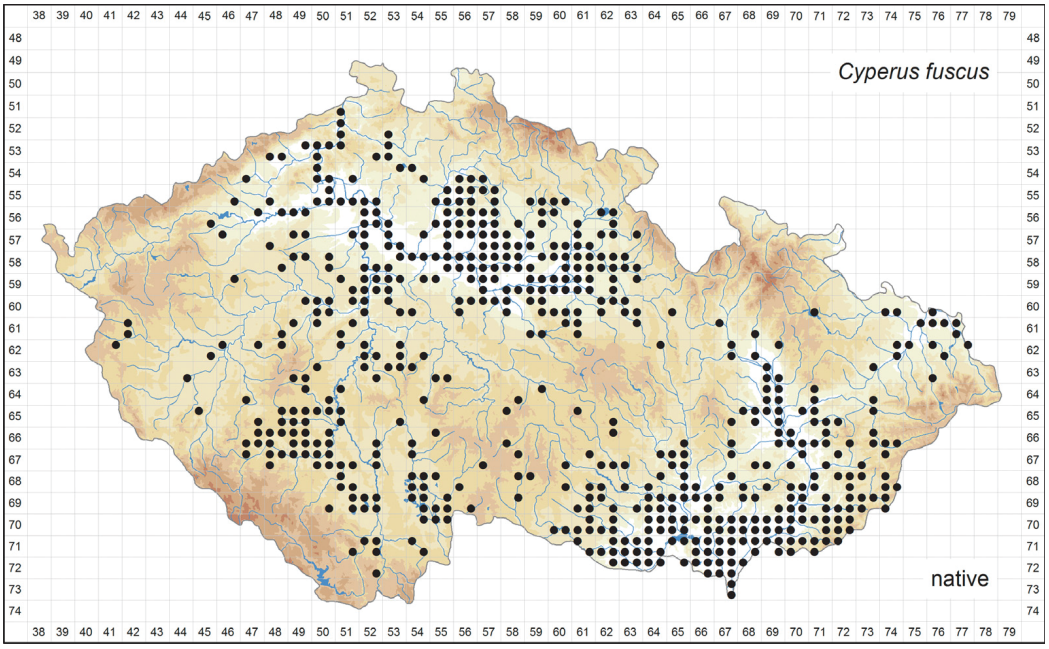

Fig. 38. – Distribution of *Cyperus fuscus* in the Czech Republic (576 occupied quadrants). Prepared by Kateřina Šumberová, Pavel Dřevojan, Zdenka Hroudová & Pavel Kúr.

Cyperus fuscus (Fig. 38)

Cyperus fuscus is a Eurasian wetland annual plant distributed mainly in warm temperate and Mediterranean parts of Europe, northern Africa and western Asia, with its northern distribution limit in southern Scandinavia. Eastwards it is scattered throughout the temperate zone of continental Asia (at high altitudes reaching the subtropical zone), being more frequent in floodplains of large rivers and around lakes. It is also known from North America where it is considered as introduced (Lampe 1996). *Cyperus fuscus* prefers mineral-rich calcareous soils, especially in northern parts of its distribution range (Hejný 1960). In the Czech Republic it is mainly confined to the Bohemian Cretaceous Basin, southern Moravia and some parts of the Carpathians (e.g. Bílé Karpaty Mts). *Cyperus fuscus* is a typical component of the vegetation of temporarily exposed bottoms of various water bodies, growing over a broad range of habitats, including fishponds, fish storage ponds, oxbows, river beds, sand pits, wet depressions in arable fields and disturbed places in wet meadows. In areas formed by acidic, mineral-poor bedrock, such as fishpond landscapes of southern Bohemia, the species was reported as rare until the 1950s, occurring mainly in eutrophic water bodies in settlements (Hejný 1960, Šumberová 2013a). As a consequence of overall eutrophication and soil chemistry changes associated with fish farming intensification, in particular combined fish and duck farming and intensive liming of some ponds, the number of records has considerably increased since then (Šumberová 2003, 2013a). Despite the loss of some populations due to habitat destruction in river alluvia, the species has recently had many hundreds of localities and is classified only as vulnerable (Grulich 2012).

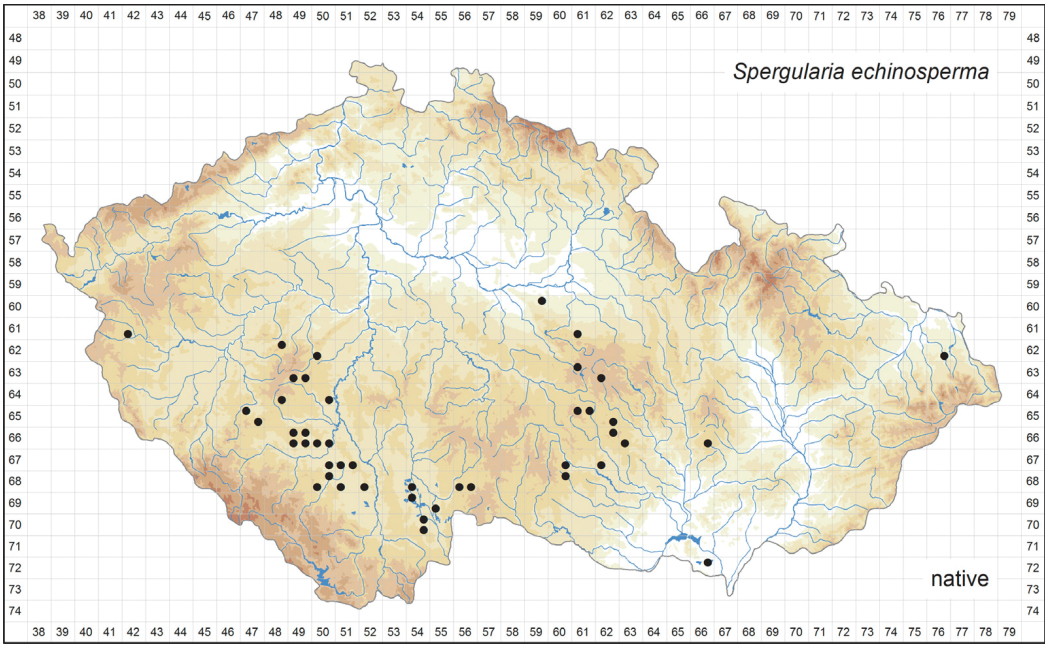

Fig. 71. – Distribution of *Spergularia echinosperma* in the Czech Republic (44 occupied quadrants). Prepared by Pavel Kúr.

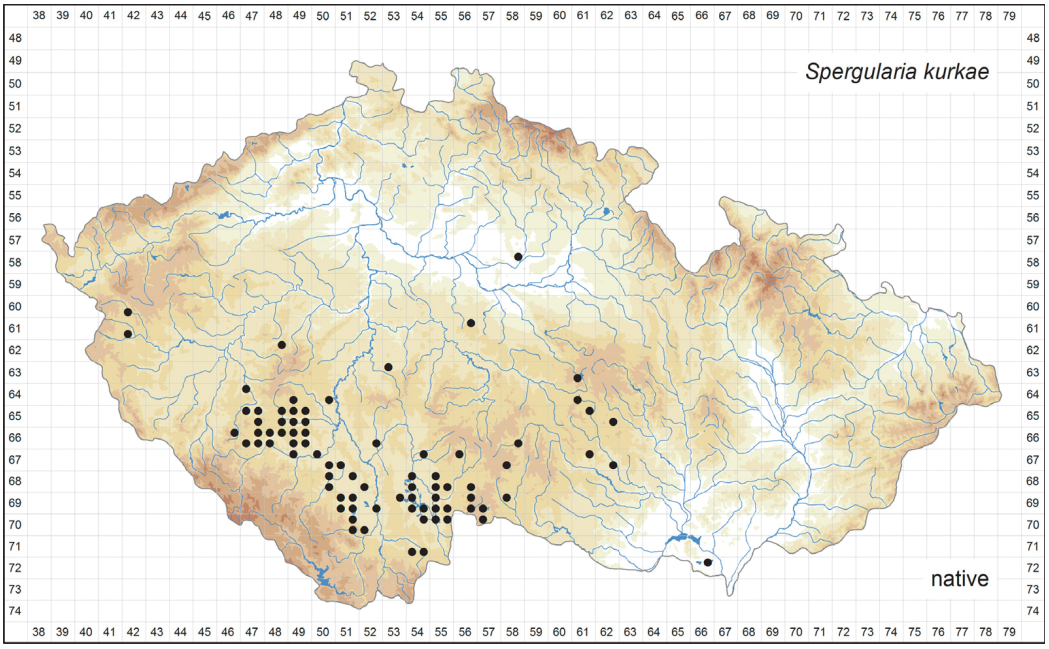

Fig. 72. – Distribution of *Spergularia kurkae* in the Czech Republic (80 occupied quadrants). Prepared by Pavel Kúr.

