Ecological hybrid speciation in central-European species of *Bolboschoenus*: genetic and morphological evaluation

Ekologická hybridní speciace středoevropských kamyšníků (*Bolboschoenus*) – genetické a morfologické zhodnocení

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Divergent natural selection is known to facilitate speciation in many taxa. The genus *Bolboschoenus* (*Cyperaceae*) is a model group for investigating ecological and homoploid hybrid speciation. Four taxa of *Bolboschoenus* occur in central Europe: the halophyte *B. maritimus* and glycophytes *B. laticarpus*, *B. planiculmis* and *B. yagara*. These species differ in their ecological niches. Such ecological and/or geographical isolation is critical for homoploid hybrid speciation. The determination of species of *Bolboschoenus* is based on morphological characters of the inflorescence and on achene shape and anatomy. On the basis of its intermediate morphology, chromosome number and ecological amplitude *B. laticarpus* is thought to be a hybrid. In order to determine the validity of morphological species and the possible hybrid origin of *B. laticarpus* we used amplified fragment length polymorphisms (AFLPs) as molecular markers and compared different genetic groups defined using STRUCTURE analysis with morphological data. The morphological classification of central-European species of *Bolboschoenus* was confirmed. Plants of heterogeneous genotypes were also found to be intermediate individuals resulting from spontaneous hybridization. Hybrid origin of *B. laticarpus*, which is genetically and morphologically intermediate between *B. yagara* and *B. planiculmis*, was elucidated. Inflorescence characters were less important for determining species than anatomical characters of achenes (widths of the exocarp and mesocarp).

Keywords: AFLP, *Bolboschoenus*, central Europe, hybridization, model-based clustering, morphometrics, speciation

Introduction

The evolutionary histories of groups of closely related species of plants have been the focus of interest of much research in recent years, especially the process of speciation (Kaplan et al. 2013, Kolář et al. 2014, 2015). Moreover, questions concerning reproductive isolating mechanisms and the genetics and genomics of speciation have been highlighted by a major European initiative as the main subjects for further research (Butlin et al. 2012). Ecological speciation is one of the main modes of speciation (Schluter 2001). It occurs as a result of reproductive isolation due to divergent selection of organisms in different environments and can arise even where species occur sympatrically (Sobel et al.

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2009). Studying microevolution within groups of closely related species differing in ecology may thus reveal the role of such environmental differences in their origin. Hybridization can result in both gene flow among plant taxa and generation of new species. Some families and genera have relatively high numbers of hybrids, with the family *Cyperaceae* being one of them (Ellstrand et al. 1996).

The European species of *Bolboschoenus* (Asch.) Palla (*Cyperaceae*) provide an example of a putative hybrid complex for which ecological selection, geographic isolation and hybridization are thought to have been important in their speciation, with the study of their genetic variation likely to shed light on their speciation processes. Of the ~14 species of *Bolboschoenus* that are distinguished worldwide (Browning & Gordon-Gray 2000, Tatanov 2007), four are native to Europe (Hroudová et al. 2007): *Bolboschoenus maritimus* (L.) Palla, *B. laticarpus* Marhold, Hroudová, Ducháček et Zákravský, *B. planiculmis* (Schmidt) T. V. Egorova, and *B. yagara* (Ohwi) Y. C. Yang et M. Zhan. Their native ranges include at least some part of Europe, with *Bolboschoenus maritimus* and *B. laticarpus* occurring mainly in Europe, whereas *B. planiculmis* and *B. yagara* reach the western borders of their ranges in central Europe and are continuously distributed through Eurasia to the Far East (Egorova & Tatanov 2003, Tatanov 2003, Hroudová et al. 2007). Central Europe is thus the area where these four taxa co-occur. Indeed, in some regions of central Europe, individuals of different species form mixed populations. In such cases, plants with intermediate morphology occur, indicating possible spontaneous hybridization. Such morphotypes are recorded frequently, especially those intermediate between *B. maritimus* and *B. planiculmis* (Ducháček 2002, Hroudová et al. 2006). Nevertheless, it is difficult to determine whether such intermediate plants have resulted from present-day spontaneous hybridization or simply represent overlapping variation in the two species' morphological characters.

The species differ in their ecological niches (Hroudová et al. 1999, 2007, Kaplan et al. 2015): *Bolboschoenus maritimus* inhabits saline habitats (mainly remnants of natural halophyte vegetation), *B. planiculmis* inhabits secondary habitats (temporarily flooded field depressions, wet meadows, arable land, inland shores of fishponds and other reservoirs, ruderal and other anthropogenic habitats), *B. yagara* occurs predominantly in the littoral of fishponds, mainly in several fishpond basins, and *B. laticarpus* has a wide habitat range, including along streams and many secondary habitats (flooded depressions in fields, wet ditches and channels, and as a weed in arable land). This obvious ecological differentiation might indicate a crucial role of environmentally-driven selection in the evolutionary process leading to speciation within the genus *Bolboschoenus*.

Current classification is based on morphological and anatomical characters of achenes, which are more reliable than inflorescence characters (Oteng-Yeboah 1974, Browning & Gordon-Grey 1993, Browning et al. 1997b, Browning & Gordon-Grey 2000). Individuals without ripe achenes cannot be determined unambiguously. *Bolboschoenus yagara* is distinguished from other species by narrowly obovate achenes, triangular in transverse section and a particularly thin exocarp. *Bolboschoenus planiculmis* has obovate achenes, concave on the abaxial side and exocarp approximately as thick as the sclerenchymatic mesocarp. *Bolboschoenus maritimus* is distinguished by lenticular achenes convex on the abaxial side, with exocarp thicker than mesocarp. *Bolboschoenus laticarpus* has trigonous achenes with thicker mesocarp than exocarp. Its achene shape varies, ranging from trigonous achenes with the edge sharp through flattened-trigonous

to slightly convex on the abaxial side. In summary, the most important characters for species determination are the shape of the achene in transverse section and the widths of the exocarp and mesocarp (Hroudová et al. 2007).

Bolboschoenus laticarpus (n = 54, 55) is a taxon that is morphologically and anatomically intermediate between *B. yagara* (n = 55) and *B. maritimus* (n = mostly 55) or *B. planiculmis* (n = mostly 54; meiotic chromosomes counted by Jarolímová & Hroudová 1998). Browning et al. (1996) hypothesize that this taxon is of hybrid origin and call it *B. maritimus* × *B. yagara*. Marhold et al. (2004) consider it to be a stable taxon of hybrid origin with possible parentage *B. yagara* × *B. planiculmis*, based on the numbers of style branches and chromosomes (Jarolímová & Hroudová 1998). Finally, hybridization in nature is most likely to occur between *B. yagara* and *B. planiculmis,* with overlapping distributions across Eurasia, than between *B. yagara* and *B. maritimus*, with distinct distributions and quite different habitats. There are no molecular studies of *Bolboschoenus* and thus study of its genetic variation provides a suitable way of elucidating the processes leading to the differences in the distributions and ecologies of the species within this genus. Moreover, molecular analyses would reveal whether *B. laticarpus* is of hybrid origin, and, if so, its likely parentage.

The AFLP method is a suitable molecular marker to generate a large number of variable loci distributed throughout the genome, and requires no previous sequence knowledge (Vos et al. 1995). This method offers powerful tools for the assessment of intraspecific variation and detection of hybrid origin (Gobert et al. 2002, Guo et al. 2006, Španiel et al. 2011, Záveská et al. 2011). We analysed populations of *B. yagara*, *B. laticarpus*, *B. maritimus* and *B. planiculmis* using AFLPs, with the objective of determining the mechanisms that resulted in inter-specific differentiation during their evolution. In particular, whether genetic variation among the species corresponds to their morphological variation, indicating gene flow during evolution, or whether species differentiation resulted predominantly from ecological (ecophysiological) speciation due to selection. Both the role of hybridization in speciation, and possible recent spontaneous hybridization between some species were also studied. We aimed to answer the following questions: (i) Does morphological variation of the species studied correspond to the genetic variation? (ii) What morphological and anatomical characters can be used to differentiate among individual species? (iii) Does spontaneous hybridization occur in natural populations of the species, and to what extent is this reflected by morphology? (iv) Is *B. laticarpus* of hybrid origin, and if so, what are its parental species?

