

Morphological, flow cytometric and cytogenetic evidence of a hybrid swarm between *Elymus hispidus* and *E. repens* in the Bílé Karpaty Mts, Czech Republic

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Abstract: Grasslands in the Bílé Karpaty Mts (Czech Republic, central Europe) are known for their extraordinary species richness and characteristic composition. At one of the local sites, the Čertoryje nature reserve, the structure of a peculiar population of *Elymus* plants (*Triticeae*) was investigated. The site is cohabited by the closely related species *E. hispidus* and *E. repens*, but a substantial percentage of the plants based on their morphology are hybrids. A combination of flow cytometric analyses (DNA content – genome size, GC content), morphometrics (36 characters) and molecular cytogenetics (genomic in situ hybridization – GISH) was used to shed light on the hybridization processes occurring within this population. One hundred and twenty-three plants were collected along 14 transects. A preliminary determination based on two discriminative morphological characters suggested that more than 30% of all individuals were hybrids. The hexaploid cytotype prevailed in the dataset (85%), but 15% of the plants were higher polyploids, namely heptaploids (7x) and octoploids (8x). Morphometric and flow-cytometric data indicated an introgressive pattern of hybridization biased towards *E. hispidus*. GISH was used to reveal the genomic composition of plants selected to cover the spectrum of genome size values. Fifteen of the 20 plants, including higher polyploids, had a hybrid genome composition with signs of successive hybridization (later than the F1 generation). There were inconsistencies between morphology, genome size and cytogenetic data. In particular, some morphologically identified hybrids had a DNA content and a genomic composition corresponding to the parental species *E. hispidus*. A shift in morphology caused the characters that in a previous extensive study were used to differentiate between *E. repens* and *E. hispidus*, to completely fail with this population. The high percentage of hybrids, unparalleled in central Europe, coupled with the occurrence of high-ploidy cytotypes and successive hybrid generations, suggest that the population is best characterized as a hybrid swarm in which interspecific hybridization is the major driving force generating progeny, which thrives at this locality and participates in successive introgressive hybridization.

Keywords: *Elymus*, flow cytometry, genome size, genomic in situ hybridization, hybrid swarm, *Poaceae*, polyploidy

Introduction

Interspecific hybridization is generally accepted as a major evolutionary force in angiosperms (Arnold 1992, Wissemann 2007, Stace et al. 2015). In some cases, it even results in speciation (Hegarty & Hiscock 2005, Rieseberg & Willis 2007, Winterfeld et al. 2014). On the other hand, hybridization can threaten the integrity of taxa and cause genetic swamping via introgression. Hybridization is frequently associated with polyploidization (whole-genome multiplication), another common mechanism in plant evolution (Soltis & Soltis 1999, Marhold & Lihová 2006, Heslop-Harrison et al. 2023). A combination of the two processes may result in the formation of complex groups of allopolyploids (Ma & Gustafson 2008). Hybridization is rather rare within sympatric populations due to genetic barriers, but once they are broken, the frequency of hybrids may increase, resulting in the formation of hybrid swarms (Glottzbecker et al. 2016).

Hybrid swarms are a specific manifestation of hybridization. They are formed by hybrids that survive and reproduce beyond the initial hybrid generation, hybridize with other hybrids and backcross with the parental species, finally leading to their preponderance in the population (e.g. Heiser 1979, Johnston et al. 2004, Krahulcová et al. 2009, Cruzan & Arnold 2012). Hybrid swarms occur in animals (*Cyprinella*; Rubidge & Taylor 2004, Ward et al. 2012, Glottzbecker et al. 2016) as well as in plants. In higher plants, they have been recorded both in woody species, such as *Pinus* (Kormutak et al. 2009, 2014), *Populus* (Keim et al. 1989), *Rhododendron* (Yan et al. 2017, 2019) and in herbaceous plants, such as *Aconitum* (Sutkowska et al. 2013), *Senecio* (Oberprieler et al. 2010), *Viola* (Krahulcová et al. 1996), *Iris* (Johnston et al. 2003, 2004, Cruzan & Arnold 2012), *Dactylorhiza* (Jacquemyn et al. 2016), *Diphysastrum* (Hanušová et al. 2014), *Pilosella* (Krahulcová et al. 2009), *Helianthus* (Heiser 1979) and *Ipomopsis* (Aldridge 2005). In grasses, hybrid swarms or complexes of introgressants are recorded in *Spartina* (Anttila et al. 2000), *Oryza* (Majumder et al. 1997, Bolaji et al. 2012), *Tribolium* (Visser & Spies 1994) and *Elymus* (Szczepaniak et al. 2007). Although hybrid swarms are considered to be an outstanding type of population, whose frequency is usually low (Lowe & Abbott 2015), their evolutionary significance cannot be disregarded, because they lead to the generation of evolutionary novelties (Floate et al. 2016). Complex heteroploid hybrid swarms can generate unique individuals such as aneuploids or $2n + n$ hybrids arising from unreduced gametes; they also undergo exceptional types of hybridization such as polyhaploid parthenogenesis (Krahulcová et al. 2009, Rosenbaumová & Krahulec 2015). A remarkable tendency to form complex allopolyploid aggregates (involving introgressants and hybrid swarms) occurs in the tribe *Triticeae*. Prominent examples include the genera *Triticum* (Waines & Barnhart 1992, Eilam et al. 2008, Kawahara 2009), *Aegilops* and *Elymus* s.l. (Dewey 1984, Löve 1984). Species of *Elymus* are also the subject of this study.

The genus *Elymus* comprises 22 species in Europe (Melderis 1980), three of which (*E. repens*, *E. hispidus*, *E. caninus*) naturally occur in Czechia (Kaplan et al. 2019). *Elymus repens* (L.) Gould [syn.: *Triticum repens* L., *Elytrigia repens* (L.) Nevski, *Agropyron repens* (L.) P. Beauv.] is a common species growing in a wide range of habitats, from wet to dry, and from natural ones, such as steppes and open woodlands, to arable land, where it is a common weed. *Elymus hispidus* (Opiz) Melderis [syn.: *Agropyron hispidum* Opiz in Bercht. et Seidl, *Elytrigia intermedia* (Host) Nevski, *Agropyron intermedium* (Host)

P. Beauv., *Thinopyrum intermedium* (Host) Barkworth et D. R. Dewey] is rarer than *E. repens*, occurring in a narrower spectrum of habitats, which are usually dry: It preferably occurs in steppic regions and on rocky slopes (classes *Koelerio-Corynephoretea* and *Festuco-Brometea*), but also in shrub communities of the alliance *Prunion fruticosae* (*Rhamno-Prunetea*) and in open woodlands (*Quercetea pubescentis*). It also occurs in fully anthropic habitats in communities of the class *Artemisietea vulgaris* (Chytrý 2007, 2009, 2013). This species also grows in the margins of fields and ditches along roads in areas where its more natural habitats occur.

These species hybridize in Czechia, especially in disturbed habitats (Mahelka et al. 2007). The hybrid is named *Elymus ×mucronatus* (Opiz) Conert [syn.: *Agropyron mucronatum* Opiz, *Elytrigia mucronata* (Opiz) Prokudin] and is characterized by an intermediate genome size and morphology (Mahelka et al. 2005, 2007, Urfusová et al. 2021a). The direction of hybridization is markedly asymmetric among hybrids from Czechia, as cpDNA identified *E. hispidus* as the maternal parent in 61 out of 63 cases (Mahelka et al. 2007). Urfusová et al. (2021a) studied the morphology and DNA content of both species of *Elymus* over a wider geographical scale by examining 1,081 plants from 302 central-European populations. They demonstrated that the species hybridized, that the hybridization was introgressive towards *E. hispidus*, and that genome size and morphological data were strongly correlated.