Spergularia echinosperma (Fig. 71)

Spergularia echinosperma is a central-European endemic (Friedrich 1979, Dvořák 1990). A recent critical revision of herbarium collections (Kúr et al., in prep.) has confirmed its presence in the Czech Republic, Germany, Austria and Slovakia only. It is confined to vegetation of annual wetland herbs on periodically exposed bottoms of freshwater reservoirs. The primary habitat of *S. echinosperma* includes alluvial pools and sandy banks of rivers; the species, however, most frequently occurs in secondary habitats, mainly exposed bottoms of fishponds (Friedrich 1979, Dvořák 1990). In the Czech Republic *S. echinosperma* is most frequent in areas with many fishponds, i.e. southern, south-western and eastern Bohemia. The species prefers pond bottoms with lower trophic levels and a sandy substrate, which may be covered with a thin layer of mineral mud (Kúr et al., in prep.). It is currently threatened by the intensification of fishpond management and is classified as endangered (Grulich 2012). *Spergularia echinosperma* has unresolved taxonomy, and it probably comprises two intraspecific taxa. A taxonomic study of this species, employing molecular markers (Kúr et al. 2014), is currently in progress (Kúr et al., in prep.). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia kurkae (Fig. 72)

Spergularia kurkae is a newly recognized species, which was described by Dvořák (1989) as a hybrid between *S. echinosperma* and *S. rubra* but has not been listed in any flora or checklist except for the Flora of the Czech Republic (Dvořák 1990) since then. Recent studies have proved that *S. kurkae*, although truly being of hybrid origin, is a stabilized, morphologically and ecologically well-separated species (Kúr et al. 2012, Kúr et

al., in prep.). The species occurs mainly in central Europe (Czech Republic, Germany and Austria), although outposts in Switzerland and France possibly exist (the taxonomic identity of these plants needs to be further investigated; Kúr et al., in prep.). It is confined to vegetation of annual wetland herbs on periodically exposed bottoms of freshwater reservoirs. The typical habitats of the species are alluvial pools, river banks, and, above all, fishponds and fish storage ponds. In the Czech Republic *S. kurkae* is most frequent in areas with many fishponds, i.e. southern, south-western and eastern Bohemia. The species has a wider ecological niche than *S. echinosperma* and can very rarely and for a short time survive outside pond bottoms (e.g. in pond sediment deposits). Its current threat level is unknown; herbarium records show that it is approximately twice as common as *S. echinosperma*. Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia marina (Fig. 73)

Spergularia marina is a nearly cosmopolitan halophilous species occurring in coastal and inland salt marshes of Europe, Asia, northern and southern Africa, North and South America, Australia and New Zealand (Hultén & Fries 1986, Meusel & Jäger 1992, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). It is not clear in which parts of its distribution range the species is indigenous and where it has been introduced. In the Czech Republic *S. marina* used to grow relatively frequently in natural saline habitats in north-western Bohemia and southern Moravia. An isolated occurrence was around mineral springs in the Soos National Nature Reserve in western Bohemia. Since World War II the species has declined considerably as a result of habitat destruction and changes in landscape management. Today it survives at a few localities only (two sites in north-western Bohemia and about ten sites in southern Moravia). However, the species has been recently found to be rapidly spreading along roads that are treated by de-icing salts during the winter. In Austria and Germany the spread of *S. marina* on road verges has been known since the 1970s (Friedrich 1979, Hohla & Melzer 2003, Hetzel 2006). In the Czech Republic the species occurs most frequently along motorways, especially in colder areas where the application of de-icing salts is more intense, and is rare in warm and dry areas. There is also a noticeable decreasing gradient in the species' abundance from the west of the country to the east. The indigenous populations are currently classified as critically threatened (Grulich 2012). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia media (Fig. 74)

Spergularia media is an obligate halophyte native to coastal and inland salt marshes of Eurasia and North Africa. It has been introduced to North and South America, Australia, New Zealand and southern Africa (Hultén & Fries 1986, Meusel & Jäger 1992, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). In the Czech Republic *S. media* used to grow naturally in saline habitats in north-western Bohemia (three localities only) and southern Moravia (a few dozens of localities). It was also introduced to the ore yard of the ironworks in Polanka nad Odrou, north-eastern Moravia, in the 1960s (Kilián & Krkavec 1962; misidentified as *S. salina*). Since World War II the species has declined dramatically as a result of habitat destruction and changes in landscape management.

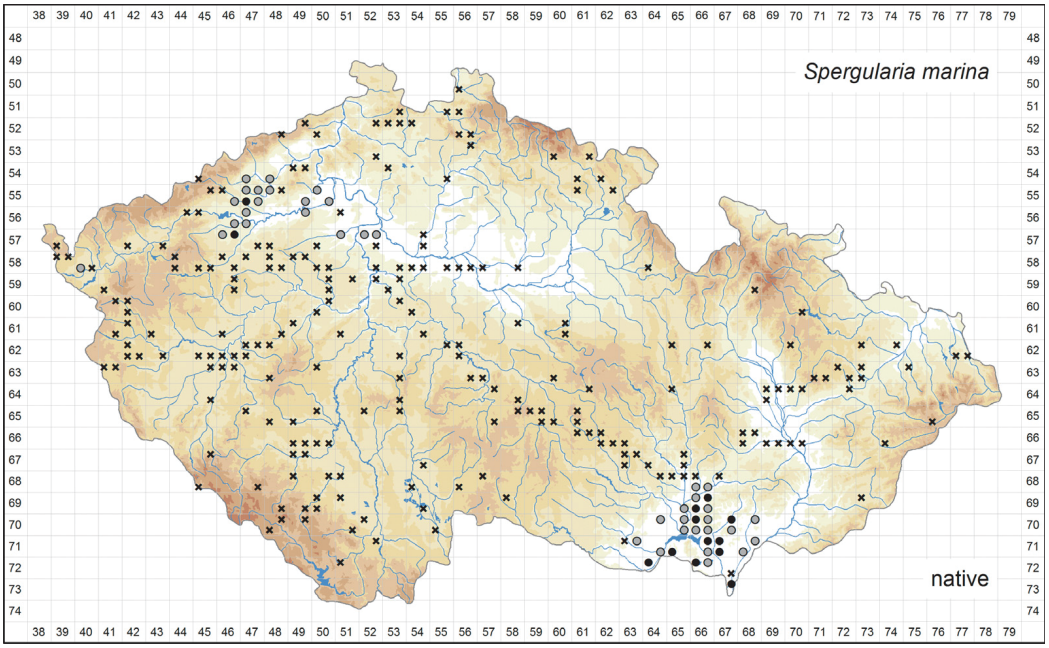

Fig. 73. – Distribution of *Spergularia marina* in the Czech Republic: ● native, at least one record in 2000–2016 (13 quadrants), ● native, pre 2000 records only (38 quadrants), × alien (224 quadrants). Prepared by Michal Ducháček & Pavel Kúr.

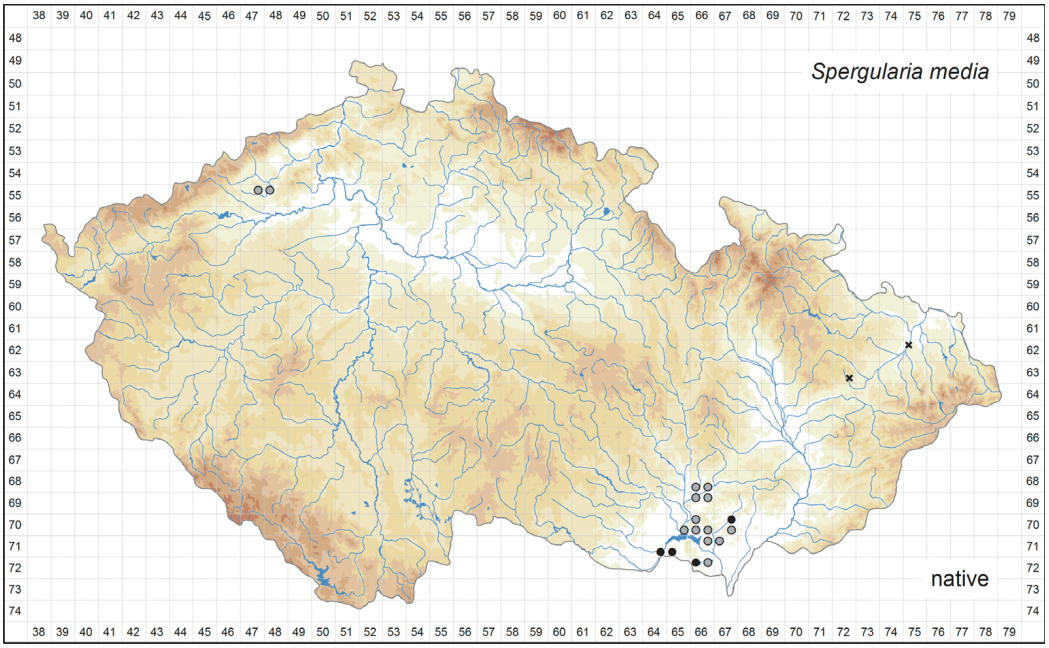

Fig. 74. – Distribution of *Spergularia media* in the Czech Republic: ● native, at least one record in 2000–2016 (4 quadrants), ● native, pre 2000 records only (14 quadrants), × alien (2 quadrants). Prepared by Michal Ducháček & Pavel Kúr.