Materials and methods

Plant material

In order to include all the variation in European taxa of *Bolboschoenus*, samples of four species of *Bolboschoenus* (*B. maritimus*, *B. laticarpus*, *B. planiculmis* and *B. yagara*) from 36 natural populations in the Czech Republic, Slovakia, Austria and Hungary were collected during 2010–2011 (Appendix 1). Species were pre-identified in the field using identification keys and the nomenclature follows Hroudová et al. (2007). When mixed populations were found (rarely the case) only individuals morphologically resembling a particular taxon were collected. The number of individuals sampled for AFLPs (as well

as inflorescences and achenes) was increased in order to collect all species in the same way. *Bolboschoenus maritimus* material from natural populations was complemented with material from plants cultivated from seeds (collected in the wild in Germany, France and Iran) in an experimental garden of the Institute of Botany ASCR in Průhonice (49°59.69'N, 14°34.00'E). For this cultivation, for each population, descendants of particular individuals were used in order to reflect intrapopulation genetic and morphological variation. The results of earlier work (Hroudová et al. 1998a) confirmed that the morphological characteristics recorded in natural populations of *Bolboschoenus* persisted after transfer into cultivation, i.e. plants from cultivation corresponded to plants from natural habitats.

Material for AFLP analyses (altogether 279 leaf samples), morphological analyses (855 inflorescences) and anatomical measurements (875 achenes) was collected using the following procedures. For AFLP analyses, undamaged parts of fresh leaves usually from 10 individuals per population (whenever possible, depending on population size) were sampled randomly and immediately dried in silica gel. Due to the extensive damage to leaves by rusts that occurs when the inflorescences are fully developed and prevent the isolation of DNA suitable for AFLP analyses, we collected fresh young leaves from different individuals (but in the same populations) than those used for morphometrics. In addition, the breaking up of inflorescences and spikelets at maturity made it impossible to obtain both inflorescences and ripe achenes from the same individuals. Whole well-developed inflorescences of 25 individuals in each population were collected and 25 ripe achenes randomly chosen for the morphological analyses. Samples were always collected at least 10 m apart in order to minimize collection from within the same clone. Voucher specimens are deposited in the Charles University Herbarium (PRC).

AFLP analysis

Total genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek). DNA pellets were dissolved in 40 µl of Elution Buffer D. DNA concentration was then measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and DNA was diluted to 50 ng·µl^{-1}.

AFLP analysis (Vos et al. 1995) was carried out using the AFLP Core Reagent Kit I (Invitrogen) and the AFLP Pre-Amp Primer Mix I (Invitrogen), following the manufacturer's instructions as modified by Záveská et al. (2011) and then further modified to yield the procedure described below. Total genomic DNA of about 50 ng was restricted for 12 h at 37° C with 0.5 U each of *Eco*RI and *Mse*I restriction enzymes (Invitrogen) and 1 µl 5 × reaction buffer (Invitrogen) in a total volume of 5 µl. Adaptors were ligated for 12 h at 37° C by adding 4.8 µl adaptor/ligation solution (Invitrogen) and 0.2 U T4 DNA ligase (Invitrogen) to the digested DNA (total volume 10 µl). Preamplification reactions (total volume 5 µl) contained 0.5 µl of restricted/ligated DNA, 4.0 µl Pre-Amp Primer Mix I, 0.5 µl 10× buffer for RedTaq JumpStart (Sigma) and 0.1 U RedTaq JumpStart DNA polymerase (Sigma). After preamplification, DNA was 10× diluted with water. Four primer combinations (selected after an initial screening of 72 primer combinations) were used for selective amplification: *Eco*RI-ATC-(6-FAM)/*Mse*I-CAA, *Eco*RI-AAG-(VIC)/*Mse*I-CTC, *Eco*RI-AAC-(NED)/*Mse*I-CAG, *Eco*RI-ACA-(PET)/*Mse*I-CAT. Selective amplification was done using 2.3 µl of the diluted preamplification mixture, 1 µl 10×

buffer for RedTaq, 0.2 μM dNTP, 0.5 pmol EcoRI-selective fluorescence-labelled primer, 2.5 pmol Mse I-selective primer and 0.2 U RedTaq JumpStart DNA polymerase (Applied Biosystems) in a total volume of 10 μl. Selective amplification products were mixed with GeneScan LIZ 600 (Applied Biosystems) size standard and electrophoresed on an ABI 3130xl Avant Genetic Analyzer (Applied Biosystems) in the DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague). Altogether, 279 samples from 36 populations were analysed. The whole AFLP procedure was repeated for 10% (29) of the samples and the error rate assessed by comparisons of identical samples (Bonin et al. 2004).

Molecular data analyses

AFLP data were analysed using GeneMarker software v1.8 (SoftGenetics LLC, PA, USA) and transferred into a binary data matrix. Only well-scorable, unambiguous fragments were recorded. Bayesian non-hierarchical clustering was performed in STRUCTURE 2.3.2.1 (Pritchard et al. 2000) to explore the genetic structure of the whole dataset, to define AFLP groups and assess the degree of admixture among species. The admixture model was used and independent allele frequencies were assumed. As AFLPs are dominant markers, a recessive allele model was used. The number of clusters (K) ranged from 1 to 10. For each K, ten runs were done to determine the stability of the results. The length of the burn-in period was set to 100,000 and the MCMC chains after burn-in were run through an additional 1,000,000 replicates (Falush et al. 2007). All computations were done on the freely available Bioportal computer cluster (University of Oslo, http://www.bioportal.uio.no). The R-script (R Development Core Team 2008) Structuresum-2009 (Ehrich et al. 2007) was used to summarize the output files and to calculate similarity coefficients between the replicate runs (Nordborg et al. 2005) and ΔK (Evanno et al. 2005). The optimal number of groups (K) was the one with consistent results over ten repeats, high similarity coefficient and highest ΔK. The software CLUMPP 1.1.1 (Jakobsson & Rosenberg 2007) and Distruct (Rosenberg 2004) were used to create graphical outputs for selected Ks. Samples with low admixture (up to 15%; Rossi et al. 2009, Roulier et al. 2013) were classified as members of one of the K AFLP groups and populations were treated as "pure" when all individuals were clearly assigned to the same group. Such populations were treated as "pure" also for analyses based on inflorescences and measurements of achenes (see below). Highly admixed individuals (more than 15%), i.e. genetically intermediate between the AFLP groups, were passively projected onto ordination diagrams in successive analyses (i.e. PCoA, PCA, CDA; Koutecký 2015; see below). A matrix of pair-wise Jaccard's similarity coefficients (Jaccard 1908) was used for the calculation of the principal coordinate analysis (PCoA) implemented in Canoco 5 (ter Braak & Šmilauer 2012) and for construction of neighbour-network in SPLITSTREE v.4.11.3 (Huson & Bryant 2006). In the graphical outputs of these two analyses, AFLP groups were coloured according to STRUCTURE results, with highly admixed individuals remaining in black or represented by grey symbols. To explore partitioning of genetic variation within and among species and populations, two analyses of molecular variance (AMOVAs, Excoffier et al. 1992; implemented in FAMD 1.3, Schlüter & Harris 2006) were performed: (i) three-level analysis (among species, among populations within species, within populations), (ii) two-level analysis for each species separately (among

populations, within populations). Only populations with all samples classified to AFLP groups were used for AMOVA calculations. Even though our sampling design was not intended to determine clonal structure within populations, we assessed genetic variability of each population by calculating the number of genotypes (Ng), Nei's gene diversity (DNei) and percentage of polymorphic markers (%poly) using the R script AFLPdat (Ehrich 2006).