Although the predominant cytotype in both species in Czechia and central Europe is hexaploid, additional ploidy levels, including hepta- (7x) and nonaploids (9x), are recorded (Mahelka et al. 2005, 2007, Urfusová et al. 2021a). Gene flow between hexaploid and nonaploid (9x) cytotypes is likely to have occurred, based on the existence of intermediate ploidy levels among the progeny of one nonaploid mother plant (Mahelka et al. 2007).

Studies on the cytogenetics of species of *Elymus* in Czechia have found that both species are allopolyploids combining in their genomes subgenomes from different species. The allohexaploid *E. repens* harbours the subgenomes of *Pseudoroegneria* and *Hordeum* (haplomes St + H; Mahelka & Kopecký 2010) whereas the subgenomes of the allohexaploid *E. hispidus* can be distinguished using probes for *Pseudoroegneria*, *Dasypyrum* and *Aegilops* (haplomes St + V + D; Mahelka et al. 2011, 2013). Hybrid genotypes combine the subgenomes (chromosomes) of both parental species (Paštová et al. 2019).

The third species of *Elymus* known to occur in Czechia, *E. caninus* (L.) L., is tetraploid, and despite its co-occurrence with both *E. repens* and *E. hispidus*, there is no evidence of its heteroploid hybridization with either of the other species (Urfusová et al. 2021a). In addition to the three naturally occurring *Elymus* species, the neophyte *Elymus obtusiflorus* (DC.) Conert has been recorded at five localities. Its detailed distribution and hybridization potential are unknown.

In the Bílé Karpaty Mts, situated in eastern part of the country, *E. hispidus* grows in different habitats unlike other, ‘ordinary’ populations. Specifically, it occurs in dry, mesic, and even seasonally wet grasslands and mesic oak woodlands. The bedrock in this area is a flysch of Mesozoic age (Pechanec & Jongepierová 2008), which strongly influences habitat conditions on a fine scale, creating wet and dry places and acid- and base-rich deep soils subject to landslides, often influenced by periodically appearing water springs. In this region, there are complexes of extremely rich meadows with scattered trees, mainly *Quercus robur*, *Q. petraea* and *Tilia cordata*. In spite of the smooth relief

and deep soils, these grasslands are thought to have a continuous connection with early Holocene communities influenced by human activity (Hájková et al. 2011, Roleček et al. 2014). At one locality, named the Čertoryje reserve, a preliminary study indicated the existence of unusual variation in the cytotypes of both *E. hispidus* and *E. repens* and their hybrids, as the first three randomly collected samples included three different chromosome numbers (A. Krahulcová, unpublished). Together with a high percentage of morphologically ambivalent genotypes, these aspects made the Čertoryje grassland reserve attractive and triggered this study aimed at addressing the following questions: (i) What is the extent of hybridization in the Čertoryje *Elymus* population and how does it correspond with other central-European populations of *Elymus*? (ii) How does the morphological pattern of plants from the Čertoryje population correspond with the ploidy level, genome size and genomic composition of *Elymus* plants?

Material and methods

Because the Čertoryje population is outstanding in some aspects, such as its high percentage of morphologically ambivalent genotypes, it was compared with a previously acquired broad-scale morphological and flow cytometric dataset for central-European populations (Mahelka et al. 2007, Urfusová et al. 2021a).

Sampling and determination of the plants

In the Čertoryje reserve, 123 plants were collected in the years 2015 and 2016 along 14 transects. The distance between the transects was 50 m and individuals were collected at 20-m intervals (Fig. 1, Supplementary Table S1). All plants were transferred to the Botanical Garden of the Faculty of Science at Charles University in Prague, where they were cultivated for further investigation. For each plant, an herbarium voucher of the above-ground part was established and one fresh leaf was collected for flow cytometric analyses. The herbarium vouchers of all samples were deposited in the Charles University herbarium (PRC).

The determination of plants was based on two main morphological characters that are used to distinguish the species under study (Melderis 1980, Kaplan et al. 2019, Urfusová et al. 2021a): the presence of cilia on the leaf sheath margins (present in *E. hispidus*, absent in *E. repens*), and the shape of the glumes (obtuse or truncate in *E. hispidus*; pointed and awned in *E. repens*).

Genome size estimation

The holoploid genome size (sensu Greilhuber et al. 2005) of the plants was estimated using flow cytometry utilizing two fluorescent dyes: propidium iodide (PI) staining was used to estimate the absolute genome size of plants (2C-DNA content, AGS) and DAPI staining to estimate the relative genome size of plants (RGS). *Pisum sativum* L. ‘Ctirad’ was used as an internal standard (2C = 9.09 pg; Doležel et al. 1998). The protocols of Doležel et al. (2007) were used along with Otto buffers (Otto 1990), only slightly modified as described in Macková et al. (2018). Analyses of PI-stained nuclei (of 123 plants in total) were done using a Partec CyFlow SL flow cytometer (Partec GmbH, Münster, Germany)

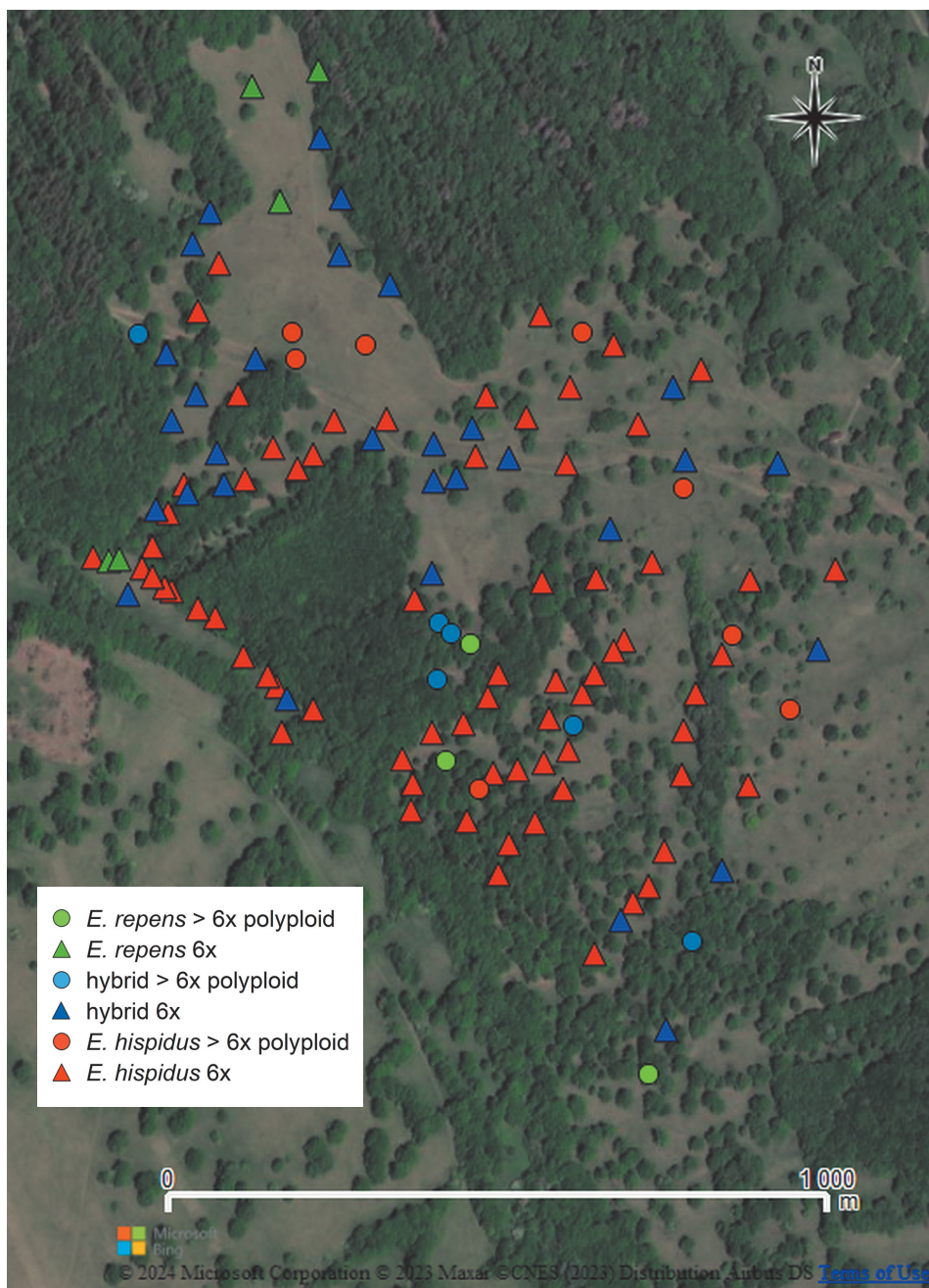


Fig. 1. Location and cytotype structure of 123 plants of *Elymus repens*, *E. hispidus* and their hybrids at the Čertoryje nature reserve. Plants were determined on the basis of two morphological characters used to distinguish *E. repens* and *E. hispidus* and their hybrids. Ploidy levels were determined using chromosome counts and flow cytometry. Note the presence of high polyploids in both species as well as their hybrids.

equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). DAPI-stained nuclei of 120 plants were analysed using flow cytometry (some of the plants died in the experimental garden) on a CyFlow ML device (Partec GmbH, Münster, Germany) equipped with a UV LED light source with a wavelength of 365 nm. All measurements using PI (5,000 nuclei) were repeated on a different day, the results were considered only if the difference between the two results did not exceed 3% and if the coefficient of variance within individual samples was less than 5%. Samples stained with DAPI (3,000 nuclei) were analysed only once. The resulting histograms were analysed using FloMax (version 2.4d, Partec, Münster, Germany). Absolute and relative genome sizes were visualized as boxplots in the Microsoft Office Excel spreadsheet program. One-way ANOVA followed by Tukey's HSD test (in PAST 2.17; Hammer et al. 2001) was used to ascertain the significance of genome size differences between the species. To make this data comparable with those of Mahelka et al. (2007), the AGS values were calibrated by analysing some of the plants studied by Mahelka et al. (2007) on both of the flow cytometers used in this study.

The application of both methods (PI and DAPI) enabled the calculation of the AT/GC ratio. GC-rich DNA is a hallmark of both coding DNA and DNA thermostability (Šmarda & Bureš 2012). It varies from 41.0% to 47.2% in *Poaceae* (Šmarda et al. 2008), so it is comparable between species or among different ploidy levels within species. To trace this characteristic in the samples, the GC content of 120 samples was calculated (104 hexaploids and 16 higher polyploids) according to Šmarda et al. (2008) and Šmarda & Bureš (2012).

Chromosome counts

The chromosomes of 27 plants, selected to cover the genome size range of morphologically unequivocally identified taxa, hybrids and outliers, were counted, which provided calibrated references for the AGS values provided by flow cytometry. Chromosome counts were obtained for four *E. repens*, six *E. hispidus* and 17 hybrid plants (Table 1).

Root tips of cultivated plants were collected and pretreated as described in Kopecký et al. (2005). Root tips were placed in ice water for 26 h, fixed in a 3:1 mixture of absolute ethanol and glacial acetic acid at 37 °C for 7 days, stained with 1% acetocarmine for 2 h and squashed in a drop of 45% acetic acid on clean microscope slides. Mitotic metaphase spreads were prepared from root tips following the protocol of Masoudi-Nejad et al. (2002). Chromosome spreads were stained with lacto-propionic orcein or 4,6-diamidino-2-phenylindole (DAPI) in Vectashield Antifade Mounting Medium (Vector Laboratories). Slides with chromosomal spreads were evaluated under an Olympus BX61 microscope equipped with epi-fluorescence illumination and an Olympus DP73 camera. CellSens v. 1.16 (Olympus) and Zoner Photo Studio 18 software were used to process images.

Distance-based morphometrics

Morphometric analyses were primarily done to investigate whether the observed pattern in the flow cytometric measurements is mirrored by the morphology of plants and to trace potential morphological shifts caused by hybridization at this particular site. For these purposes, plants from the Čertoryje population (only hexaploid plants were included)

Table 1. Chromosome counts and holoploid (absolute) genome sizes (AGS) of 27 *Elymus* plants from the Čertoryje population, which represent the variation in AGS observed within the population. They were classified based on two morphological characters that can be used to discriminate between *E. repens* and *E. hispidus*. The samples are ordered based on their AGS values.

Plant ID	Morphological determination	AGS [pg/2C]	Chromosome number [2n]
T2P2	<i>E. repens</i>	24.9	42
T1P3	hybrid	25.7	42
T1P2	hybrid	25.9	42
T3P10	hybrid	26.0	41
T2P4	hybrid	26.1	42
T2P6	hybrid	26.5	42
T4P7	hybrid	26.5	42
T14P2	hybrid	26.5	41
T3P12	hybrid	26.6	41
T3P9	hybrid	26.7	42
T12P2	hybrid	28.4	42
T7P5	hybrid	28.7	42
T13P12	hybrid	28.8	41
T3P7	hybrid	28.8	42
T6P5	hybrid	29.1	42
T2P1	<i>E. hispidus</i>	28.1	42
T2P8	<i>E. hispidus</i>	28.5	42
T2P9	<i>E. hispidus</i>	28.8	42
T14P8	<i>E. repens</i>	30.2	49
T14P6	hybrid	30.3	49
T9P1	hybrid	30.5	49
T9P6	<i>E. repens</i>	30.8	48
T10P7	<i>E. repens</i>	30.8	49
T4P5	<i>E. hispidus</i>	32.7	49
T13P10	<i>E. hispidus</i>	32.8	46, 47, 48
T4P4	<i>E. hispidus</i>	33.2	49
T1P1	hybrid	35.4	56

were analysed utilizing three datasets: (i) Čertoryje plants alone, (ii) Čertoryje plants together with 330 *Elymus* plants from a previous central-European study (Urfusová et al. 2021a) and (iii) Čertoryje plants together with 20 ‘pure’ *E. repens* plants from localities without the occurrence of *E. hispidus* and the hybrid from the central-European study (Urfusová et al. 2021a). The objective of including these samples of *E. repens* was to eliminate the potential effect of the lack of *E. repens* samples in the Čertoryje dataset.

Thirty-six morphological characters (11 semi-qualitative, nine binary and 16 quantitative) were measured or scored for all specimens of fully developed plants (Table 2). Characters were measured using a pair of digital callipers (accuracy 0.01 mm; Proteco) and a stereo microscope (Olympus SZX12, Tokyo, Japan). Minute characters (mainly on spikelet) were photographed using a digital camera (Olympus E-M 5II, objective Olympus DF Plapo 1XPF) attached to a stereo microscope and subsequently measured in ImageJ 1.49v (Schneider et al. 2012). Multivariate statistical analyses (principal component analysis, PCA) were processed in R v. 3.2.3 (R Core Team 2013) using the packages ade4, class, permute, scatterplot3d, vegan and mass, and the set of functions in MorphoTools (Koutecký 2015). For each quantitative character, univariate statistics (the minimum,

Table 2. List of morphological characters used in the quantitative analysis of form. Binary characters: 0 – absent, 1 – present; scale: 1 – obtuse or oblong, 2 – pointed and/or with awn on the ridge, 3 – acute and/or with awn at the tip.