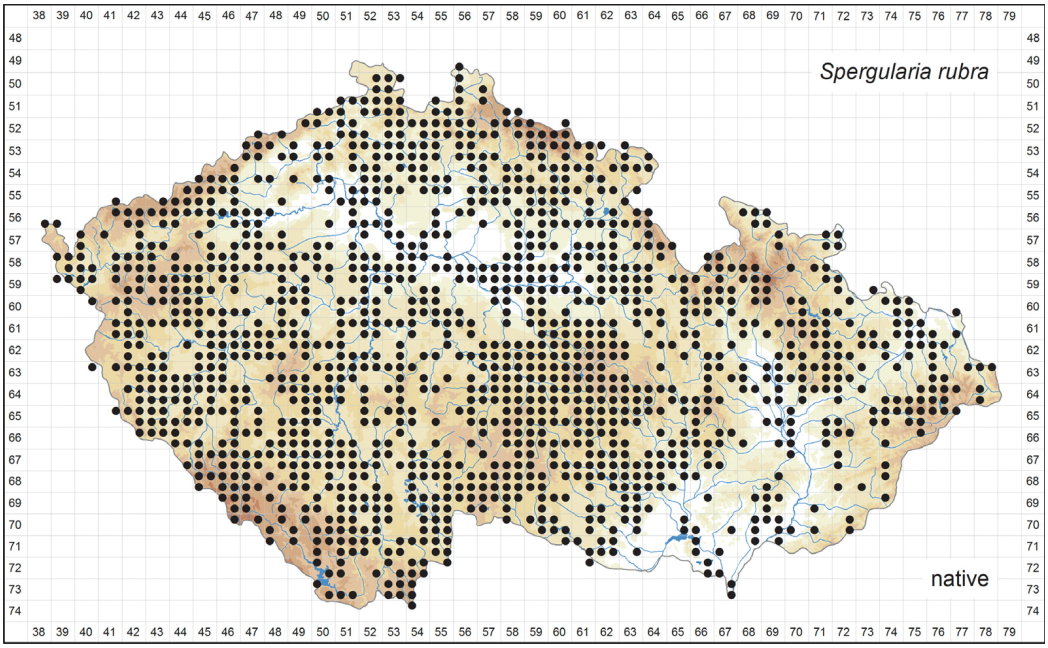

Fig. 75. – Distribution of *Spergularia rubra* in the Czech Republic (1395 occupied quadrants). Prepared by Pavel Kúr & Michal Ducháček.

Today it survives at four localities in southern Moravia only. *Spergularia media* has also been recently found at three sites on motorway verges (motorways D1 in north-eastern Moravia and D2 in southern Moravia). The species has been known from this type of habitat from Austria too (Hohla & Melzer 2003, Adler et al. 2008, Fischer et al. 2008), but its establishment and spread along road verges is slow as it is adapted to less disturbed habitats (Scott & Davison 1982). The species is currently classified as critically threatened (Grulich 2012). Because of frequent misidentifications, the distribution map was based solely on revised herbarium specimens and our own field records.

Spergularia rubra (Fig. 75)

Spergularia rubra is a cosmopolitan species native to Eurasia and introduced to North and South America, southern Africa, Australia and New Zealand (Hultén & Fries 1986, Monnier & Ratter 1993, Hartman & Rabeler 2005, Adams et al. 2008). Its assumed primary habitats are river banks and alluvial pools, but it has successfully spread to various types of secondary habitats. It prefers disturbed sandy sites, like footpaths, field margins or road verges, avoiding calcareous soils (Friedrich 1979, Hartman & Rabeler 2005). In the Czech Republic *S. rubra* is widespread throughout the country. Most of the gaps in the distribution map are due to under-recording but some may be true absences caused by the lack of suitable habitats or the dominance of base-rich and heavy soils.

See www.preslia.cz for Electronic Appendices 1–87

Acknowledgements

Producing distribution maps would not be possible without the effort of innumerable field botanists who often devoted a lot of their time, and frequently also much of their lives, to collecting plant distribution records and plant specimens for herbaria. We are grateful to all who made this study possible and helped us in any way. Curators of the herbaria visited allowed us to study their specimens and collect records. Administrators of the integrated databases, particularly Jan Štěpánek, Milan Chytrý, Dana Michalcová and Karel Chobot as representatives of the major ones, agreed to share plant distribution records. Pavel Dřevojan, Petr Filippov and Zdenka Hroudová contributed with records to produce maps. The South-Bohemian branch of the Czech Botanical Society kindly provided data from the regional floristic database of Václav Chán. Regional collaborators, particularly Jan Doležal, Radek Štencl, Leo Bureš, Pavel Dřevojan, Lenka Pivoňková, Daniel Koutecký, David Hlisnikovský, Radim Paulič, Rudolf Hlaváček, Luděk Čech, Zuzana Mruzíková, Jiří Brabec, Věra Hromádková, Milan Štech, Jan Blahovec, Jan Košnar, Romana Prausová, Tomáš Svačina, Hana Galušková, Petr Koutecký, Tomáš Tichý, Karel Fajmon, Josef Komárek, Vladimír Melichar, Věra Samková, Ester Ekrtová, Petr Lepší, Petr Petřík, Martin Lepší, Pavel Lustyk, Jiří Kocián, Sylvie Pecháčková, Čestmír Ondráček, David Půbal, Hana Houzarová, Petra Juřáková, Milan Kotilínek, Martin Dančák, Michal Juříček, Dagmar Zábranská, Jana Janáková, Josef Albrecht, Milan Marek and Jaroslav Zámečník commented on early versions of maps

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Souhrn

Druhá část ze série publikací věnovaných rozšíření cévnatých rostlin v České republice obsahuje síťové mapy a doprovodné komentáře k 87 taxonům z rodů *Antennaria*, *Aposeris*, *Astragalus*, *Avenula*, *Bidens*, *Carex*, *Cenchrus*, *Centunculus*, *Convallaria*, *Crocus*, *Cryptogramma*, *Cyperus*, *Dryopteris*, *Gladiolus*, *Gratiola*, *Helictochloa*, *Hierochloë*, *Lindernia*, *Maianthemum*, *Myriophyllum*, *Notholaena*, *Nymphoides*, *Radiola*, *Schoenoplectus*, *Sisyrinchium*, *Spergularia*, *Tillaea*, *Veratrum* a *Veronica*. Základem jsou údaje získané excerpcí herbářů a literatury, terénní zápisy a nálezy dostupné v databázích, které prověřili taxonomičtí experti. Mnohé taxony patří mezi vzácné nebo ohrožené rostliny a jsou proto zařazeny na Červeném seznamu. Mezi skupiny rostlin zvláště zasažené změnami nebo úplným zničením biotopů patří psamofyty. *Astragalus arenarius*, *Hierochloë odorata* a *H. repens* jsou kriticky ohrožené druhy, které ustoupily zejména v důsledku převodu písčin na ornou půdu, těžbě písku, zalesňování, změnám v obhospodařování krajiny a eutrofizace prostředí následovanou sukcesí. Všechny tři jmenované druhy se dnes vyskytují na malém počtu lokalit a jejich populace jsou většinou velmi chudé. Další skupinou ohroženou kvůli vazbě na specifická stanoviště jsou konkurenčně slabé mokřadní jednoletky, jako jsou *Centunculus minimus*, *Cyperus flavescens*, *C. michelianus*, *Lindernia procumbens*, *Radiola linoides* a *Tillaea aquatica*. Ty se nejčastěji vyskytují na obnažených dnech rybníků nebo řečiští toků, v opuštěných pískovnách a na extenzivně obhospodařovaných vlhkých písčitých polích. Ačkoliv některé byly v minulosti i hojnější, všechny výrazně ustoupily v důsledku intenzifikace hospodaření na rybnících, zejména následkem přihnojování a omezení pravidelného letnění rybníků, a dále v důsledku rozsáhlých změn ve využívání krajiny. Dnes se tyto druhy vyskytují jen na malém počtu posledních lokalit, mnohdy nepravidelně, s delšími periodami absence, a jsou proto řazeny mezi kriticky ohrožené taxony. Článek přináší i první mapu rozšíření středoevropského endemita *Spergularia kurkae*, který byl jako samostatný druh rozlišen teprve nedávno. Dříve vzácné druhy *Astragalus asper*, *Schoenoplectus supinus* a *Veronica pumila* dnes patří mezi taxony na území ČR vyhynulé. Naproti tomu dříve vzácný druh *Spergularia marina*, která se vyskytoval jen na několika přirozených slaniskách, se v důsledku zimního solení silnic rozšířil po většině území ČR. Revize herbářového dokladu ke starému literárnímu údaji o výskytu zavlečeného druhu *Astragalus alopecuroides* ukázala, že se ve skutečnosti jedná o druh, jehož správné jméno je *A. alopecurus*. Mezi další přechodně zavlečené nebo jen lokálně zdomácnělé druhy, jejichž rozšíření je podrobně zpracováno v tomto článku, patří *Bidens pilosus*, *Cenchrus echinatus*, *Gratiola neglecta* a *Lindernia dubia*, které jsou dokumentovány jen z malého počtu lokalit. *Bidens connatus* byl donedávna velmi vzácný, ale v poslední době se začal šířit na nově uvolněná stanoviště po extrémní povodni v roce 2002. Typickým případem zdomácnělých neofytů jsou *Veronica filiformis* a *V. peregrina*, které se již vyskytují na několika až mnoha stovkách lokalit v různých částech ČR. Invazní druhy zastupuje *Bidens frondosus*, který se začal intenzivněji šířit ve 30. letech 20. století a dnes je široce rozšířený a běžný. Celkový obraz rozšíření jednotlivých zpracovávaných taxonů poskytují mapy, konkrétní floristické údaje odrážející odlišné trendy v různých oblastech a v různých obdobích jsou uloženy v databázi Pladias a dostupné v elektronických přílohách. Každou mapu doprovází textový komentář, který obsahuje nástin celkového rozšíření, výčet nejčastějších stanovišť a stručnou charakteristiku rozšíření v České republice, případně i doplňující informace k taxonomii, biologii, změnám v rozšíření a míře ohrožení.

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Paper IV

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

Kúr P., Košnar J., Koutecký P., Tremetsberger K. & Štech M. (2016): *Preslia 88: 391–407*.

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

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

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Kúr P., Košnar J., Koutecký P., Tremetsberger K. & Štech M. (2016): Origin of *Spergularia ×kurkae*, a hybrid between the rare endemic *S. echinosperma* and its widespread congener *S. rubra*. – Preslia 88: 391–407.