Morphometric analyses

The structure of inflorescences and the morphology and pericarp anatomy of achenes are taxonomically important characters for determining species of *Bolboschoenus*. The selection of characters for species differentiation was based on the literature (Hroudová et al. 1997, 1998b, 2002, 2007, Ducháček 2002). Morphological and anatomical characters are given in Table 1 and Fig. 1. Morphometric analyses were done in order to evaluate the fit of morphological characters to genetically well-defined AFLP groups and reveal correspondence of taxonomic concept with genetic analysis. In order to obtain suitable sections of achenes for repeated measurements of pericarp anatomy, particular achenes were destroyed so the same achenes could not be used for further measurements. Therefore, it was necessary to analyse the four data sets separately. The first data set contained information on seven morphological characters (e.g. numbers and lengths of peduncles and spikelets) of 855 inflorescences. The second data set consisted of information on two morphological characters (achene length and width) of 875 achenes. The third data set included data on two morphological characters (achene width and thickness in transverse section) of 875 achenes. The fourth data set included information on six pericarp anatomical characters of 875 achenes. Morphological characters of inflorescences were measured using a digital calliper (first data set). Width and length of achenes were measured on whole achenes using a dissecting microscope with a digital camera to record images (second data set). Subsequently, width and thickness were obtained by cutting achenes with a razor in the widest part (third data set). Finally, we prepared the achenes so that anatomical characters of the pericarp (fourth data set, width of exo-, meso- and endocarp in the narrowest and in the widest parts) could be recorded. Achenes of *Bolboschoenus* are hard to cut; therefore, we had to cut off the base of achenes and put them in water for one week to soften them. Anatomical and morphological characters remained unchanged (Hroudová et al. 1997). Cross sections of achenes 20 μm thick were obtained using Shadon Cryotome® microtome (77200226, Shandon Sci., Astmoor Runcorn, UK). Olympus BX50 microscopes with digital camera Olympus E 10 were used to record images. We measured all the achene characters using ImageJ software (National Institute of Health, USA).

The first data set of inflorescence characters and fourth data set of anatomical characters of achenes we subjected to multivariate morphometric analyses. These data sets were tested using the Shapiro-Wilk statistic for normality and non-parametric Spearman correlation coefficients were computed to investigate the correlations between the characters. Characters that were highly correlated with one another (exceeding 0.95 – length of peduncles / length of spikelets) were excluded from further analyses. Principal component analysis (PCA) with both individual plants and population means as operational units was done using Canoco 5 (ter Braak & Šmilauer 2012) for initial insight into the

Table 1. – List of morphological characters used in the study. * Highly correlated character that was excluded from the total analyses.

Inflorescence characters	Achene characters
Number of peduncles	Length/width of achenes
Number of spikelets	Width/thickness of achenes
Length of peduncles	Anatomical characters of pericarp
Length of spikelets	Width of exocarp at the widest part
Number of spikelets/ number of peduncles	Width of mesocarp at the widest part
Length of peduncles/ number of spikelets	Width of endocarp at the widest part
*Length of peduncles / length of spikelets	Width of exocarp at the narrowest part
	Width of mesocarp at the narrowest part
	Width of endocarp at the narrowest part

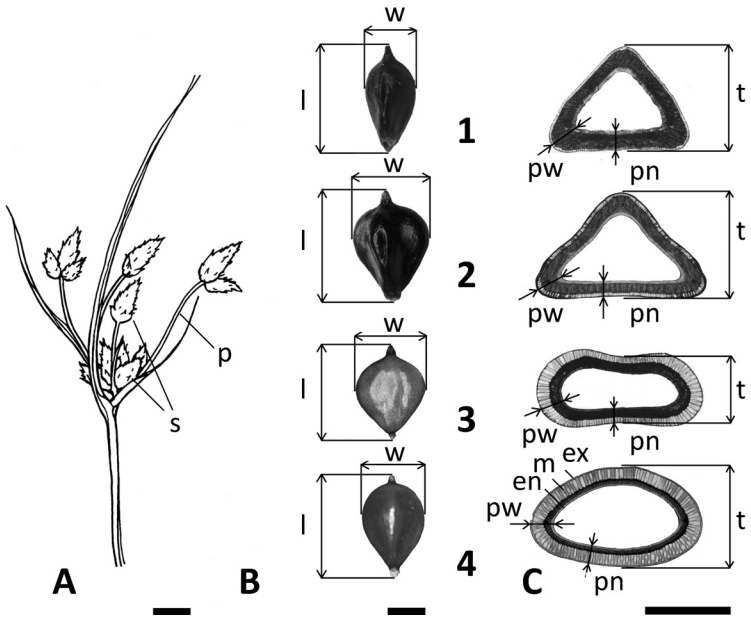

Fig. 1. – Morphological and anatomical characters: (A) inflorescence, (B) achenes, (C) achene anatomical characters in cross sections. 1, *Bolboschoenus yagara*; 2, *B. laticarpus*; 3, *B. planiculmis*; 4, *B. maritimus*. Scale bars: 1 cm (A), 1 mm (B, C). p, peduncle; s, spikelet. l, length of achene; w, width of achene. t, thickness of achene; pw, width of pericarp layers at the widest part; pn, width of pericarp layers at the narrowest part; ex, exocarp; m, mesocarp; en, endocarp. (A) Del. Z. Hroudová. (B), (C) Photo S. Píšová.

overall morphological variation. Canonical discriminant analysis was used to determine variation among predetermined AFLP groups (species) and the most important characters for their differentiation using R-script Morphotools (Koutecký 2015). In consequence, highly admixed individuals were passively projected. Classificatory discriminant analyses in R-script Morphotools were used to compute the percentage of genetically "pure" (i.e. admixed up to 15%) individuals correctly assigned to predefined AFLP groups. These individuals subsequently served as the training data set for determination of populations with higher admixtures. A one-way ANOVA procedure, Kruskal-Wallis test

(Kruskal & Wallis 1952), Tukey HSD multiple comparison test and graphic representation by box plots were used to determine statistical differences among AFLP groups in the second (length/width ratio) and third data sets (width/thickness ratio) in R package multcomp (Hothorn et al. 2008). Admixed individuals were also additionally displayed for demonstration.

Results

AFLP analysis

The four selective primer combinations generated 122 AFLP markers (all polymorphic), ranging in size from 100–500 bp. The technical error rate based on repeated AFLP analysis of 29 samples was 4.47%. STRUCTURE analysis yielded highly consistent results (i.e. similarity coefficient for ten runs) for K = 2–4; higher K values did not converge towards the same outcomes. Results for K = 2 discriminated two morphological groups generally corresponding to *B. yagara* and *B. planiculmis*. The species *B. maritimus* was not distinguished from *B. planiculmis*. Samples determined as *B. laticarpus* were approximately a 50:50 admixture of both groups (Fig. 2A). K = 3 discriminated AFLP groups corresponding to species: *B. yagara*, *B. planiculmis* and *B. maritimus*. The putative hybrid *B. laticarpus* was an even admixture of *B. yagara* and *B. planiculmis*. Result with highest ΔK, K = 4 discriminated all four species: *B. yagara* (81 samples), *B. laticarpus* (57 samples), *B. planiculmis* (78 samples) and *B. maritimus* (32 samples). Most of the individuals of *B. laticarpus* were classified in an independent group (species). Nevertheless, some individuals were not differentiated as putative hybrids of *B. yagara* and *B. planiculmis*. In all analyses, the majority of the samples were clearly (i.e. with an up to 0.15 assignment probability to another group; ad hoc setting for dealing with admixtures) placed in one of the four AFLP groups. However, some of the individuals were highly admixed (i.e. with at least a 0.15 assignment probability) and assumed to be hybrids or results of introgression between AFLP groups. For the better differentiation of these admixed samples they were split into three groups in the graphs (Fig. 2A: group A – 10 samples between *B. yagara* and *B. laticarpus*, group B – seven samples between *B. laticarpus* and *B. planiculmis* and group C – 14 samples between *B. planiculmis* and *B. maritimus*). Moreover, two samples from Dolní Věstonice were an admixture of more than two groups (putative hybrid individuals of *B. yagara* × *B. planiculmis* with admixture of *B. maritimus*).

Combination of the first two PCoA axes (explaining 18.1% and 4.9% of the variability; Fig. 2B) and a neighbour-net diagram (Fig. 2C) largely confirmed the STRUCTURE results. In both analyses, *B. laticarpus* was in an intermediate position between the presumed parental species (*B. yagara* and *B. planiculmis*). The majority of admixed individuals were also placed between the AFLP groups.