Character	Units	Abbreviation
ratio of length of spikelet without awn to length of 4th spike rachis internode	–	LSTwtA.L4IR
ratio of length of spikelet without awn to width of spikelet	–	LSTwtA.WST
ratio of length of spikelet with awn to length of 4th spike rachis internode	–	LSTwA.L4IR
ratio of length of spikelet with awn to width of spikelet	–	LSTwA.WST
ratio of length of lower glume to length of 4th spike rachis internode	–	LG1.L4IR
ratio of length of lower glume to length of spikelet without awn	–	LG1.LSTwtA
ratio of length of lower glume to length of spikelet with awn	–	LG1.LSTwA
ratio of length of upper glume to length of lower glume	–	DG1.2
ratio of length of spikelet with awn to length of spikelet without awn	–	LSTwtA.LSTwA
ratio of length of spike without awn to length of spike with awn	–	LSwtA.LSwA
presence of hairs on leaf sheath margins	binary 0/1	CSM
presence of awn on glume	binary 0/1	AG
presence of awn on lemma	binary 0/1	AL
grey colour of leaves	binary 0/1	GL
hairiness of another leaf sheath other than upper and lower	binary 0/1	HALS
hairiness of upper leaf sheath	binary 0/1	HLSU
hairiness of leaves	binary 0/1	HL
length of 2nd upper stem internode	mm	L2UI
length of 2nd spike rachis internode	mm	L2IR
length of 4th spike rachis internode	mm	L4IR
length of hairs on margins of leaf sheaths	mm	LC
length of spikelet without awn	mm	LSTwtA
length of spikelet with awn	mm	LSTwA
length of spike with awn	mm	LSwA
length of glume awn	mm	LAG
length of lemma awn	mm	LAL
length of lower glume	mm	LG1
width of 2nd upper leaf	mm	W2L
width of spikelet	mm	WST
number of hairs on leaf sheaths/1 mm		NoC
number of spikelets		NS
number of leaf sheaths with hairs		NoLSwC
ratio of number of leaf sheaths with hairs to number of leaves	–	NoLSwC:NL
number of leaves		NL
shape of glume	scale 1, 2, 3	SG
shape of lemma	scale 1, 2, 3	SL

maximum, average and the 25th and 75th percentiles) and correlations with other characters using Spearman's correlation (for data not normally distributed) were calculated. Characters that were not variable (one character – hairiness on upper leaf sheath; Table 2) or were strongly correlated (i.e. having a correlation coefficient greater than 0.95) were omitted (three characters – the presence of awns on glumes, the number of leaf sheaths with hairs and the length of upper glume). Characters that were not normally distributed were transformed to a logarithmic scale. Redundancy analysis (RDA; van den Wollenberg 1977) with a Monte Carlo permutation test (999 permutations), with the purpose of examining the morphology and genome size of plants, was performed in Canoco 5 (ter Braak & Šmilauer 2012).

Genomic in situ hybridization (GISH)

Genomic in situ hybridization (GISH) was used on a set of 20 plants in order to investigate their genomic composition. The set of plants selected largely overlaps with the set selected for chromosome counting (Table 1). We only omitted the parental species, because they were analysed elsewhere (Mahelka & Kopecký 2010, Mahelka et al. 2011). Three additional plants could not be analysed, because of the poor quality of the root tips used in the analysis. Based on previous cytogenetic studies on *Elymus* species from Czechia (see the Introduction), selected genotypes in this study were analysed using probes for *Pseudoroegneria spicata*, *Hordeum bogdanii*, *Dasypyrum villosum* and *Aegilops tauschii* (for probe details, see below). The probes included the subgenomes of both allopolyploid species as well as their hybrid, which enabled the determination of whether the genotypes investigated were of the hybrids or one of the parental species.

At least five metaphase spreads for each of the 20 plants were analysed. Using the Biotin-Nick Translation Kit or the DIG-Nick Translation Kit (Roche, Indianapolis, IN), the total genomic DNA of the following species was labelled: *Pseudoroegneria spicata* (Pursh) Á. Löve (USDA accession identifier PI563869), *Hordeum bogdanii* Wilensky (Leibniz Institute of Plant Genetics and Crop Plant Research GenBank, Gatersleben, accession identifier BCC 2063), *Dasypyrum villosum* (L.) P. Candargy (PI639751) and *Aegilops tauschii* Coss. (PI542278). Seeds of the accessions were kindly provided by the Germplasm Resources Information Network (GRIN) of the United States Department of Agriculture (USDA). In situ hybridization and detection were done as described in Mahelka et al. (2011) under conditions of 77% stringency. The first round of GISH was done using probes for *Pseudoroegneria* and *Hordeum*. After photographing the metaphases, slides were washed and reprobbed with probes for *Dasypyrum* and *Aegilops*. Slides were viewed under an Axio Imager Z.2 Zeiss microscope (Zeiss) equipped with Cool Cube 1 camera (Metasystems, Altlüßheim, Germany). ISIS software v. 5.4.7 (Metasystems) and Adobe Photoshop software were used for image processing. Reprobing of the slides was applied following Schwarzacher & Heslop-Harrison (2000).

Results

Species structure in the Čertoryje population

Based on a preliminary morphological investigation using two characters to discriminate between plants, the collection of 123 samples from the Čertoryje population consisted of 78 plants of *E. hispidus*, eight of *E. repens* and 37 hybrids (Fig. 1). Thus, the percentage of hybrids reached 30% for all of the *Elymus* plants sampled.

Flow cytometry and chromosome counts

Using flow cytometry, AGS of all 123 plants was analysed (Figs 2 and 3). DNA-hexaploids prevailed (105 plants, 85%), but AGS analyses indicated 18 plants (15%) with a higher DNA content, pointing to the existence of ploidy levels greater than 6x. Chromosome counts for seven of these plants confirmed the presence of heptaploids and octoploids (Table 1). Among the hexaploids, AGS ranged between 24.40 and 24.89 pg for *E. repens*, 27.77 and 29.72 pg for *E. hispidus* and 25.71 and 29.69 pg for the hybrid. The average 2C

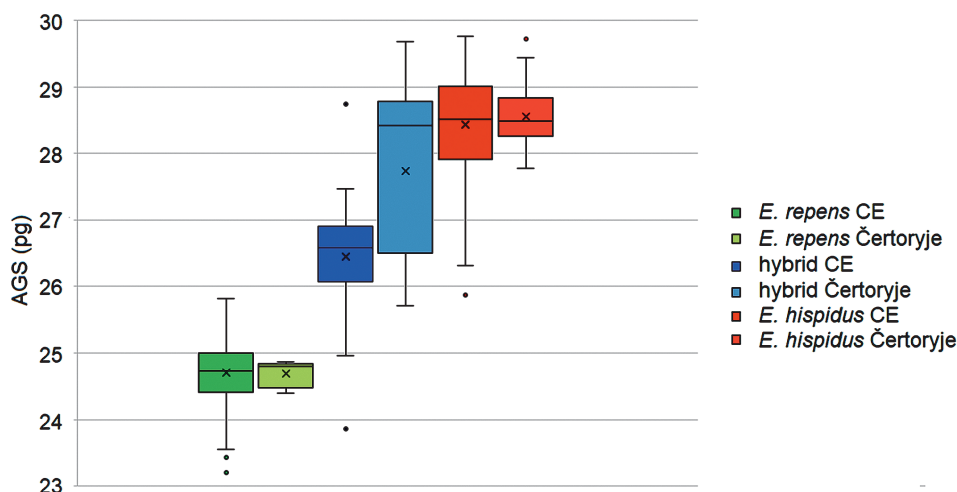


Fig. 2. Box plot comparing absolute genome sizes (AGS) of *Elymus* plants from the Čertoryje nature reserve with the values obtained for 294 central-European populations (Urfusová et al. 2021a). Plants were determined on the basis of two morphological characters used to distinguish *E. repens* and *E. hispidus*.

values ($\pm$ SD) of the species studied were 24.69 ± 0.20 pg for *E. repens*, 28.55 ± 0.39 pg for *E. hispidus*, and 27.74 ± 1.27 pg for the hybrids. AGS values differed significantly between hexaploid *E. hispidus* and *E. repens* (ANOVA: $F_{1,73} = 469.2$, $P < 0.001$) and between hexaploid *E. repens* and the hybrid (ANOVA: $F_{1,34} = 28.25$, $P < 0.001$).