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,

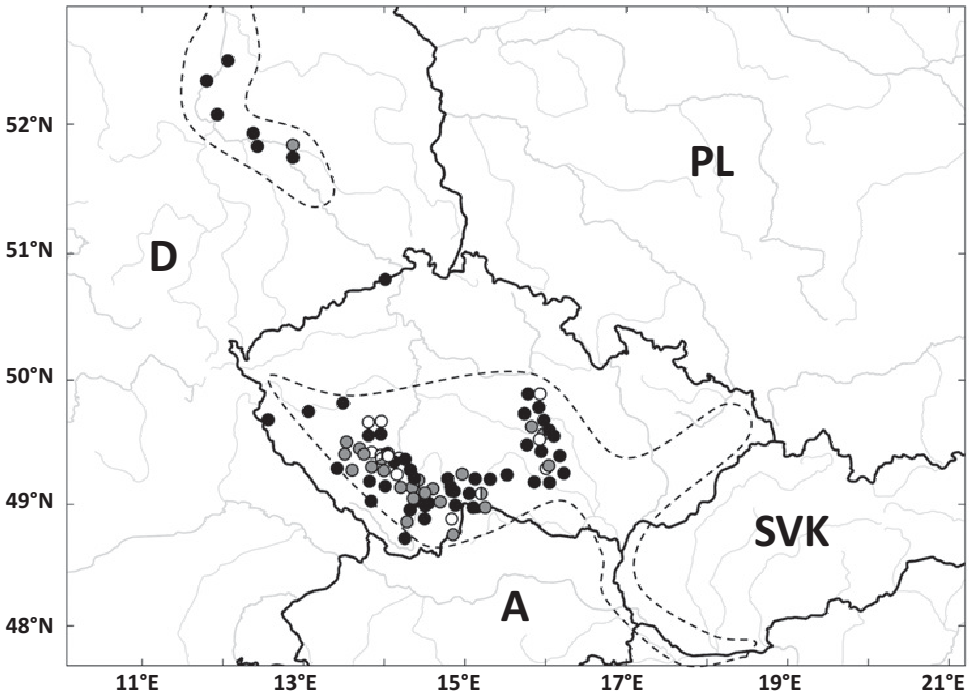

Fig. 1. – Distributions of the populations of *Spergularia echinosperma* (white), *S.* ×*kurkae* (grey) and *S. rubra* (black) studied. The dashed lines denote the distribution of *S. echinosperma* (compiled from the review of herbarium specimens; P. Kúr et al., unpubl.). The distribution of *S. rubra* 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

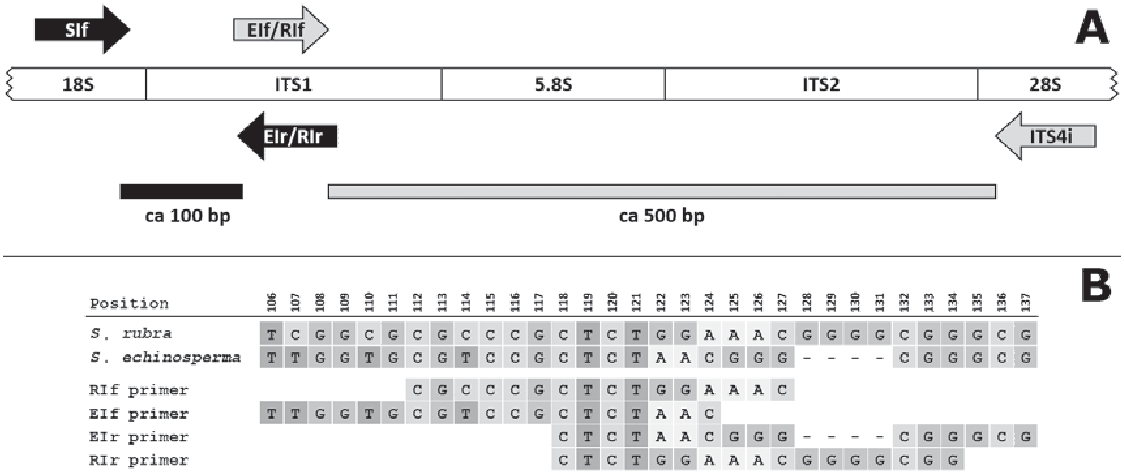

Fig. 2. – (A) Scheme of the primers used in the repeat-specific amplification of *Spergularia echinosperma* and *S. rubra*, with approximate lengths of the PCR products. (B) Variable site in the ITS sequences of *S. echinosperma* and *S. rubra* targeted by the repeat-specific PCR primers.

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).

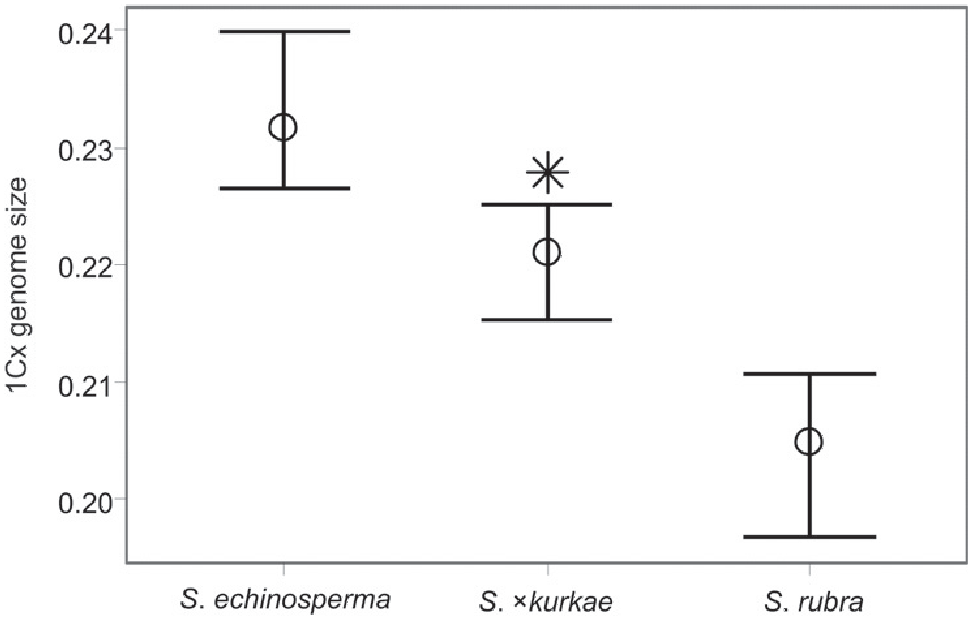

Fig. 3. – Range plot of the equivalents of the 1Cx values calculated from the genome sizes based on DAPI staining for *Spergularia echinosperma*, *S.* ×*kurkae*, and *S. rubra* expressed in terms of a ratio with the 1C value of the internal standard *Glycine max*. Midpoint = median; error bar = min–max.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

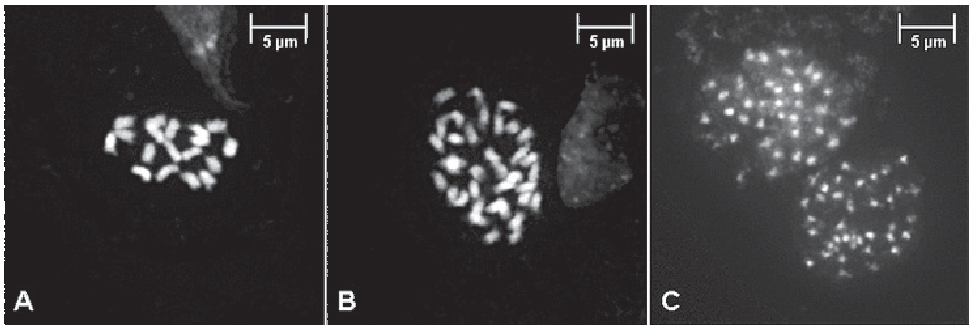

Fig. 4. – Mitotic chromosome spreads of *Spergularia echinosperma* (A; 2n = 18), *S.* ×*kurkae* (B; 2n = 36) and *S. rubra* (C; 2n = 36).

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*.

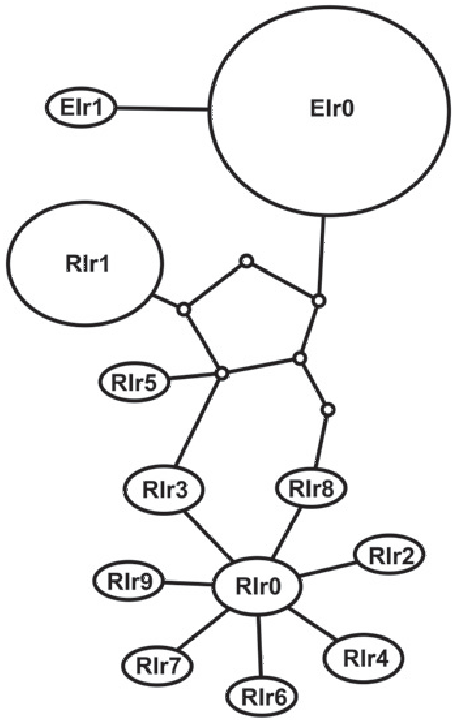

Fig. 5. – Ribotype network based on the sequences obtained from repeat-specific amplification with SIf/EIr and SIf/RIr primers. The size of the ovals indicate the relative frequencies of particular ribotypes in our data. The empty circles represent hypothetical missing haplotypes.