The AMOVA for separate AFLP groups indicate that AFLP groups (species) are well differentiated, as 49.6% of the total variation was attributed to the differences among groups (Table 2). About 18.5% of the variation was among populations within species and the rest (31.9%) to differences among individuals within populations.

The estimates of number of genotypes, Nei's gene diversity and percentage of polymorphic markers for each population are shown in Appendix 1. Maximum genetic diversity

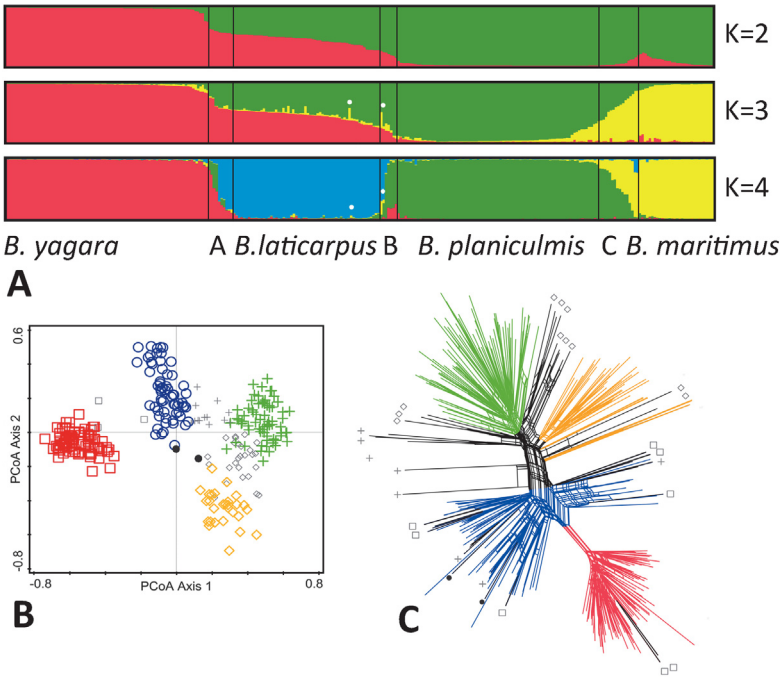

Fig. 2. – (A) Bar plot showing Bayesian assignment probabilities using software STRUCTURE for two, three and four clusters (K = 2–4) based on 122 AFLP loci and 279 individuals of *Bolboschoenus*. Four genetic groups associated with the species were recorded plus intermediate individuals (groups A, B, C; white dots – two samples of *B. laticarpus* with admixture of *B. maritimus*) between them. (B) Principal coordinate analysis (PCoA) using Jaccard's similarity coefficient. The first two axes explain 18.1% and 4.9% of the variation. Colours indicate AFLP groups detected by STRUCTURE and passively projected admixed individuals (red square, *B. yagara*; blue circle, *B. laticarpus*; green cross, *B. planiculmis*; yellow diamond, *B. maritimus*; grey square, group A; grey cross, group B; grey diamond, group C; black dots – two samples of *B. laticarpus* with admixture of *B. maritimus*). (C) Neighbour net diagram of 279 individuals of *Bolboschoenus*. Colours indicate AFLP groups detected by STRUCTURE (red lines, *B. yagara*; blue lines, *B. laticarpus*; green lines, *B. planiculmis*; yellow lines, *B. maritimus*; black lines, admixed samples: grey square, group A; grey cross, group B; grey diamond, group C; black dots – two samples of *B. laticarpus* with admixture of *B. maritimus*).

Table 2. – Analysis of molecular variance (AMOVA) of AFLP data (N = 247 individuals).

Grouping	Source of variation	d.f.	Sum of squares	Variance components	% of total variance
4 species	Among species	3	12.98	0.06	49.63
	Among populations/ within groups	32	6.87	0.02	18.46
	Within populations	227	9.51	0.04	31.91
B. yagara	Among populations	9	0.76	0.01	15.64
	Within populations	73	2.43	0.03	84.35
B. laticarpus	Among populations	8	3.49	0.06	65.35
	Within populations	61	1.87	0.03	34.64
B. planiculmis	Among populations	10	2.45	0.03	35.25
	Within populations	71	3.52	0.05	64.75
B. maritimus	Among populations	5	1.14	0.03	32.65
	Within populations	26	1.70	0.07	67.35

was recorded for the *B. maritimus* populations HU (DNei = 0.29, %poly = 68.03%) and B (DNei = 0.23, %poly = 63.93%). Minimum genetic diversity was recorded for populations NE (DNei = 0.11, %poly = 17.21%) of *B. laticarpus*, and TO (DNei = 0.13, %poly = 19.67%) of *B. yagara*. No species-specific markers were found. The populations sampled were highly diverse and most plants had different genotypes. Investigation of clonal reproduction, however, was not the main goal of this study and further research is needed.

Morphometric analyses

A principal component analysis (PCA) based on mean values of inflorescence characters (first data set, 855 individuals × 6 characters) revealed four overlapping groups partially separated along the first axis (number and length of peduncles, Fig. 3A). Individuals of the putative hybrid taxon *B. laticarpus* were placed between its supposed parental taxa, *B. yagara* and *B. planiculmis*. Additionally, passively projected admixed individuals were scattered within and between these groups. PCA eigenvectors are given in Table 3. PCA based on anatomical characters of achenes (fourth data set, 875 individuals × 6 characters) revealed four well-separated AFLP groups along the first axis. This differentiation is attributable to exocarp and mesocarp widths at narrowest part. The *B. laticarpus* was also placed between its parental taxa and the admixed individuals were scattered within and between these groups (Fig. 3C, Table 4). The samples from AFLP group C were the most variable.

The CDA, based on the morphological characters of inflorescences, was done using four predefined AFLP groups and passively projected admixed individuals. It also revealed partial overlaps between groups (first data set, Fig. 3B). The individuals of *B. laticarpus* and *B. planiculmis* were slightly separated along the first canonical axis and those of *B. yagara* and *B. maritimus* along the second axis. Moreover, the individuals of the hybrid taxon *B. laticarpus* were placed between their parental taxa as in the PCA. The third axis (not shown) explained 4.6% of the variability and did not provide a differentiation that differed from that provided by the first and second axes. The characters most highly correlated with the canonical axes were: number of peduncles, number of peduncled spikelets, number of sessile spikelets/number of peduncled spikelets, length of sessile spikelets/length of peduncles of peduncled spikelets, length of peduncled spikelets and number of peduncled spikelets/number of peduncles of peduncled spikelets (Table 3). The CDA based on the anatomical characters of achenes (fourth data set) separated individual AFLP groups (species) even better (with less overlap). Admixed individuals were situated within and between them (Fig. 3D). The third axis (not shown) explained only 2.8% of the variability and did not provide a differentiation that differed from that provided by the first two axes. The most important characters for differentiation of separate taxa were exocarp and mesocarp widths (Table 4).