Morphological hybrids formed two distinct groups based on AGS determined by flow cytometric analysis (Figs 3 and 4). Samples from one group (samples 9–22 in Fig. 3) had intermediate AGS values between the two parental taxa, the AGS values of the other group (samples 23–43 in Fig. 3) overlapped those for *E. hispidus* (Figs 3 and 4). Representative plants from both groups were identified as candidates for the GISH analysis used to reveal their genomic composition in order to verify their hybrid status (see below).

The GC content was on average 44.94% (min. 44.26%, max. 46.84%; Fig. 3, Supplementary Figs S1 and S2). Whereas the GC content of *E. repens* and *E. hispidus* plants differed (ANOVA including higher polyploids: $F_{2,116} = 3.716$, $P = 0.027$; ANOVA excluding higher polyploids: $F_{2,100} = 3.814$, $P = 0.025$), no difference was recorded between the GC content of hybrids and their parents.

Morphometrics

Hexaploid plants from the Čertoryje population were analysed in three ways (see Material and methods). In the principal component analysis (PCA) carried out solely for *Elymus* plants from the Čertoryje nature reserve, the first and second axes explained 26.3% and 14.9% of the variation, respectively (Fig. 5). With the combined dataset composed of plants from Čertoryje and central-European populations, the first and second component axes explained 31.0% and 13.5% of the variation, respectively (Fig. 6). When 20 *E. repens* plants were added to the Čertoryje dataset, the first and second component axes explained 37.4% and 11.6% of the variation, respectively (Fig. 7). All PCAs revealed partial separation of groups along the first axis, but a certain level of overlap of species with their hybrids is apparent in all three datasets (Figs 5, 6 and 7). It is obvious

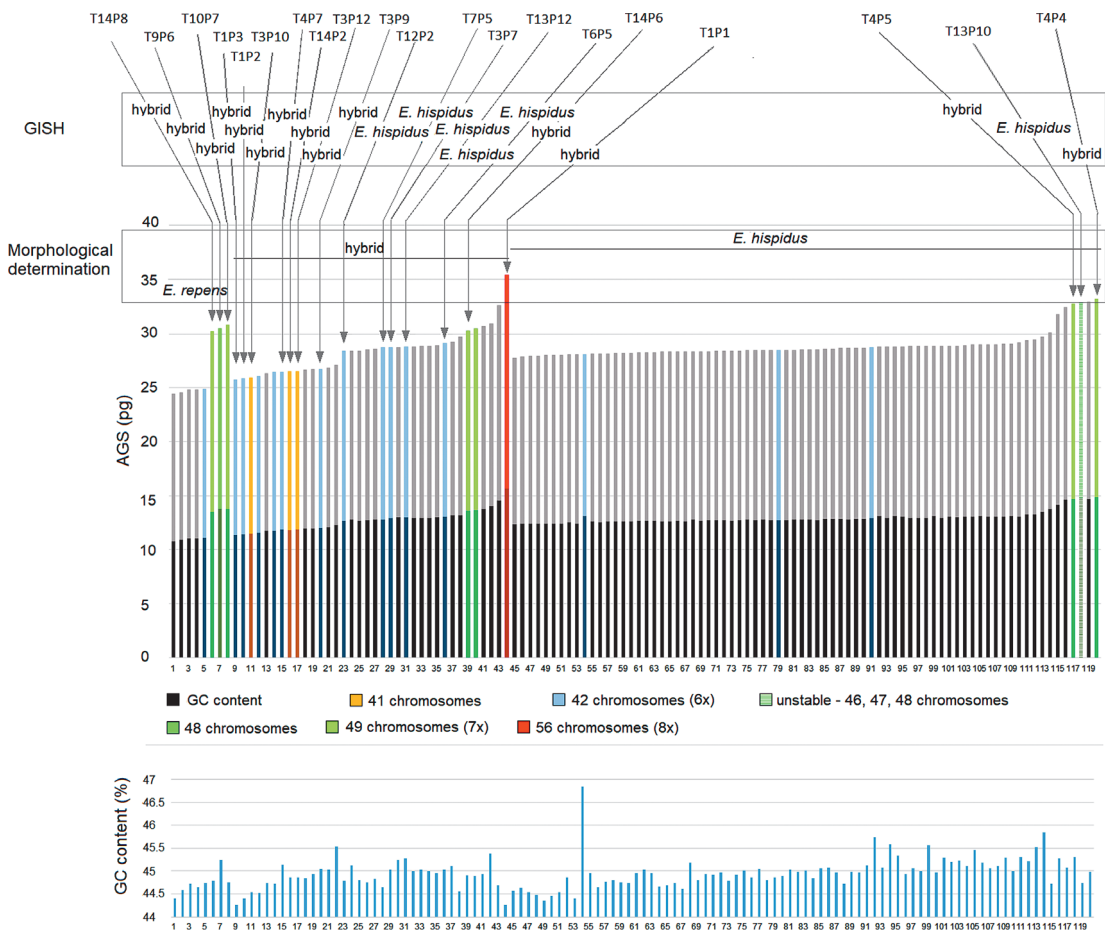


Fig. 3. GC content and absolute genome sizes (AGS) of 120 *Elymus* plants from the Čertoryje nature reserve, including high polyploids. GC content is displayed in absolute values (black bars in the upper panel) and relative values (bottom panel). Plants with verified chromosome counts are marked by different colours. All plants were determined morphologically and a subset of plants was analysed using genomic in situ hybridization (GISH).

from the combined Čertoryje + central-European dataset that the hybrids from Čertoryje are closer to *E. hispidus* (Fig. 6).

The PCA yielded somewhat different results for each dataset. For the Čertoryje dataset analysed alone, the ratio of the length of lower glume to length of 4th spike rachis internode, the presence of an awn on the glume and the ratio of length of spikelet with awn to length of 4th spike rachis internode were the most highly correlated characters on the first axis. In the combined dataset (Čertoryje + central-European populations), the most highly correlated characters were the ratio of length of lower glume to length of 4th spike rachis internode, shape of glume and number of hairs on leaf sheaths/1 mm (see Supplementary Table S2 and S3 for the table of eigenvectors). In the Čertoryje dataset with added *E. repens* plants, the highly correlated characters were the ratio of the length

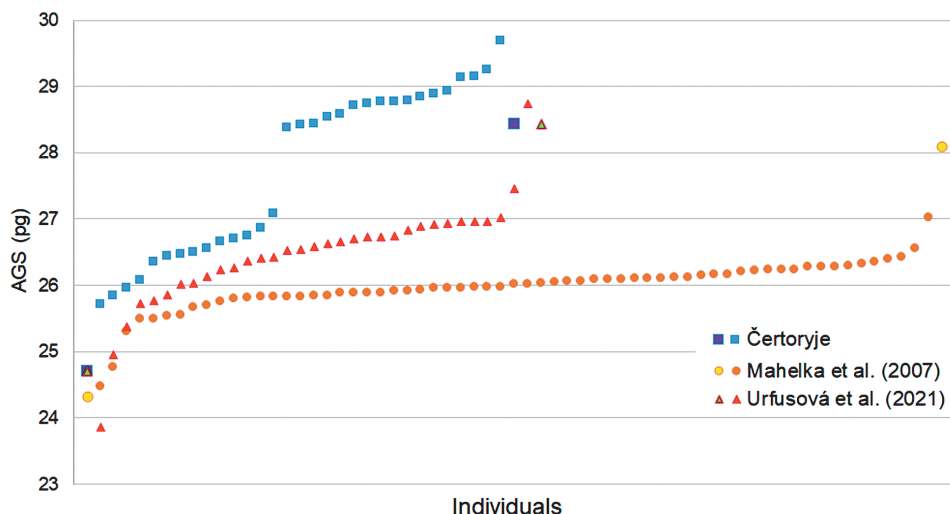


Fig. 4. A pooled analysis of the absolute genome sizes (AGS) of hexaploid hybrids from the Čertoryje nature reserve and hybrids reported in Mahelka et al. (2007) and Urfusová et al. (2021a). The AGS values were corrected by adding the difference caused by the different flow cytometers used. The first symbol on each line is the average value for the parental species *Elymus repens* and the last is the average value for *E. hispidus*.

of lower glume to length of 4th spike rachis internode, length of 4th spike rachis internode and the ratio of length of spikelet with awn to length of the 4th spike rachis internode (eigenvectors are given in Supplementary Table S4). Thus, only one character (the ratio of length of lower glume to length of 4th spike rachis internode) was common to all three datasets. This indicates certain inconsistency between the morphological patterns of plants from Čertoryje and other central-European populations (see Discussion).