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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Taxonomy and evolutionary diversification of the Central European endemic *Spergularia echinosperma* (Caryophyllaceae)

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Abstract

The patterns of morphological variation and distribution of a rare Central European endemic *Spergularia echinosperma* were investigated. Morphometric analyses revealed the existence of two distinct morphotypes differing mainly in seed color, which was either brown or black. The differences in seed color were also supported by differences in some other morphological characters. Revision of 28 herbarium collections from the Czech Republic and Germany revealed clear geographic separation of the morphotypes. The black-seeded morphotype occurred nearly exclusively in drained fishponds in the southwestern part of the Czech Republic. The brown-seeded morphotype was found in drained fishponds in the eastern part of the Czech Republic and in alluvial pools and river deposits of the Elbe in Germany. We hypothesize that the black-seeded morphotype may have been indigenous in former natural lakes which were widespread in the south-western part of the Czech Republic and were frequently transformed into fishponds. The brown-seeded morphotype may have its origin in river alluvia of the Elbe in Germany and possibly also of other rivers in the Czech Republic. As the two morphotypes are morphologically well-delimited and have an almost completely vicariant distribution, we propose their formal taxonomic treatment and describe the brown-seeded morphotype as *S. echinosperma* subsp. *albensis*.

Introduction

There are relatively few vascular plants endemic to Central Europe outside high mountains. *Spergularia echinosperma* (Čelakovský 1881: 867) Ascherson & Graebner (1893: 517) belongs to the long-recognized ones (Čelakovský 1881, Friedrich 1979, Dvořák 1990). It is a species confined to the threatened vegetation of annual wetland herbs bound to exposed bottoms of freshwater reservoirs (class Isoëto-Nano-Juncetea). The species has two main centers of distribution—Germany where *S. echinosperma* grows in alluvial pools and river banks along the Elbe (Friedrich 1979, Jäger 2011, Brück et al. 2012), and the Czech Republic where it grows exclusively in secondary habitats, especially drained fishponds (Friedrich 1979, Dvořák 1990, Kaplan et al. 2016). It is also marginally distributed in Austria (Fischer et al. 2008) and Slovakia (Dvořák 1979, Goliašová 2012).

Spergularia echinosperma has been long considered a taxonomically critical species (Jage 1974, Dvořák 1990, Suda et al. 2007). The reasons were its morphological similarity to a

widespread congener *S. rubra* (Linnaeus 1753: 423) Presl & Presl (1819: 94), for which *S. echinosperma* was frequently confused, and the alleged presence of gene flow between these two species. Recent studies (Kúr et al. 2012, Kúr et al. 2016), however, demonstrated that although an interspecific hybrid between *S. echinosperma* and *S. rubra* exists—*S. kurkae* Dvořák (1989: 320)—it is a stable hybrid species reproductively isolated from the parents. It was also proven that all the three species are morphologically well-delimited.

The main morphological characters discriminating *S. echinosperma* from the other species are those on seeds (Kúr et al. 2012). However, there has been contradictory information in the literature as for the actual values of some seed characters in *S. echinosperma*. The most conspicuous is the difference in the indicated seed color. Czech authors describe *S. echinosperma* as possessing black seeds (Dostál 1989, Dvořák 1990, Kúr et al. 2012), including the description of this taxon by Čelakovský (1881). German plants, on the other hand, are characterized by (dark) brown seed color (Jage 1974, Friedrich 1979). Our study of the herbarium material of *S. echinosperma* indeed confirmed the presence of two groups differing in seed color.

The taxonomic value of this character in *S. echinosperma* remains unknown. In the genus *Spergularia*, seed characters are generally very important, and they are vital for the determination of some species (Monnier & Ratter 1993, Hartman & Rabeler 2005). It is therefore possible the varying seed morphology in *S. echinosperma* may reflect an unrevealed taxonomic structure of this Central European endemic.

In this study we combined an extensive revision of herbarium material and morphometric analyses of selected specimens. Our aims were to investigate correlations of seed color with other morphological characters and to map the distribution of particular morphotypes. Specifically, we asked the following questions: (1) What is the pattern of morphological variation in *S. echinosperma*? (2) Is the morphological variation correlated with different environmental conditions and/or geographical regions?

Materials and Methods

Plant material from 21 public herbaria (B, BRNM, BRNU, CB, CBFS, DR, GAT, HAL, JE, LIM, LIT, MJ, MNVD, OLM, OP, PL, PR, PRA, PRC, STU, ZMT) and 7 personal herbaria (H. Jage, J. Komárek, J. Zámečník, L. Čech, P. Kúr, R. Paulič, Z. Kaplan) from the Czech Republic and Germany was revised (Electronic Appendix 1). The type of habitat was recorded for each locality based on the description on the herbarium label.

A subset of 114 plants from 15 populations were used for the morphometric analyses (1–19 individuals per population; see Table 1 for the exact localities and acronyms of the populations used in the text). Only mature plants with ripe capsules were used.

In total, 14 quantitative and 7 derived ratio characters were used (Table 2). Diagnostic characters used by Kúr et al. (2012) and other potentially informative characters based on our field experience were included. Seed color was used as a classificatory variable. All the individuals could be unambiguously classified into two groups, one possessing black and the other brown seeds (Fig. 1). Special caution was given to evaluating well-developed seeds only.

The data were processed by multivariate statistical analyses. Quantitative characters that deviated most from a normal distribution in each of the pre-defined groups were log-transformed to improve normality (Table 2). Principal component analysis (PCA) was used to visualize the

TABLE 1. List of the populations of *Spergularia echinosperma* used for the morphometric analyses. The geographic coordinates are presented in the WGS 84 format.

Label	Locality	Latitude	Longitude	Number of plants	Date	Collector	Seed color
Bleddin	Distr. Wittenberg, Bleddin: oxbow lake called "Bleddiner Riß", exposed margin	51.79411	12.79542	7	26.6.1982	H. Jage	brown
Gallin	Distr. Wittenberg, Gallin: scour upstream the ferry in the village, the right bank of the Elbe	51.83680	12.75619	4	11.9.1967	H. Jage	brown
Hodemysl	Dist. Příbram, Hoděmyšl: bare bottom of the Velký hoděmyšlský fishpond	49.61600	13.87864	7	21.6.2011	P. Kúr	black
Hrachoviste	Dist. Jindřichův Hradec, Hrachoviště: bare bottom of the Hrachovišťský fishpond	48.92864	14.76408	10	26.6.2011	P. Kúr	black
Kojatin	Dist. Třebíč, Kojatín: bare bottom of the Kojatínský fishpond	49.24172	16.00847	6	6.6.2011	P. Kúr	brown
KWurf	Distr. Roßlau, Klieken: oxbow lake called "Kurzer Wurf" WSW of the town	51.88031	12.32558	7	9.9.1989	H. Jage	brown
Malobor	Dist. Strakonice, Sedlice: bare bottom of the Malobor pond	49.36678	13.97556	7	25.6.2008	P. Kúr	black
Mlynhor	Dist. Strakonice, Drahonice: bare bottom of the Mlýnský horní fishpond	49.19467	14.08694	7	25.6.2011	P. Kúr	black
Parezny	Dist. Žďár Nad Sázavou, Bohdalov: bare bottom of the Pařezný fishpond	49.47744	15.85317	1	4.6.2011	P. Kúr	brown
Pratau	Distr. Wittenberg, Pratau: oxbow lake N of the town, E of the F2 road (near the "Bude 100")	51.85115	12.64539	7	7.10.1963	H. Jage	brown
Priesitz	Distr. Wittenberg, Priesitz: the Old Elbe ca 1 km NE of the town; sandy-muddy margin of the oxbow lake	51.70727	12.83715	7	12.10.1971	H. Jage	brown
Skopec	Dist. Písek, Nová Ves u Protivína: bare bottom of the Skopec fishpond	49.23108	14.25231	10	25.6.2011	P. Kúr	black
Svihov	Dist. Chrudim, Švihov: bare bottom of the Švihov fishpond	49.84264	15.85931	10	5.6.2011	P. Kúr	brown
Tangermunde	Distr. Stendal, Tangermünde: right bank of the Elbe opposite the town, under the road bridge.	52.56491	11.98564	5	14.10.1963	H. Jage	brown
Terlicko	Dist. Karviná, Těrlicko: exposed margin of the Těrlicko water reservoir	49.74336	18.49515	19	25.10.2012	H. Jage	brown

overall pattern of morphological variation in the data (CANOCO 5; Šmilauer & Lepš 2014). To find out which characters significantly separated the seed color groups, canonical discriminant analysis (CDA) was applied. The significance of individual characters was tested using both marginal effects (i.e., when a character is alone in the model) and unique contributions of the characters (i.e., the addition of each character into the model with all other characters) (Koutecký 2015). Forward selection of characters was employed to detect the combination of characters most contributing to the separation of the groups. The threshold significance level was set to $\alpha = 0.05$ and a Monte-Carlo permutation test (1000 permutations) used. The predictive ability of the selected characters was tested by classificatory discriminant analysis based on the posterior group membership probabilities and cross-validation using whole populations as leave-out units. The percentage of misclassified samples in each group served as a measure of the predictive ability.

TABLE 2. Morphological characters used in the morphometric analyses and summary of their values for the black-seeded (41 individuals) and brown-seeded (73 individuals) *S. echinosperma* morphotypes. The numbers denote (minimum–)10th percentile/**mean**/90th percentile(–maximum). Characters log-transformed prior to the multivariate analyses are marked with an asterisk.