The classificatory DA based on morphological characters of inflorescences (first data set) correctly assigned 77.4% of the individuals to predefined AFLP groups (80.3% of *B. yagara*, 62.7% *B. laticarpus*, 75.4% *B. planiculmis*, 85.0% *B. maritimus*). The classificatory DA based on anatomical characters of achenes (fourth data set) was more successful and correctly assigned 96.4% individuals (98.2% of *B. yagara*, 98.7% *B. laticarpus*, 88.8% *B. planiculmis*, 100% *B. maritimus*). The rest of the samples (3.6%) were misclassified in other groups. A part of population NZ was misclassified in the *B. maritimus* group. Populations with a high admixture were analysed for species determination with

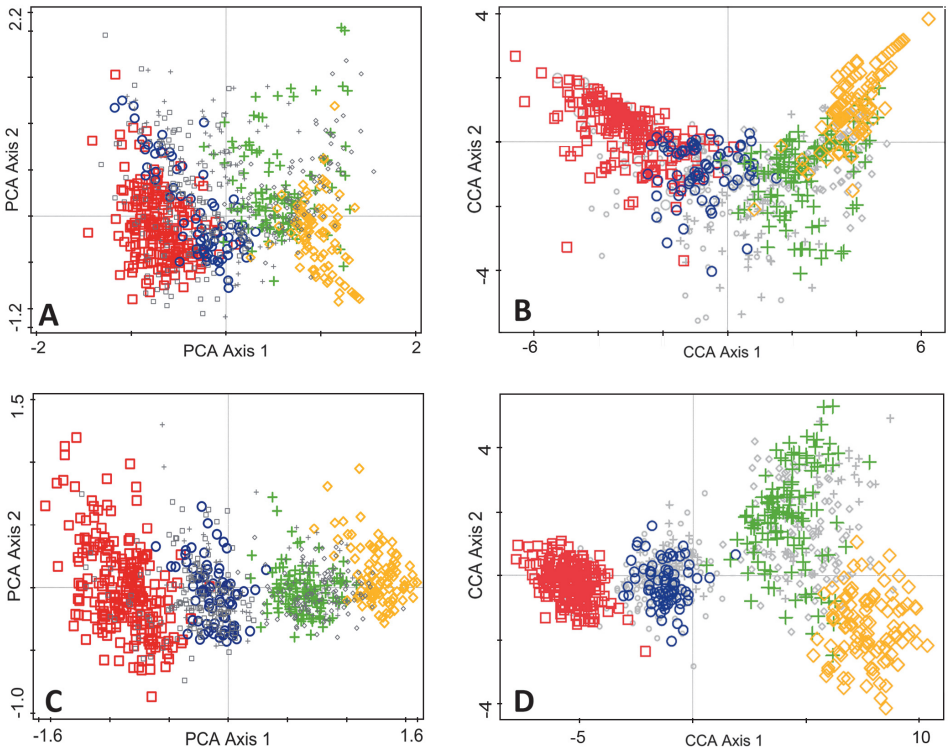

Fig. 3. – (A) Principal component analysis (PCA) based on six morphological characters of the inflorescences of 855 individuals of *Bolboschoenus*. The first two axes explain 55.1% and 22.7% of the variation. (B) Canonical discriminant analyses (CDA) of 855 individuals of *Bolboschoenus* based on six morphological characters of inflorescences. The first two components explain 29.8% and 12.6% of the variation. (C) Principal component analysis (PCA) based on six anatomical characters of 875 achenes. The first two axes explain 78.4% and 8.3% of the variation. (D) Canonical discriminant analyses (CDA) of 875 individuals based on six anatomical characters on achenes. The first two components explain 32.1% and 19.1% of the variation. Colours indicate AFLP groups detected by STRUCTURE and passively projected admixed individuals (red square, *B. yagara*; blue circle, *B. laticarpus*; green cross, *B. planiculmis*; yellow diamond, *B. maritimus*; grey square, group A; grey cross, group B; grey diamond, group C).

four predefined AFLP groups as a training set. Population KR (group A) was classified in the *B. yagara* group, while populations BO, DP, LI, LD-L, SK4 and AU2, DV_L (group B) were classified in the *B. laticarpus* group. In addition, populations DI, JK and DJ-PL, TD (group C) were classified in the *B. planiculmis* group. Samples of intermediate populations SK-KU and B were partially classified in the *B. planiculmis* and *B. maritimus* groups. (Appendix 1, CDA species).

The variation in the length/width ratio (second data set, Fig. 4A) is significantly different among species (Kruskal-Wallis test, $H = 410.18$, $df = 3$, $P < 0.0001$) as well as differences in width/thickness ratio (third data set, Fig. 4B, Kruskal-Wallis test, $H = 440.19$, $df = 3$, $P < 0.0001$). The four AFLP groups were well separated based on Tukey HSD multiple comparison test ($P < 0.001$). Admixed individuals (groups A, B and C) are also displayed between AFLP groups for comparison.

Table 3. – Results of morphometric analyses based on characters of inflorescences. The three highest PCA eigenvector and total canonical structure values are presented in bold.

Character	PCA 1	PCA 2	PCA 3	CDA 1	CDA 2	CDA 3
Number of peduncles	**–0.945**	0.004	0.084	**0.689**	0.193	–0.074
Number of spikelets	–0.706	0.626	0.241	0.237	–0.326	0.035
Length of peduncles	**–0.955**	0.052	0.210	**0.588**	–0.119	0.226
Length of spikelets	0.481	–0.278	0.830	–0.169	0.325	0.022
Number of spikelets / number of peduncles	0.369	**0.858**	0.066	–0.176	**–0.570**	–0.566
Length of peduncles / number of spikelets	–0,797	–0.392	–0.033	0.481	–0.172	0.228

Table 4. – Results of morphometric analyses based on anatomical characters of achenes. The three highest PCA eigenvector and total canonical structure values are presented in bold.

Character	PCA 1	PCA 2	PCA 3	CDA 1	CDA 2	CDA 3
Width of exocarp at the widest part	**0.921**	0.119	–0.084	**0.650**	0.606	0.428
Width of mesocarp at the widest part	**–0.910**	–0.165	0.055	–0.358	–0.020	**0.784**
Width of endocarp at the widest part	–0.766	0.620	0.165	–0.181	–0.045	0.297
Width of exocarp at the narrowest part	0.907	0.208	–0.084	0.512	**–0.603**	0.423
Width of mesocarp at the narrowest part	**–0.932**	–0.130	0.099	–0.432	0.277	0.240
Width of endocarp at the narrowest part	–0.866	0.109	–0.488	–0.261	0.037	0.355

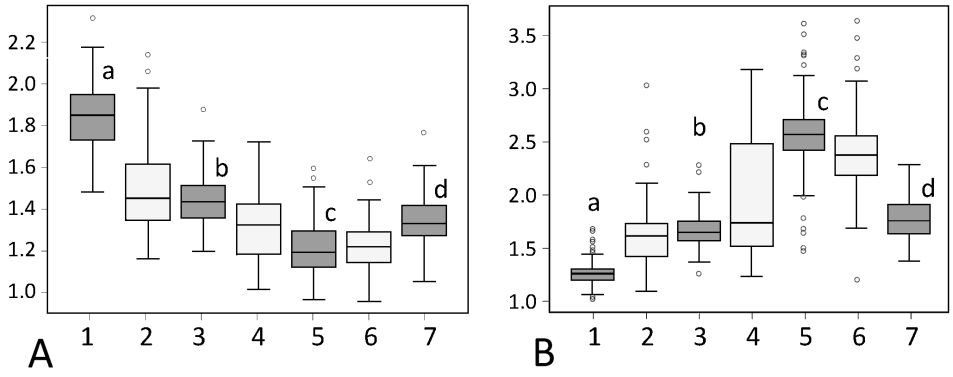

Fig. 4. – (A) Box plot showing the distribution of the ratio length to width of achenes (N = 875). (B) Box plot showing the distribution of the ratio width to thickness of achenes (n = 875). Different letters above boxes indicate statistical differences between four AFLP groups based on a Tukey HSD multiple comparison test ($P < 0.001$). The admixed groups are also displayed. 1, *B. yagara*; 2, group A; 3, *B. laticarpus*; 4, group B; 5, *B. planiculmis*; 6, group C; 7, *B. maritimus*.

Discussion

In this study, we focused on speciation in central-European species of *Bolboschoenus*. Molecular variation and morphological characters were compared and hybrid status of *B. laticarpus* assessed. Although we did not search for morphologically intermediate individuals the specimens of the species studied include both individuals that were morphologically typical and intermediate between species. Intermediate individuals were recorded especially between *B. planiculmis* and *B. maritimus*, and impossible to determine unambiguously using a classificatory function. The STRUCTURE analysis of AFLP data revealed genetic groups that mostly corresponded to morphologically defined species. Similar to other studies (Rossi et al. 2009, Roullier et al. 2013) individuals with posterior assignment probabilities above 0.85 (i.e. with less than 15% admixture) were assigned to a specific AFLP group (corresponding to a particular species), while individuals with higher assignment probabilities were considered to be admixed (i.e. hybrids). We found four AFLP genetic groups corresponding to morphological and anatomical differences in the achenes. The current classification of European species of *Bolboschoenus* was thereby confirmed, and in accordance with previous studies we accept the traditional morphological concept (Marhold et al. 2004, Hroudová et al. 2005, 2006, 2007).