The relationship between AGS and morphology in hexaploid *Elymus* plants from Čertoryje was visualized using RDA and a simple plot with AGS and PC1 as axes (Supplementary Figs S3 and S4). Both indicated a partial separation of *Elymus* species (especially *E. repens*) and an overlap of some of the hybrids with *E. hispidus*. In addition, the morphological characters of plants from Čertoryje were compared with plants from other central-European populations. In particular, the usefulness of the recently recognized morphological characters for identifying *Elymus* species (Urfusová et al. 2021a) was tested, which revealed a shift in some characters in the Čertoryje dataset (Supplementary Figs S5–S9). The three characters emerging from the PCA for the Čertoryje population indicated that the hybrids from the Čertoryje population are morphologically intermediate between *E. hispidus* and hybrids from central-European populations (Supplementary Figs S5, S8 and S9). Altogether, the data show that the hybrids and *E. hispidus* plants from Čertoryje are morphologically closer to each other than either is to its central-European counterpart, which supports the scenario of backcrossing and introgressive hybridization.

Genomic *in situ* hybridization

Using GISH, the genome composition of 20 genotypes was identified. The results are summarized graphically in Fig. 8.

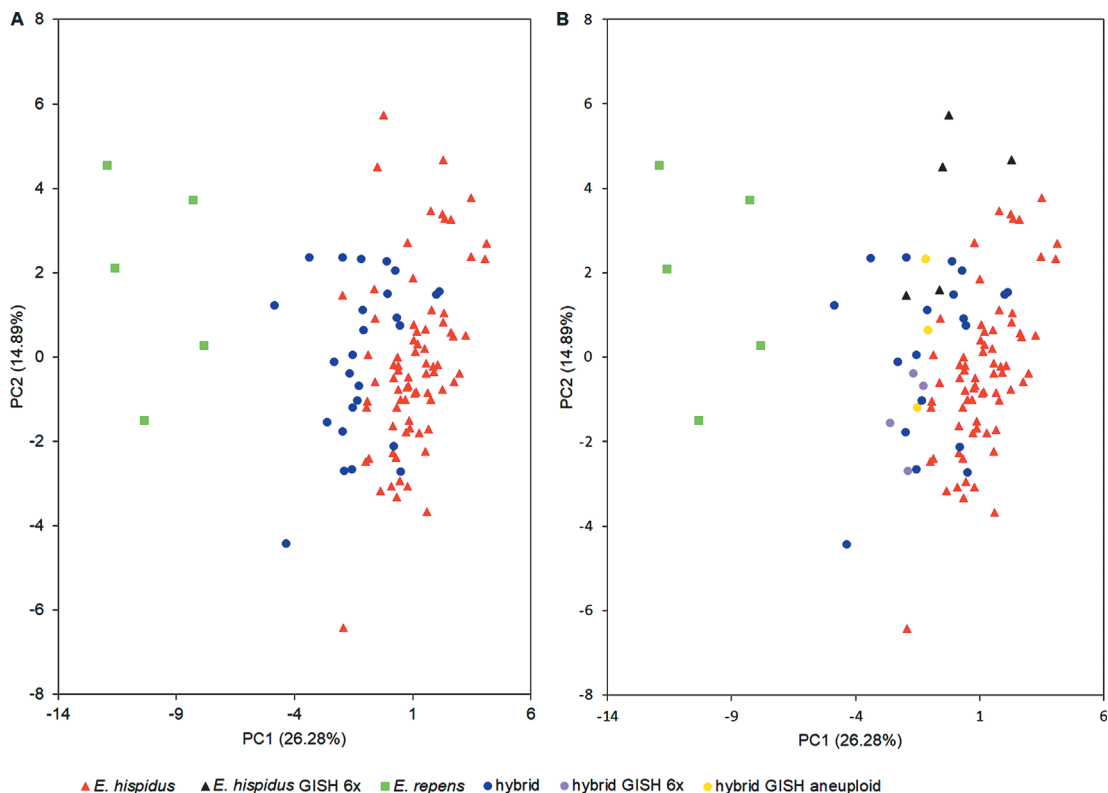


Fig. 5. Principal component analysis of morphological characters of *Elymus* plants from the Čertoryje nature reserve (hexaploids only). The two panels present results for plants classified based on the two main morphological characters (A) and genomic in situ hybridization (B).

Genotypes T3P7, T6P5, T7P5, T12P2 and T13P12. – These five genotypes are hexaploid with $2n = 6x = 42$ chromosomes. GISH revealed that they consisted of one subgenome (i.e. 14 chromosomes) corresponding to *Dasypyrum*, one subgenome corresponding to *Aegilops* and one corresponding to *Pseudoroegneria*. The subgenome of *Hordeum* seemed to be absent in these genotypes. Based on GISH, these genotypes were most likely those of *E. hispidus*.

Genotypes T1P2 and T1P3. – These two genotypes are hexaploid ($2n = 6x = 42$) having one chromosome set from each of the *Dasypyrum*, *Aegilops* and *Hordeum* genomes and three chromosome sets from *Pseudoroegneria*. They are likely to be hybrids of hexaploid *E. repens* (28 *Pse* + 14 *Hor* chromosomes) $\times$ hexaploid *E. hispidus* (14 *Pse* + 14 *Aeg* + 14 *Das* chromosomes). One chromosome of *Pseudoroegneria* had a translocation from *Dasypyrum* (a distal half of one arm) in both genotypes, indicating the existence of post-hybridization generations.

Genotypes T3P10, T3P12 and T14P2. – These genotypes are similar to the previous T1P2 and T1P3 genotypes, but are numerical aneuploids with 41 chromosomes. Three complete sets (21 chromosomes) belong to *Pseudoroegneria*, one set to *Hordeum*, one set to *Aegilops*, and six chromosomes to *Dasypyrum*. Like the previous two genotypes

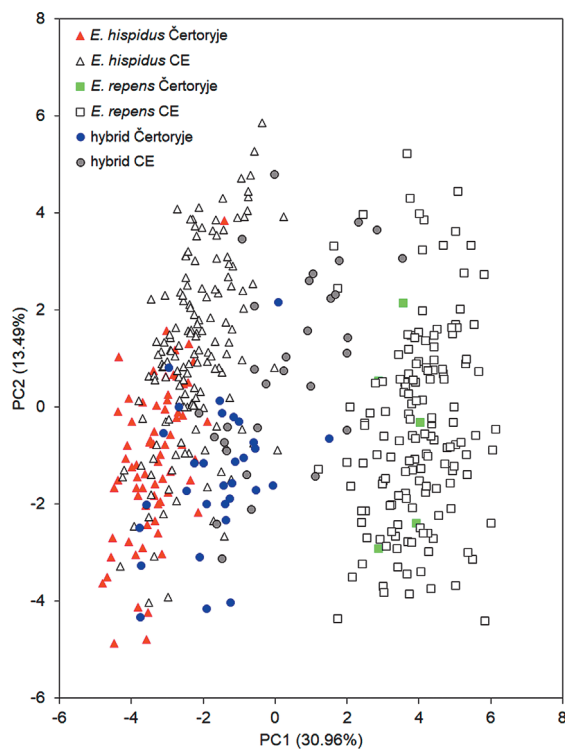


Fig. 6. Principal component analysis of a combined morphometric dataset including *Elymus* plants from the Čertoryje nature reserve and central-European populations (Urfusová et al. 2021a). Only hexaploid cytotypes were included.