Acronym	Character [units]	Seed color group	
		brown	black
Cap-K	capsule-sepal length ratio	(1.00–)1.13/**1.26**/1.56(–1.41)	(1.07–)1.13/**1.21**/1.39(–1.33)
CapsLeng	capsule length [mm]	(2.4–)3.0/**3.2**/4.1(–3.5)	(2.6–)2.8/**3.1**/3.7(–3.5)
FrPedLen*	length of the fruit pedicel adjacent to the capsule [mm]	(1.7–)2.4/**4.1**/9.1(–6.7)	(3.8–)4.1/**5.6**/11.1(–7.5)
InterLen*	length of the internode adjacent to the measured leaf [mm]	(3.33–)6.47/**11.43**/21.27(–16.83)	(5.43–)7.90/**11.94**/25.07(–18.97)
Int-Leaf*	internode length/leaf length ratio	(0.43–)0.77/**1.02**/1.65(–1.34)	(0.72–)0.91/**1.39**/3.42(–1.87)
KLength*	sepal length [mm]	(2.1–)2.3/**2.6**/3.4(–2.8)	(2.1–)2.3/**2.6**/3.2(–3.0)
LeafLeng*	leaf length [mm]	(5.3–)6.4/**11.5**/21.0(–17.6)	(5.0–)5.7/**9.1**/15.6(–12.7)
LeafRat*	leaf length/width ratio	(10.4–)15.6/**22.8**/38.5(–32.5)	(13.2–)14.8/**21.5**/62.0(–26.9)
LeafWidt	leaf width [mm]	(0.2–)0.4/**0.5**/0.8(–0.6)	(0.1–)0.2/**0.5**/0.8(–0.7)
LengSeed	seed length [µm] (Fig. 7)	(323–)377/**412**/507(–467)	(336–)357/**397**/473(–448)
PapHei	papilla height [µm] (Fig. 7)	(12–)15/**18**/23(–21)	(16–)17/**19**/24(–21)
PapNum	number of papillae on one quarter of the seed circumference (papillae density)	(5–)8/**12**/19(–15)	(10–)13/**15**/20(–18)
PapRat	ratio of the papilla upper part ("head") width and papilla lower part ("neck") width (papilla shape)	(1.01–)1.10/**1.24**/1.55(–1.42)	(1.04–)1.08/**1.21**/1.50(–1.35)
Ped-Cap*	pedicel/capsule length ratio	(0.57–)0.73/**1.27**/2.28(–2.10)	(1.21–)1.39/**1.80**/3.25(–2.34)
PlHeight*	height of the longest stem [cm]	(4–)5/**8**/16(–12)	(5–)6/**8**/12(–10)
SeedRat	seed length/width ratio	(1.09–)1.22/**1.29**/1.51(–1.38)	(1.23–)1.26/**1.34**/1.48(–1.42)
StemWidth*	stem width [mm]	(0.4–)0.4/**0.6**/1.3(–0.7)	(0.2–)0.3/**0.5**/0.8(–0.6)
StpLt	stipule length [mm]	(0.9–)1.1/**1.3**/1.8(–1.6)	(0.9–)1.1/**1.2**/1.5(–1.4)
StpRT	stipule length/width ratio	(0.48–)0.60/**0.72**/1.01(–0.86)	(0.58–)0.63/**0.74**/0.90(–0.83)
StpWd	stipule width [mm]	(1.3–)1.6/**1.9**/2.5(–2.2)	(1.3–)1.5/**1.7**/2.0(–1.8)
WidtSeed	seed width [µm] (Fig. 7)	(242–)289/**321**/383(–358)	(252–)265/**297**/341(–338)

The discriminant analyses were computed using the MorphoTools scripts (Koutecký 2015) in R 3.2.3 (R Development Core Team 2015).

We also reanalyzed the data by classification trees that create a hierarchical classification based on univariate splits that can then be visualized as an easily interpretable tree diagram (Breiman et al. 1984). The function rpart (package rpart) in R 3.2.3 (R Development Core Team 2015) was used. The minimum split parameter (minsplit) was set to 1 and the initial complexity parameter (cp) to 0.001. A cross-validation using the populations as the leave-out subsamples was used to assess the optimal tree complexity, instead of random subsamples as implemented in the original method (Venables & Ripley 2002). The resulting tree was selected on the basis of the 1-SE rule (Venables & Ripley 2002).

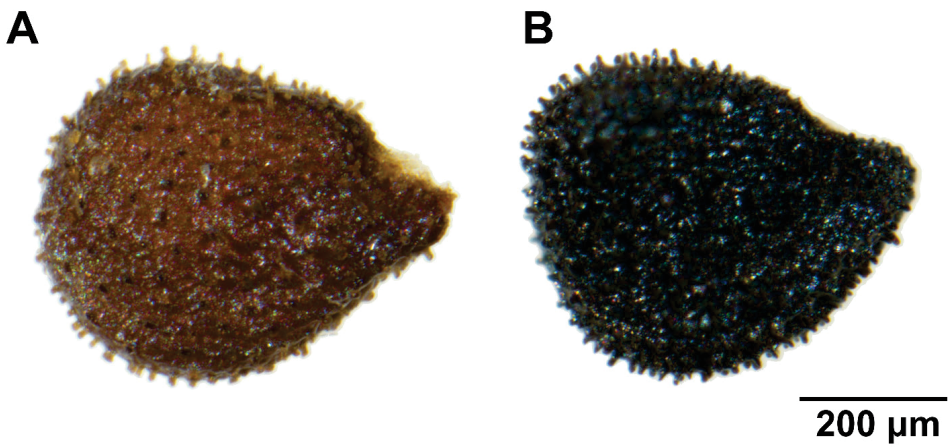

FIGURE 1. A typical seed of the brown-seeded (A) and black-seeded (B) morphotype of *Spergularia echinosperma*.

Results

Using all 21 morphological characters, PCA did not produce any clear and distinct clusters of plants. However, the black-seeded and brown-seeded plants separated slightly along the second ordination axis (Fig. 2).

In spite of this, CDA identified some characters significantly separating the groups (Table 3). The 7 best predictors selected by forward selection were mainly seed characters (papillae

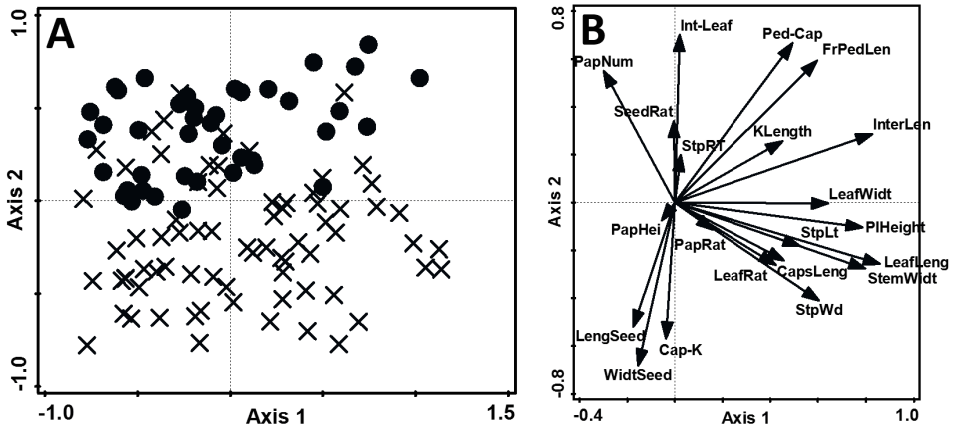

FIGURE 2. PCA of individuals of *Spergularia echinosperma*: (A) distribution of individuals in the ordination space (circles – black seeded plants, X-marks – brown-seeded plants), (B) fit of the 21 morphological characters to the ordination axes. The first and the second ordination axes explain 24.3% and 16.4% of the total variation, respectively.

density, papilla head/neck ratio, papilla height, seed width) although there were also some well-discriminating characters on the vegetative parts (i.e. pedicel/capsule length ratio, stipule width, and internode/leaf length ratio). The predictive ability of the selected characters was 84% of correctly classified samples (Fig. 3, Table 4).

The final classification tree selected had 3 terminal nodes (complexity parameter cp = 0.1). It confirmed the high discrimination power of pedicel/capsule length ratio. The second character selected was leaf length, contrary to the CDA forward selection (Fig. 4). The overall predictive power of this model was lower with 65% of correctly classified samples (Table 4).

The revision of herbarium specimens showed that the black-seeded morphotype is, with two exceptions, restricted to the southwestern part of the Czech Republic (especially the South Bohemian fishpond basins). The exceptions were one locality in Germany (Rathenow) and one locality in the eastern part of the Czech Republic (Kadolecký fishpond near Křižanov). The brown-seeded morphotype, on the other hand, occurs in the eastern part of the Czech Republic (especially in the Bohemian-Moravian Highlands) and Germany (along the Elbe) only (Fig. 5).

The localities of *S. echinosperma* were found in four distinct types of habitats – fishponds, river reservoirs, alluvial pools, and river banks (Table 5). Nearly all localities from the Czech Republic came from drained fishponds. An exception was one population from an exposed

TABLE 3. Morphological characters tested in the forward selection with their conditional and marginal effects, unique contributions, and their contributions to the canonical axis (biplot scores).