Important morphological and anatomical characters for species determination

The first genetic group, with the least genetic variation corresponded to the species *B. yagara* (= *B. maritimus* subsp. *maritimus* with narrow fruits – Hroudová et al. 1998b or *B. fluviatilis* subsp. *yagara* – Browning et al. 1997a, Hayasaka & Ohashi 2002). The inflorescence characters partially differentiating this species are the higher number of peduncles, higher number of peduncled spikelets and lower ratio of sessile/peduncled spikelets. Ducháček (2002) extensively investigated the morphological variation of inflorescence characters and mentions the same characters for species differentiation. However, he also reports partial overlaps among species. We found that the shape of achenes (recorded here as the length/width and width/thickness ratios) was more important than inflorescence characters for determining species. For example, achenes of *B. yagara* were narrow, elongated and triangular in transverse section. Not even the shape of achenes was sufficient for species differentiation. The most reliable distinguishing characters proved to be anatomical characters: very thin exocarp and thick mesocarp. This study confirmed their importance, first emphasized by Browning & Gordon-Gray (1993), and subsequently used in determination keys by Hroudová (2002) and Hroudová et al. (2007).

The second genetic group included individuals of the putative hybrid *B. laticarpus* noted as *B. maritimus* subsp. *maritimus* with wide fruits (Hroudová et al. 1998b), *B. maritimus* × *B. yagara* (Browning et al. 1996) and *B. yagara* × *B. koshewnikowii* (Hroudová 2002). Inflorescence characters were intermediate between those of *B. yagara* and *B. planiculmis*, as in Ducháček (2002) and Marhold et al. (2004). *Bolboschoenus laticarpus* differed from *B. yagara* mainly in having a lower number of branches and peduncled spikelets. Achenes were somewhat wider than those of *B. yagara*, elongated and trigonous with a somewhat thicker exocarp (see below for more details about hybrid origin of *B. laticarpus*).

The third genetic group included the most variable species *B. planiculmis* (*B. koshewnikowii* in Kozhevnikov 1988 or *B. maritimus* subsp. *compactus* in Hroudová et al. 1998b). Inflorescences consisted of sessile spikelets, sometimes accompanied by a few peduncled spikelets. This species had very wide and short achenes, concave to flat dorsally. The most reliable character was exocarp approximately as thick as the mesocarp. Ducháček (2002) considers *B. planiculmis* to be a readily distinguishable species. Nevertheless, he, like Hroudová et al. (2006), mentions individuals intermediate between *B. planiculmis* and *B. maritimus* for which determination is impossible.

The fourth group included populations of *B. maritimus* from coastal and inland saline habitats (*B. maritimus* s. str.) with head-like inflorescence consisting of sessile spikelets and a few peduncled spikelets. This species was distinguished by having a thicker exocarp than mesocarp. Achenes were wide, short and convex dorsally. However, achenes may also sometimes be lenticular like those of *B. planiculmis* (Ducháček 2002, Hroudová et al. 2007).

In summary, morphological characters of inflorescences appear to be less reliable than the morphological and anatomical characters of achenes. In particular, we consider exocarp and mesocarp widths and achene shape in transverse section to be the most important characters for species determination.

Hybrid origin of Bolboschoenus laticarpus

Hybridization influences plant evolution, inducing diversification and speciation and affecting genetic variation (Lihová et al. 2007). The frequency of spontaneous hybridization is higher in certain families and genera such as *Bolboschoenus* (Ellstrand et al. 1996). *Bolboschoenus laticarpus*, which is assumed to be of hybrid origin because of its intermediate morphology (Browning et al. 1996, Hroudová 2002, Marhold et al. 2004) is in the second genetic group. All samples of *B. laticarpus* were intermediate between *B. yagara* and *B. planiculmis*, with genetic information from both parental taxa (almost in the ratio 50:50 for division K = 3, Fig. 2A). Backcrossing with the parental species is rare and was recorded only for *B. planiculmis* in population SK4 and SK_Ma. Although *B. laticarpus* is closer to *B. yagara* in inflorescence structure (compound inflorescence with sessile and peduncled spikelets) and in fruit shape and anatomy (trigonous fruits with thicker mesocarp than exocarp), an overall comparison of its morphological and anatomical characters indicates it occupies an intermediate position between *B. yagara* and *B. planiculmis*.

The chromosome number of *B. yagara* is n = 55, *B. planiculmis* n = 54 (only exceptionally n = 55), while in *B. laticarpus* both n = 55 and n = 54 are recorded (Jarolímová & Hroudová 1998). This is in agreement with the parentage of *B. laticarpus* determined in this study, because it is unlikely that *B. maritimus* with n = 55 could be the second parent.

The STRUCTURE result for division K = 4 indicate that *B. laticarpus* is a stable hybridogenous species. It differs from other European species of *Bolboschoenus* by its wide ecological amplitude and in inhabiting a broad range of habitats (Marhold et al. 2004), which is reflected in its role in plant communities. This species appears to occur very frequently in central Europe, spreading along rivers, (e.g. association *Phalarido-Bolboschoenetum laticarpi* Passarge 1999 corr. Krumbiegel 2006; Hroudová et al. 1999, 2009) in river floodplains and also as a weed in arable land (Hroudová et al. 2007). Such

success contrasts with that of many interspecific hybrids that are less successful than their parental taxa (Yakimowski & Rieseberg 2014). Indeed, interspecific hybridization sometimes even leads to sterility, which is not recorded for *B. laticarpus* (Moravcová et al. 2002). However, in other cases gene combinations can give rise to hybrids that are fitter than their parental taxa, able to inhabit unoccupied niches, and with the origins of some hybrid species connected with habitat disturbance in man-influenced landscapes (Schemske 2000). In the case of *B. laticarpus*, hybridization led to an increase in fitness, which enabled it to inhabit a wide range of habitats including secondary ones (e.g. arable land) (Hroudová et al. 2014). The life-history characteristics of *B. laticarpus* correspond to the adaptive traits associated with the formation of new species during evolution (Ellstrand et al. 1996): (i) outcrossing, (ii) developmental and ecological flexibility, (iii) perennial habit and vegetative reproduction.

The question regarding the centre of origin of *B. laticarpus* remains. Its current distribution is mainly in central Europe, along some rivers and even reaches the sea coast (Hroudová et al. 2007). This indicates its possible origin in central Europe, where the areas of distribution of *B. yagara* and *B. planiculmis* also overlap and mixed populations occur (e.g. AU2). However, in contrast to *B. laticarpus*, the continuous distributions of both its parental taxa extend eastwards through Russia to the Far East (Egorova & Tatanov 2003, Tatanov 2003, Hroudová et al. 2007), and plants very similar morphologically to European *B. laticarpus* occur in Japan, under the name *Bolboschoenus fluviatilis* subsp. *yagara*, type B (Hayasaka & Ohashi 2002), in Kazakhstan and East Asia (Tatanov 2007). This suggests a possible polytopic origin of this hybrid species.

Recent hybridization among species

The percentage of species that hybridize varies among families, but around 25% of plant species hybridize with at least one other species (Mallet 2007). Hybridization undoubtedly is an important process in *Bolboschoenus* evolution and a means of adapting to habitat conditions. Hybridization occurred not only in the past [leading to the establishment of the stable hybridogenous *B. laticarpus* with a different ecological niche, area of distribution (Hroudová et al. 2007) and special biological traits (Hroudová et al. 2014); see above] but also recently. Distributions of central-European species of *Bolboschoenus* overlap, which provides the opportunity for hybridization. We recorded 31 individuals (11%) that were placed between AFLP groups, reflecting a genetic admixture, presumably a result of spontaneous hybridization. These genetically intermediate groups (A, B, C) differ in their representation. The least numerous is the group between *B. laticarpus* and *B. planiculmis* (2%). Slightly more numerous is the admixture group between *B. yagara* and *B. laticarpus* (4%), and it is not excluded that some backcrossing between them has occurred. Finally, we recorded many admixtures (5%) intermediate between *B. planiculmis* and *B. maritimus*; in some cases whole populations consisted of admixtures. However, such admixed populations (e.g. population B) were morphologically clearly identified as *B. maritimus* (Ducháček 2002).