(T1P2 and T1P3), it is likely that these three genotypes are also hybrids between hexaploid *E. repens* and *E. hispidus*, with one chromosome of *Dasypyrum* being eliminated. In genotype T14P2, a *Pse/Das* translocation was detected, indicating the existence of post-hybridization generations.

Genotypes T3P9 and T4P7. – These two genotypes are genomically similar to the previous ones, are balanced aneuploids, meaning that they have a genome composition of 22 *Pse* + 7 *Hor* + 7 *Aeg* + 6 *Das* chromosomes, all being hexaploid ($2n = 6x = 42$). In genotype T3P9, one chromosome of *Pseudoroegneria* carried a translocation (almost an entire arm) from *Dasypyrum*. Like the previous genotypes, these two genotypes were most likely hybrids between hexaploid *E. repens* and *E. hispidus*. Again, chromosomal irregularities (the chromosomal substitution and the translocation) indicate the existence of post-hybridization generations.

Genotype T9P6. – This is another (numerically) aneuploid genotype, in this case with 48 chromosomes, having seven *Dasypyrum*, seven *Aegilops*, seven *Hordeum* and 27 *Pseudoroegneria* chromosomes. One *Hordeum* chromosome has a large translocation (almost an entire arm) from *Pseudoroegneria*. In addition, two *Pseudoroegneria* chromosomes have terminal translocations from *Hordeum* (one on the long arm and one on the short arm).

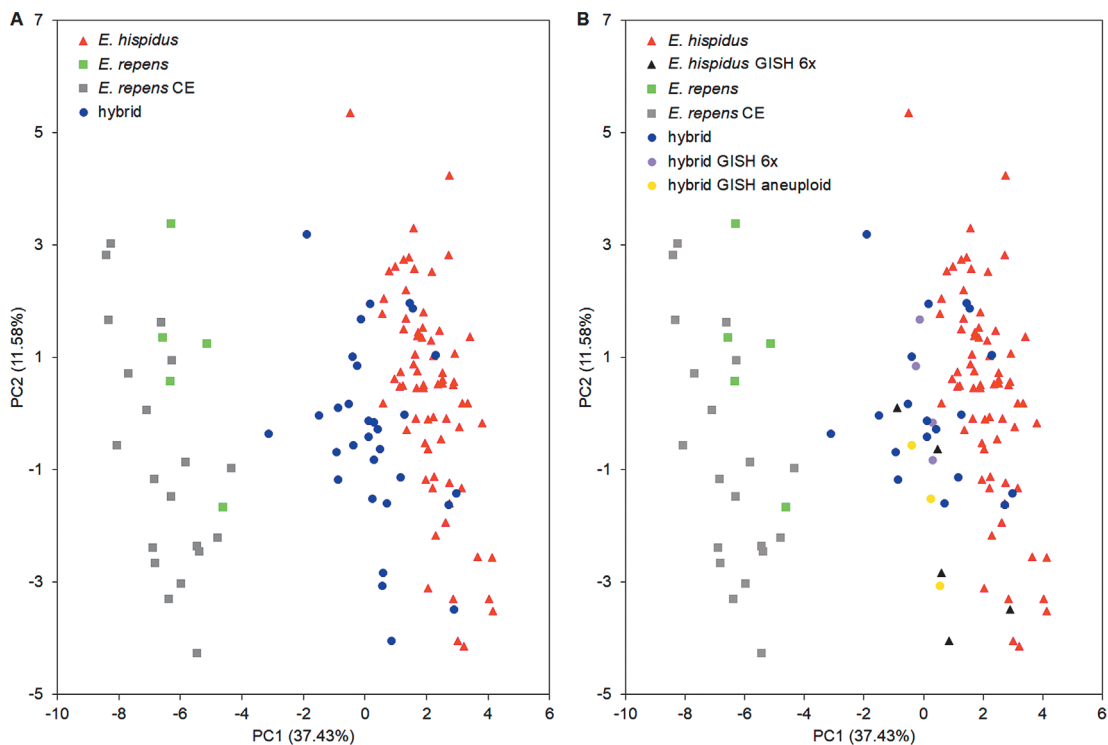


Fig. 7. Principal component analysis of plants from the Čertoryje nature reserve, supplemented with 20 *Elymus repens* plants from other central-European localities. *Elymus repens* plants were added to avoid potential bias due the few samples of *E. repens* from Čertoryje. Only hexaploid cytotypes were included. The two panels present the results of the analyses of plants determined based on the two main morphological characters (A) and some plants determined based on genomic in situ hybridization (B).

Genotypes T10P7, T14P6 and T14P8. – These genotypes are heptaploid ($2n = 7x = 49$) with their genomes composed of four progenitors: GISH using probes from *Dasypyrum*, *Aegilops* and *Hordeum* revealed a dispersed signal over seven chromosomes each, while the remaining 28 chromosomes were labelled by the probe for *Pseudoroegneria*. Two genotypes (T14P8 and T10P7) have four translocations based on GISH: (i) One segment from *Pseudoroegneria* translocated to the distal region of the short arm of one chromosome of *Dasypyrum*. It is likely this translocation was inherited from the parental species *E. hispidus* (Mahelka et al. 2011). (ii) One segment from *Hordeum* translocated to the short arm of a *Pseudoroegneria* chromosome. (iii) One segment from *Hordeum* translocated to the long arm of a *Pseudoroegneria* chromosome. (iv) One segment from *Pseudoroegneria* translocated to the short arm of a *Hordeum* chromosome. As in the previous genotype T9P6, this genotype is a hybrid between *E. repens* and *E. hispidus*. The translocations involving three subgenomes out of four, excluding translocation (i), are indicative of the existence of post-hybridization generations.

Genotypes T4P4 and T4P5. – These two genotypes are heptaploid ($2n = 7x = 49$). However, their genome composition differs from the previous genotypes: 14 and 14 chromosomes are labelled with probes for *Dasypyrum* and *Aegilops*, respectively, whereas the