Character	Conditional effects		Marginal effects		Unique contributions		Biplot scores
	F	p	F	p	F	p	
PapNum	60.8	0.005	60.8	0.005	17.1	0.001	0.426
Ped-Cap	30.8	0.005	36.9	0.005	7.4	0.005	0.332
StpWd	19.7	0.005	23.3	0.005	2.3	0.146	-0.263
PapRat	13.7	0.005	2.0	0.200	12.6	0.001	-0.077
WidtSeed	8.8	0.005	18.8	0.005	3.4	0.064	-0.237
PapHei	6.3	0.015	7.8	0.005	22.3	0.001	0.152
Int-Leaf	4.9	0.020	30.2	0.005	0.3	0.639	0.300
FrPedLen	n. s.		25.9	0.005	7.9	0.003	0.278
StemWidth	n. s.		12.9	0.005	3.2	0.071	-0.196
SeedRat	n. s.		11.3	0.005	2.2	0.141	0.183
LeafLeng	n. s.		10.6	0.005	0.0	0.972	-0.178
StpLt	n. s.		8.0	0.015	0.4	0.539	-0.154
Cap-K	n. s.		7.5	0.010	0.0	0.920	-0.149
CapsLeng	n. s.		6.5	0.020	3.4	0.065	-0.139
LeafWidt	n. s.		5.4	0.030	0.0	0.925	-0.127
LengSeed	n. s.		4.7	0.035	2.2	0.140	-0.118
StpRT	n. s.		1.2	0.275	0.8	0.384	0.060
LeafRat	n. s.		1.0	0.305	0.4	0.568	-0.056
InterLen	n. s.		0.5	0.505	0.1	0.785	0.038
PlHeight	n. s.		0.2	0.690	6.3	0.011	-0.023
KLength	n. s.		0.0	0.940	0.0	1.000	-0.002

margin of a river reservoir. In Germany, *S. echinosperma* occurred nearly exclusively in alluvial pools or river banks along the Elbe River. An exception was the only German locality of black-seeded *S. echinosperma* which was located in the vicinity of the river Havel, ca 20 km off the Elbe. The type of habitat was unfortunately not specified on the herbarium label.

TABLE 4. Summary of the classification matrices of the black-seeded and brown-seeded morphotypes of *Spergularia echinosperma* resulting from the classificatory discriminant analysis and classification trees.

Classificatory discriminant analysis				Classification trees			
observed	black	brown	Total	observed	black	brown	Total
predicted				predicted			
black	37 (90.2%)	14 (19.2%)		black	24 (58.5%)	23 (31.5%)	
brown	4 (9.8%)	59 (80.8%)		brown	17 (41.5%)	50 (68.5%)	
Percent correct	90.0%	80.8%	84.2%	Percent correct	58.5%	68.5%	64.9%

TABLE 5. Absolute frequencies of habitat types in which *Spergularia echinosperma* was recorded, summarized across morphotypes and countries.

Country	Czech Republic		Germany	
Seed color	black	brown	black	brown
fishpond	47	63	.	.
reservoir	.	1	.	.
alluvial pool	.	.	.	31
river bank	.	.	.	34
unknown	.	.	1	.

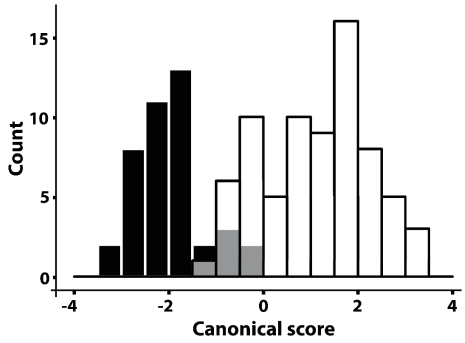

FIGURE 3. Distribution of the canonical scores from CDA for the black-seeded (black) and brown-seeded (white) morphotype of *Spergularia echinosperma*.

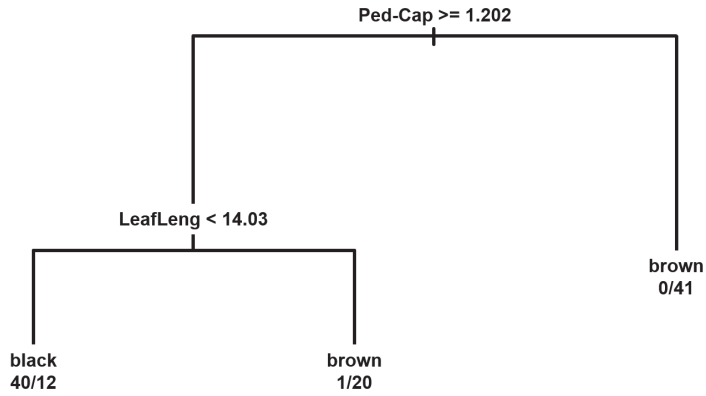

FIGURE 4. Classification tree of the individuals of the black-seeded and brown-seeded morphotype of *Spergularia echinosperma*. If a character value matches the classification rule, the determination continues to the left branch, otherwise to the right branch. Lengths of the branches correspond to the relative discriminatory powers of the respective rules. The group names at the terminal nodes indicate the predicted classification of a particular node, whereas the numbers separated by slashes indicate actual membership of samples classified to a particular node (black/brown).

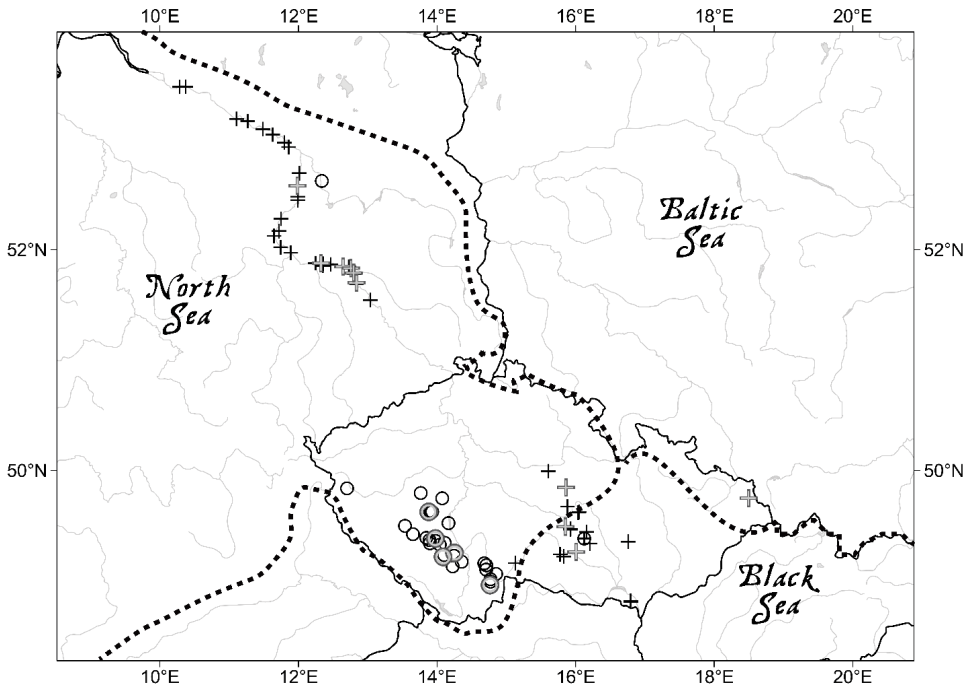

FIGURE 5. Distribution of the herbarium specimen records of the black-seeded (circles) and brown-seeded (crosses) morphotype of *Spergularia echinosperma*. Populations used for morphometric analyses are highlighted in gray. The dashed lines denote the main European drainage divides.

Discussion

We confirmed that the observed differences in seed color in *Spergularia echinosperma* are correlated with the differences in some other morphological characters. Most of them are related to seed morphology, but there are also some well-discriminating vegetative characters. Even if the determination of the morphotypes without the information about the seed color is not fully reliable, their morphological separation seems to be well-supported.

The morphotypes display a clearly vicariant distribution. The presence of brown-seeded *S. echinosperma* in Germany is in accordance with the descriptions of this species by German authors (Jage 1974, Friedrich 1979). From the Czech Republic, however, only the black-seeded morphotype has been reported so far (Dostál 1989, Dvořák 1990, Kúr 2012).

In this moment, we can only speculate about the origin and migration history of the different lineages of *S. echinosperma*. The black-seeded morphotype may have its origin in natural lakes which existed in South and South-West Bohemia and were frequently transformed into fishponds during the Middle Ages and the Early Modern Age (Chvojka et al. 2010, Pokorný 2015). The brown-seeded morphotype, on the other hand, may be indigenous to periodically exposed substrates in river alluvia. This is easily conceivable in the case of the Elbe where the species still grows in this type of habitats. The same may hold true for the populations of brown-seeded *S. echinosperma* in the eastern part of the Czech Republic. In this region, natural lakes were nor common (Chlupáč et al. 2002), and alluvial pools seems to be a more probable primary habitat for *S. echinosperma*. Periodically exposed alluvial pools still occur in this region (especially along the Morava River), but they were vastly destroyed in the 20th century. Unfortunately, there are no historical records of *S. echinosperma* from these habitats in this region to corroborate the indigenous status of the species in this region.

Even if the two morphotypes of *S. echinosperma* have probably evolved in different regions, they nearly complete vicariance is surprising. As the Bohemian populations of black-seeded *S. echinosperma* lie within the Elbe river catchment, one would expect their presence at the lower reaches of the Elbe too. This could be explained by different ecological adaptations of the morphotypes. The black-seeded morphotype may be adapted to the management of the South Bohemian fishponds where there is only a relatively short and unpredictable period of substrate exposure during the spring, which makes a strong selection pressure on shortening life cycle and the presence of primary seed dormancy (Šumberová et al. 2005). In contrast, alluvial pools in river floodplains are usually exposed for a longer period in late summer and fall, and their water regime is more predictable probably relaxing existing selection pressures (Šumberová 2011). Differences in seed dormancy may also be the direct cause of different seed morphology. Seeds of the black-seeded morphotype probably have thicker testa than those of the brown-seeded morphotype, which is a trait that is related to increased seed dormancy (Bewley et al. 2012).