The comparison of populations of *B. maritimus* from highly saline areas (e.g. sea coasts) and localities with lower salinity (some inland saline marshes) revealed genetical differences. Samples from habitats with a high salinity were genetically pure, even though the two localities were distantly remote from one another (France, Iran). On the

other hand, some inland samples (from areas where the distribution of *B. maritimus* and *B. planiculmis* overlapped) contained admixtures of *B. planiculmis* genotypes. *Bolboschoenus planiculmis* and *B. maritimus* occur most often together in inland freshwaters or slightly saline habitats (e.g. in southern Moravia, southern Slovakia, Lower Austria: Ducháček 2002, Hroudová et al. 2007, 2014), and intermediate morphotypes are frequently found there. We suppose that these intermediate morphotypes are spontaneous hybrids that cannot arise in areas where only *B. maritimus* occurs in habitats with high salinity (sea coast, inland salt lakes). Nevertheless, introgression or backcrossing can only be properly studied using variable codominant microsatellite markers (e.g. Snow et al. 2010).

The finding that the genotypes of some of the plants morphologically similar to *B. maritimus*, had admixtures of *B. planiculmis* genotypes (e.g. in populations B and HU), may account for some irregularities and changes in plant occurrence. The admixture of *B. planiculmis* genotype may influence their biological traits, e.g. survival in a wider range of habitats (not saline or slightly saline). This might account for the occurrence of *B. maritimus* in some unusual habitats, small fishponds, sand pits or depressions in fields.

On the other hand, hybridization between *B. yagara* and *B. laticarpus* is probably less frequent. Although both species are usually fully fertile and sometimes occur together their seedlings rarely become established. Their seeds germinate and seedlings establish only on the water-saturated exposed bottoms of fishponds (Hroudová et al. 1996). We suppose that their limited generative reproduction due to unstable habitat conditions results in prevalence of clonal growth and also in a limited occurrence of recent spontaneous hybrids of these species. Moreover, two individuals appeared to be an admixture of three genetic groups. These plants originated from mixed populations of *B. laticarpus* and *B. planiculmis* (population DV), and thus we interpret their occurrence as resulting from backcrossing between *B. laticarpus* and *B. planiculmis*, with an admixture of *B. maritimus*.

Although the aim of this study was not to determine the clonal structure of populations and our sampling design did not allow us to properly address clonal structure, we found some indications that species might differ in their degree of clonality. Populations were highly diverse and most individuals belonged to separate genotypes. Clones occurred occasionally in populations of *B. laticarpus* and *B. planiculmis*, but in only one population of *B. yagara*.

Conclusions and perspectives for future studies

The present study confirmed the most recent taxonomic classification of European species of *Bolboschoenus*. Based on AFLP data, four genetic groups corresponding to the species were found. Correlations of important determination characters with genetic groups were detected using canonical discriminant analysis, which confirmed the importance of morphological and anatomical characters of achenes. We consider exocarp and mesocarp widths and achene shape in transverse section to be the most reliable characters for species determination. Moreover, admixed individuals were recorded between genetic groups, indicating possible spontaneous hybridization, mostly between *B. planiculmis* and *B. maritimus*. Populations of *B. maritimus* from inland areas contained

admixtures of *B. planiculmis* genotypes, while populations of *B. maritimus* from coastal areas and habitats with a high salinity were genetically pure. Nevertheless, for a further elucidation of this pattern of variation a more detailed study of *B. maritimus* throughout its distribution is needed.

Hybrid origin of *B. laticarpus* was confirmed, with *B. yagara* and *B. planiculmis* as its parental taxa. It is genetically and morphologically intermediate and is a stable hybrid. Further study should investigate the possibility of polytopic origins of *B. laticarpus* in Japan or China and further verifying its supposed hybrid parentage.

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Souhrn

Rod *Bolboschoenus* (kamyšník, *Cyperaceae*) představuje vhodnou modelovou skupinu ke studiu ekologické a homoploidní hybridní speciace. Ve střední Evropě se přirozeně vyskytují čtyři druhy tohoto rodu: *B. maritimus* (k. přímořský), *B. laticarpus* (k. širokoplodý), *B. planiculmis* (k. polní) a *B. yagara* (k. vrcholičnatý). V rámci skupiny těchto čtyř taxonů, dříve označované jako široce pojatý druh *B. maritimus*, můžeme pozorovat odlišné ekologické nároky. Právě taková ekologická či zeměpisná izolace je při homoploidní hybridní speciaci rozhodující. Současné taxonomické členění rodu *Bolboschoenus* je založeno na morfologii květenství a tvaru a anatomii nažek. Pro jeho ověření jsme porovnali morfologická data se čtyřmi genetickými skupinami předdefinovanými na základě dat získaných pomocí molekulárního markeru AFLP (Amplified Fragment Length Polymorphism). Navíc nám tato metoda umožnila prozkoumat i předpokládaný hybridogenní původ druhu *B. laticarpus*, který je založený na jeho přechodných morfologických znacích, počtu chromozomů i široké ekologické amplitudě. Výsledky potvrdily současnou klasifikaci středoevropských kamyšníků – morfologická diferenciace z velké části odpovídá genetickým skupinám. Potvrzen byl rovněž hybridní původ druhu *B. laticarpus* jako výsledek křížení mezi druhy *B. yagara* a *B. planiculmis*. V současné době se ovšem v přírodních populacích vyskytuje i určitý podíl rostlin s heterogenním genotypem, představujících jedince vzniklé spontánní hybridizací (zejména mezi *B. maritimus* a *B. planiculmis* v oblastech, kde jsou rozšířeny oba druhy).

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Appendix 1. – List of the populations of the species of *Bolboschoenus* studied and their characteristics. AFLP group – determination of each population based on AFLP data (STRUCTURE 85%): Y – *B. yagara*, A – *B. yagara* × *B. laticarpus*, L – *B. laticarpus*, B – *B. laticarpus* × *B. planiculmis*, P – *B. planiculmis*, C – *B. planiculmis* × *B. maritimus*, M – *B. maritimus*. Nind – number of individuals analysed for AFLPs; Ng – number of genotypes; DNei – Nei's gene diversity; %poly – percentage of AFLP markers demonstrating intra-population polymorphism. N – number of inflorescences used in the morphometric analysis. CDA – species determination of each population based on classificatory discriminant analyses of anatomical characters of pericarp (Y – *B. yagara*, L – *B. laticarpus*, P – *B. planiculmis*, M – *B. maritimus*.