The absence of the black-seeded morphotype in the eastern part of the Czech Republic as well as the absence of the brown-seeded morphotype in south-western part of the Czech Republic are harder to explain as it is the same type of habitats, i.e. fishponds. This may be the result of dispersal limitation. As the majority of the populations of the black-seeded morphotype lie within a different drainage basin than do the populations of the brown-seeded morphotype, a limited diaspore exchange between the two regions seems logical. In addition, there may also exist some sort of environmental filtering. As far as we know, fishponds in the Bohemian-Moravian

Highlands, where most of the Czech localities of the brown-seeded morphotype lie, are usually dried for a longer period than the South-Bohemian fishponds (K. Šumberová, pers. comm.). This may create ecological conditions more similar to those of exposed substrates in river alluvia.

Clearly, further studies, including macrofossil analyses and the study of dormancy and germination biology, are needed to elucidate the evolution history of *S. echinosperma*. Partial insight should be provided by an ongoing genetic study (based on published microsatellite markers; Kúr et al. 2014).

Considering the obvious geographic and ecological differentiation of the two morphotypes, their taxonomic treatment as separate entities seems justified. As the morphological differences between the morphotypes are tiny, we propose treating them at the rank of subspecies. As the type of *S. echinosperma* belongs to the black-seeded morphotype (deposited in the herbarium PR, No. 374981; Kúr et al. 2012), we describe the brown-seeded morphotype as the new subspecies.

Descriptions of the new subspecies

Spergularia echinosperma subsp. *albensis* Kúr, Amarell, Jage & Štech, subspec. nova

Diagnosis:—*Spergularia echinosperma* subsp. *albensis* resembles habitually *S. echinosperma* subsp. *echinosperma*, however, it differs in having seeds with brown to dark brown testa. In addition, *S. echinosperma* subsp. *albensis* has, on average, shorter fruit pedicels, lower pedicel length / fruit length ratio and longer leaves than *S. echinosperma* subsp. *echinosperma*.

Type:—Germany, distr. Wittenberg, Priesitz: oxbow lake 1 km East by North of the church in the village; lat.: +51.7047, long.: +12.8393; 12. 10. 1971; leg. H. Jage (holotype: GLM, No. 0168069, Fig. 6; isotype: PR, No. 878041).

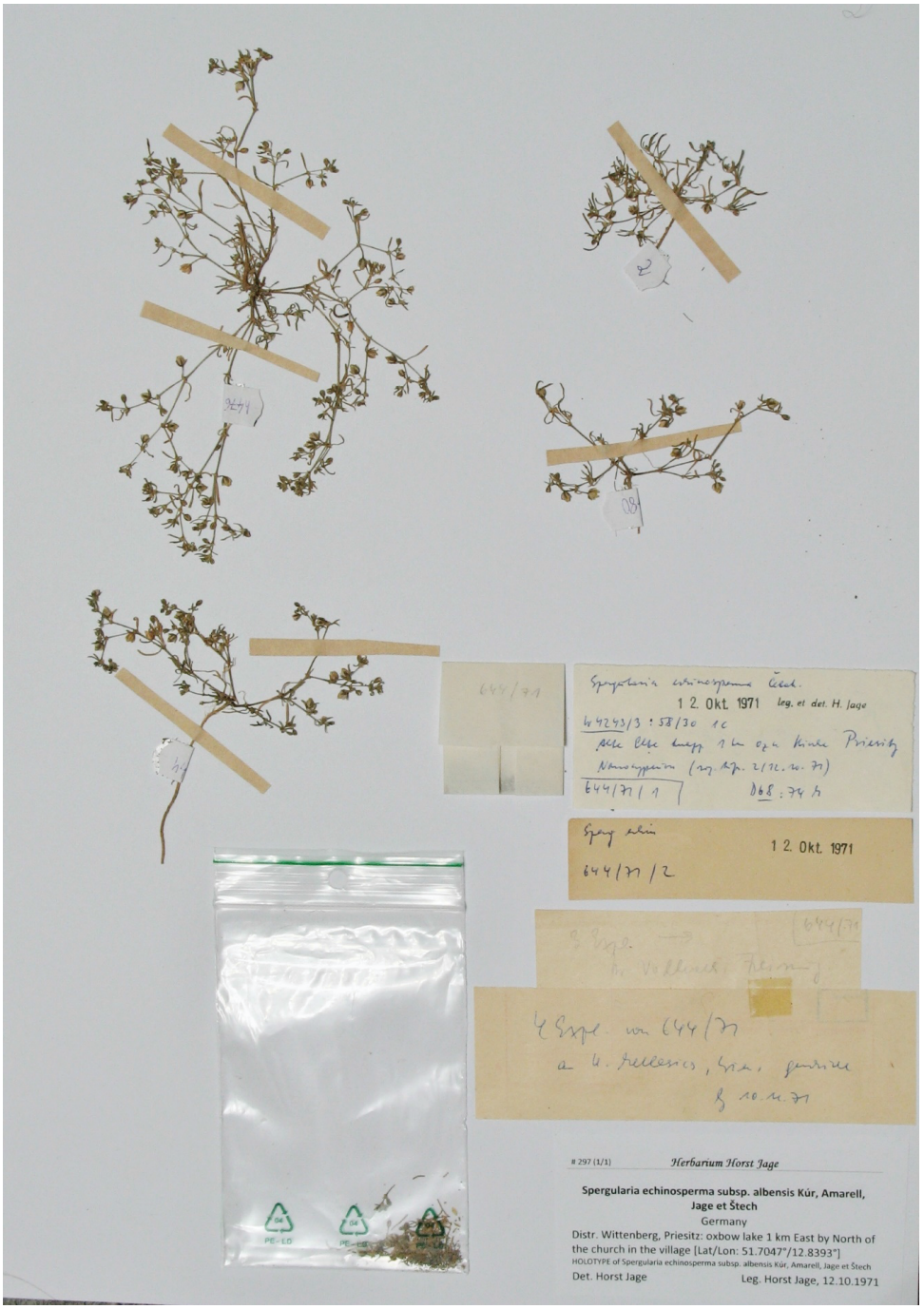

FIGURE 6. Holotype of *Spergularia echinosperma* subsp. *albensis* deposited in GLM.

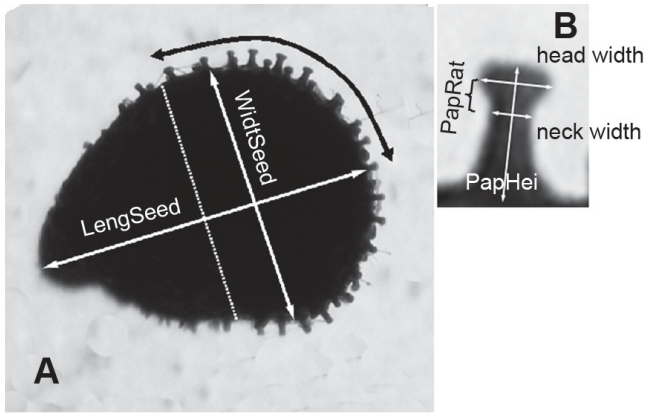

FIGURE 7. Characters measured on the seeds (A) and surface papillae (B). The black curved line specifies the part of the seed circumference where the density of papillae was determined. The longitudinal border of this part is a plane halving the vector of maximal seed length and perpendicular to it (indicated by a dotted line). The character PapRat was computed by dividing the width of the papilla head by the width of the neck.

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General conclusions

The thesis extends significantly the current knowledge of the Central-European endemic *Spergularia echinosperma*. Conclusive evidence of the existence of *S. kurkae*, an allotetraploid hybrid between diploid *S. echinosperma* and tetraploid *S. rubra*, has been presented. Our data 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 from the both parents. Although there are some indications of ongoing gene flow between *S. kurkae* and *S. rubra*, it appears to be rare, with little effect on the boundary between the taxa. In contrast, no evidence of gene flow from *S. rubra* to *S. echinosperma* was found, providing, despite concerns, no support for the threat of the genetic assimilation of *S. echinosperma*. 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

The distributions of *S. echinosperma*, *S. kurkae* and *S. rubra* in the Czech Republic have been mapped based on a large revision of herbarium specimens. The data shows that the distribution ranges of *S. echinosperma* and *S. kurkae* in the Czech Republic largely overlaps. The both species are most frequent in areas with many fishponds, i.e. Southern, South-Western and Eastern Bohemia, but *S. kurkae* is roughly twice as more abundant as *S. echinosperma*. *Spergularia rubra* is widespread thorough the country. The obtained data on morphology and distribution of the species have been applied in the *Spergularia* treatment for the upcoming new Key to the flora of the Czech Republic.

In addition, the taxonomic structure of *S. echinosperma* has been clarified. The existence of two infraspecific taxa, described at the subspecific rank, has been documented. *Spergularia echinosperma* (Čelak) A. et Gr. subsp. *echinosperma* is characterized by black seeds and is nearly exclusively distributed in South, South-West and West Bohemia. It grows exclusively in the bottoms of drained fishponds and may have its origin in natural lakes that were frequently transformed into fishponds in the past. *Spergularia echinosperma* subsp. *albensis* Kúr, Amarell, Jage & Štech differs from the nominal subspecies mainly by brown seed color and is distributed in the eastern part of the Czech Republic (Eastern Bohemia, Moravia and Silesia) and Germany. In the Czech Republic, it occurs in bottoms of drained fishponds and rarely in exposed margins of river reservoirs. In Germany, this subspecies is found exclusively in primary habitats, i.e. alluvial pools and river banks of the Elbe River.

Despite the considerable contribution of this work towards understanding the biology of *S. echinosperma* and its newly recognized congener *S. kurkae*, many unanswered questions remain. The global distributions of the both species needs to be mapped and the dubious reports of *S. echinosperma* from countries out of Central Europe examined. The unexpected vicariance of the two *S. echinosperma* subspecies also calls for further studies. Better insight into the evolutionary history and migration patterns of *S. echinosperma* will be hopefully provided with the aid of the microsatellite markers developed as a part of this thesis.

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