		AFLP group												CDA
Acronym	Locality and date of collection	Y	A	L	B	P	C	M	N_{ind}	N_g	D_{Nei}	%poly	N	species
Bolboschoenus yagara														
DZA	CZ-SB, the fishpond Zadní 1.5 km S of the village Domanín, 5 km SW of the town of Třeboň, 48°57.583'N, 14°44.983'E, 450 m, 26.7.2011	7							7	7	0.16	36.06	25	Y
HR	CZ-SB, the fishpond Hrachovišťský at western border of the village Hrachoviště, 8 km S of the town of Třeboň, 48°55.733'N, 14°45.85'E, 460 m, 20.9.2010	8							8	8	0.12	30.33	25	Y
KO	CZ-SB, north-eastern shore of the fishpond Koclířov, ca 1 km SW of the town Lomnice n. Lužnicí, 49°4.633'N, 14°42.083'E, 425 m, 16.6.2011	10							10	10	0.14	42.62	25	Y
KR	CZ-SB, the fishpond Králek 3.2 km ESE of the town Kardašova Řečice, 49°10.583'N, 14°53.767'E, 470 m, 28.7.2011	6	2						8	8	0.17	44.26	25	Y
OB	CZ-SB, the fishpond Oběšený near south-western border of the village Mláka, 8 km NE of the town of Třeboň, 49°3.4'N, 14°50.35'E, 445 m, 29.7.2010	9							10	10	0.18	49.18	11	Y
OS	CZ-SB, the fishpond Ostrý ~600 m S of the village Kolence, 5 km E of the town Lomnice n. Lužnicí; 49°5.067'N, 14°47.083'E, 420 m, 29.7.2010	10							10	9	0.11	30.33	17	Y
SH	CZ-SB, the Stehlík fishpond 500 m SE of the village Klec, 2.5 km NE of the town Lomnice n. Lužnicí, 49°5.55'N, 14°45.183'E, 420 m, 27.7.2011	9							9	9	0.16	45.08	25	Y
TI	CZ-SB, the fishpond Velký Tisý, littoral in the bay at western shore of the Lůsy penninsula, 3.3 km S of the town Lomnice n. Lužnicí, 49°4.1'N, 14°42.4'E, 420 m, 25.7.2011	9							9	9	0.13	36.06	25	Y
TO	CZ-SB, the fishpond Tobolky near the village Branná, 4 km S of the town Třeboň, 48°57.65'N, 14°46.333'E, 440 m, 28.7.2010	3							3	3	0.13	19.67	25	Y
VO	CZ-SB,the fishpond Velká Ochoz, ~2.2 km S of the town Kardašova Řečice, 49°10.167'N, 14°50.467'E, 430 m, 20.9.2010	10							10	10	0.14	33.61	25	Y

Acronym	Locality and date of collection	AFLP group							N_{ind}	N_g	D_{Nei}	%poly	N	CDA species
		Y	A	L	B	P	C	M						
Bolboschoenus laticarpus														
AU2	A, Lower Austria, the fishpond shore near bridge over railway line at eastern border of the town Bernhardsthal, 48°41.567'N, 16°52.633'E, 160 m, 4.8.2011		1	5	2				8	8	0.21	55.74	25	L
BO	CZ-NM, field depression near north-western border of the village Bohuslavice, ~6 km N of the town Mohelnice, 49°49.667'N, 16°56.167'E, 260 m, 5.8.2011		1	7					9	7	0.17	42.62	25	L
DJ-L	CZ-EB, depression in field near north-eastern border of the village Dolní Jelení, 11 km NNW of the town Vysoké Mýto, 50°3.133'N, 16°6.583'E, 280 m, 5.8.2011			5					6	6	0.16	38.52	25	L
DP	CZ-CB, Prague city, flooded depression in field at north-eastern border of the Dolní Počernice suburb, near the road to Svépravice, 50°5.45'N, 14°35.25'E, 230 m, 21.7.2010		1	3					4	3	0.14	20.49	25	L
DV	CZ-SM, wet depression in field 0.5 km W of the village Dolní Věstonice, 48°53.183'N, 16°37.817'E, 170 m, 4.8.2011			9	1				10	9	0.18	45.90	25	L
LD-L	CZ-SM, wet depression in field near the highway to Slovakia, 1.6 km NE of the town Lanžhot, 48°43.9'N, 16°58.817'E, 150 m, 1.9.2010		1						2	2	0.29	28.69	25	L
LI	CZ-CB, Prague city, wet depression in field near the fishponds in protected area „Litožnice“ ~1.5 km SSE of the railway station Běchovice, 50°4.2'N, 14°36.417'E, 230 m, 21.7.2010		3	6					9	8	0.14	36.88	25	L
NE	CZ-CB, Prague city, wet depression in field near Netluky farm-house, near the road Uhříněves – Koloděje, 50°2.667'N, 14°36.933'E, 270 m, 21.7.2010			6					6	3	0.11	17.21	25	L
SK-MA	SK, Záhorie lowland, wet depression in field N of the road Plavecký Štvrtok – Láb, ~800 m NE of the village Láb, 48°22.183'N, 16°58.85'E, 190 m, 3.8.2011			7					7	6	0.09	20.49	25	L
SK4	SK, Záhorie lowland, wet depression in field near the road Vysoká pri Morave – Záhorská ves, 2.4 km NW of the village Vysoká pri Morave, 48°20.833'N, 16°53.517'E, 180 m, 3.8.2011		1	9					10	10	0.15	38.52	25	L
Bolboschoenus planiculmis														
AU1	A, Lower Austria, shore of the small fishpond near the road Poysdorf – Herrnbaumgarten, near southern border of the village Herrnabumgarten, 48°41.317'N, 16°40.333'E, 190 m, 4.8.2011					8			8	7	0.22	51.64	25	P
DI	CZ-SM, wet depression in field near the Haraska brook, S of the Martinice farm-house, ~3 km WNW of the town Klobouky u Brna, 49°0.317'N, 16°49.35'E, 230 m, 31.8.2010				1	7			8	7	0.17	45.08	25	P

Acronym	Locality and date of collection	AFLP group							N_{ind}	N_g	D_{Nei}	%poly	N	CDA species
		Y	A	L	B	P	C	M						
DJ-PL	CZ-EB, depression in field near north-eastern border of the village Dolní Jelení, 11 km NNW of the town Vysoké Mýto, 50°3.133'N, 16°6.583'E, 280 m, 5.8.2011					7	1		8	8	0.14	43.44	25	P
JK	CZ-SM, wet depression in the meadow near the pool Kutnar, 3 km SW of the village Rakvice, 48°50.2'N, 16°47.617'E, 180 m, 29.6.2011				1	8			9	7	0.23	57.38	25	P
LD-PL	CZ-SM, wet depression in field near the highway to Slovakia, 1.6 km NE of the town Lanžhot, 48°43.9'N, 16°58.817'E, 150 m, 1.9.2010					10			6	5	0.09	20.49	25	P
NO	CZ-SM, salt marsh at the north-eastern border of the village Novosedly, 48°50.35'N, 16°29.833'E, 180 m, 31.8.2010					8			8	8	0.20	46.72	25	P
NZ	CZ-SM, wet depression in field near the southern bay of the Nesyt fishpond, 3.5 km NW of the town Valtice, 48°45.783'N, 16°43.7'E, 170 m, 31.8.2010					10			10	8	0.15	36.88	25	P, M
SK-KU	SK, Záhorie lowland, depression in field at southern border of the town Kúty, 48°39.083'N, 17°0.717'E, 190 m, 31.8.2010					3	5		8	7	0.13	36.88	25	P, M
TD	CZ-SM, salt marsh near the Trkmanský Dvůr farm-house, 3.5 km NE of the village Rakvice, 48°51.85'N, 16°50.717'E, 170 m, 28.6.2011					7	1		8	8	0.24	56.56	25	P
ZA	CZ-SM, restored salt marsh 1.2 km N of the village Terezín, 48°57.933'N, 16°56.35'E, 175 m, 31.8.2010					10			10	10	0.21	54.10	25	P
Bolboschoenus maritimus														
AZ	IR, West Azerbaijan, Makou to Buralan							7	7	7	0.16	36.06	10	M
B	CZ-NB, depression in field near the Bečovský potok brook, 1 km S of the town Bečov, near the road Bečov – Volevčice, 50°26.45'N, 13°42.567'E, 220 m, 29.8.2010				2	1	5	1	9	9	0.23	63.93	25	P, M
HU	HU, Hortobágy Puszta, salt marsh 8 km E of the town Hortobágy, 47°34.617'N, 21°15.05'E, 100 m, 19.5.2010						2	5	7	7	0.29	68.03	5	M
TE	D, Sachsen-Anhalt, surroundings of saline "slagheap", Salzstelle Teutschenthal W of the town Halle, 51°27.183'N, 11°48.933'E, 100 m							7	7	7	0.17	39.34	25	M
TU1	F, la Camarque, Rhône Delta, Tour du Valat Wildlife Reserve 1, marsh Emprunt Nord Tamarquiron, 43°30'N, 4°30'E							8	8	8	0.21	50.00	25	M
TU2	F, la Camarque, Rhône Delta, Tour du Valat Wildlife Reserve 2, marsh Relongue Nord, 43°30'N, 4°30'E							4	4	4	0.12	20.95	25	M