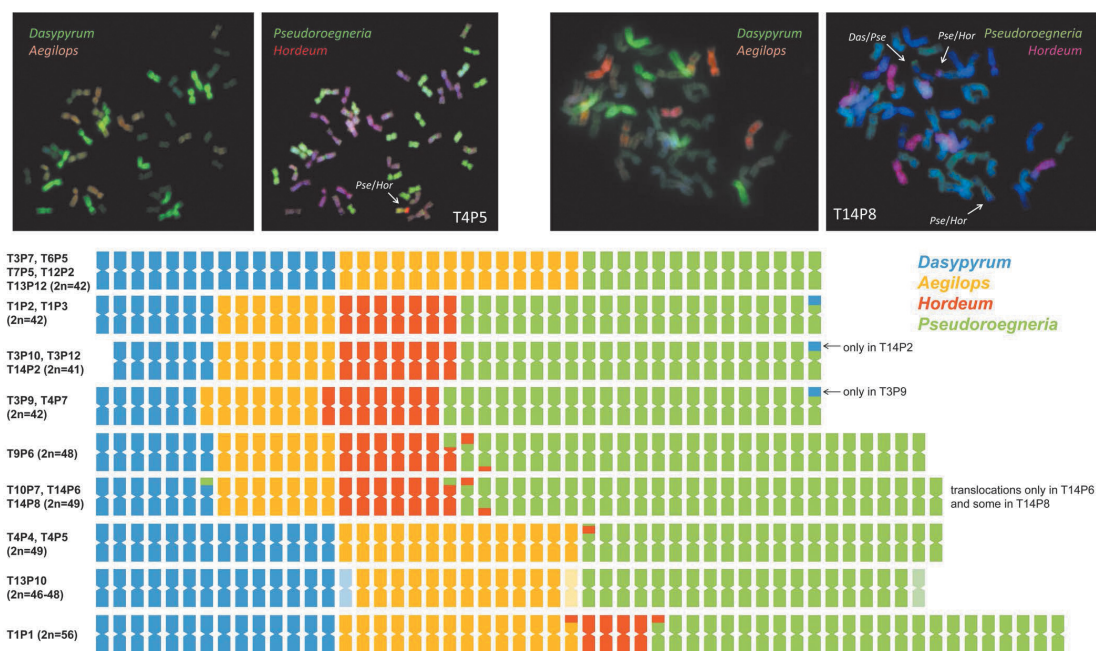


Fig. 8. Molecular cytogenetic analysis of selected genotypes of *Elymus* from the Čertoryje nature reserve. Fluorescent signals were detected after FISH with total genomic DNA of *Dasyphyrum villosum* labelled with digoxigenin (green colour), total genomic DNA of *Aegilops tauschii* labelled with biotin (red colour), and after washing and re-probing with total genomic DNA of *Pseudoroegneria spicata* labelled with digoxigenin (green colour) and total genomic DNA of *Hordeum bogdanii* labelled with biotin (red colour) in accessions T4P5 (upper left) and T14P8 (upper right). Note the translocations between *Pseudoroegneria* and *Hordeum* chromosomes (both genotypes) and between *Dasyphyrum* and *Pseudoroegneria* chromosomes (genotype T14P8), indicated by arrows. Karyotypes of all genotypes studied are drawn with all translocations indicated (bottom panel). Light-coloured chromosomes in genotype T13P10 indicate the variability of this karyotype in the cells investigated (see main text).

remaining 21 chromosomes are labelled with a probe for *Pseudoroegneria*. One small translocation from *Hordeum* was detected on one short arm of one *Pseudoroegneria* chromosome in both genotypes. This translocation seemed to be located on the same chromosome of *Pseudoroegneria* in both accessions; however, it is likely that they are not identical. In genotype T4P5, it forms most of the short arm of this particular chromosome (except the pericentromeric region, Fig. 8), while in accession T4P4 it looks like an intercalated translocation with a small terminal segment from the host *Pseudoroegneria* chromosome. The contribution from *Hordeum*, albeit small, suggests that these genotypes were most likely successive generations of a hybrid (involving *E. repens* and *E. hispidus*).

Genotype T13P10. – GISH produced contradictory results for this accession. In the several cells analysed, the genomic constitution varies consisting of 19 *Pse* + 13 *Aeg* + 14 *Das*, 20 *Pse* + 13 *Aeg* + 15 *Das* and 20 *Pse* + 12 *Aeg* + 15 *Das*. The reason for the inconsistency is unknown, but technical issues, including the poor quality of preparations, cannot be excluded. Genomically, the absence of *Hordeum* suggests that it is a higher polyploid genotype of *E. hispidus*.

Genotype T1P1. – This is the only octoploid ($2n = 8x = 56$). It consists of DNA from all four progenitors tested in this study. Probes from *Aegilops* and *Dasyphyrum* produced a dispersed signal over 14 chromosomes, whereas probes for *Pseudoroegneria* and *Hordeum* labelled 24 and four chromosomes, respectively. There are two translocations: one from *Hordeum* into the chromosome of *Pseudoroegneria* and the other one also from *Hordeum*, but into the chromosome of *Aegilops*. The origin of this octoploid genotype is not clear, but the contribution from all four progenitors indicates that it is a high polyploid hybrid involving *E. repens* and *E. hispidus*. The presence of the translocations indicates the existence of post-hybridization generations.

Discussion

Determination of plant samples and inference of hybrids

In this study, morphometrics, flow cytometry and molecular cytogenetics were used to characterize plant samples from Čertoryje (Bílé Karpaty Mts). The results provided an insight into the evolutionary processes taking place at Čertoryje. The *Elymus* species studied are routinely differentiated using two main morphological characters: the shape of the glume and the presence of cilia on the leaf sheaths margins (Kaplan et al. 2019; see also Urfusová et al. 2021a), as well as DNA content (AGS). Both the morphology and AGS of hybrids (*E. ×mucronatus*) are intermediate (Mahelka et al. 2005, Urfusová et al. 2021a). The basic determination of the plants from the Čertoryje population, therefore, was based on their morphology. Morphometric measurements and genome size analyses were carried out in order to determine whether these two variables were correlated. Unexpectedly, this revealed an incongruent pattern of AGS and morphology, as the AGS of half of the plants morphologically identified as hybrids did not exhibit intermediate values, but instead overlapped with the range of AGS for *E. hispidus* (Fig. 4). The presence of this and other abnormalities triggered the need to analyse these genotypes from a cytogenetic point of view in order to reveal their genomic composition. In this respect, GISH revealed a hybrid origin of most samples. However, the hybrid status of the genotypes T3P7, T6P5, T7P5, T12P2 and T13P12 is controversial. These genotypes have a hybrid morphology, but GISH and flow cytometry indicated they are pure *E. hispidus*. It was hypothesized that this incongruence may indicate that the genotypes are introgressants towards *E. hispidus*. The data indicate that hybrids of a later than the F1 generation occur in the Čertoryje population, so backcrossing cannot be excluded. The elimination of chromatin of one parental species can occur quickly in grasses. In *×Festulolium* hybrids (hybrids between *Festuca* and *Lolium*), if backcrossing to *Lolium* occurs in one of the earlier generations, then by the F7–F8 generation the elimination of *Festuca* is nearly complete (Kopecký et al. 2019). Therefore, the hybrid status of these genotypes cannot be confirmed using GISH at this stage, so it was inferred solely on the basis of morphology. For the purposes of this study, a hybrid is defined as a genotype that has either an intermediate morphology or an obvious contribution from both parental chromosomes based on GISH, or a combination of both characteristics.

Shift in the morphology of plants from the Čertoryje population

To put the newly obtained data from the Čertoryje population in a broader framework, it was compared with the data obtained for more than 1,000 plants from 294 central-European (CE) populations (Urfusová et al. 2021a). The advantage of such a comparative approach is that it used an identical determination system, the same flow cytometric instrument and identical elaboration of morphometrics (the same set of morphological characters processed by the same person). The only difference was the sampling method: at the Čertoryje reserve the plants were collected along transects whereas the CE dataset was based on sampling five to ten randomly selected plants per population.

In the central-European dataset, Urfusová et al. (2021a) found that the morphological characters most highly correlated with the first PCA axis (PC1 axis, including the determination characters) were the shape of glume, the number of hairs on leaf sheaths/1 mm and the ratio of the length of lower glume to length of 4th internode of the spike rachis. Adding the Čertoryje population to the large central-European dataset disrupted this pattern, as the ratio of length of the lower glume to the length of the 4th internode of the rachis was then the only character useful for the differentiation of *Elymus* plants in the combined dataset. This inconsistency indicates a shift in the morphology of plants from the Čertoryje population, where the differentiating characters singled out from the central-European dataset (Urfusová et al. 2021a) did not work. Importantly, even the main two determinant characters published in botanical keys and floras (the shape of the glume and the presence of hairs on the leaf sheaths margins; Melderis 1980, Kaplan et al. 2019) did not work at the Čertoryje reserve.

Čertoryje reserve: a site of extraordinary introgressive hybridization

Compared to other populations, the Čertoryje population had a markedly different structure. With more than 30% hexaploid hybrids and 15% higher polyploids (both percentages being based on the total number of plants), the Čertoryje population is unparalleled in terms of the frequency of hybrids and high polyploids. Among 294 central-European populations, Urfusová et al. (2021a) report 3.4% of plants to be hybrids (including high polyploids) and 1.1% out of 1,026 plants sampled were high polyploids. Mahelka et al. (2007) sampled *Elymus* populations from contrasting habitats (natural and agricultural) and report 30 hexaploid hybrids (11.2%) and four nonaploids (1.5%) among 269 plants. In that study, the frequency of hybrids was influenced by the type of habitat, as the highest frequency (16%) was recorded at a very disturbed site and no hybrid was found in any steppe like habitat. Mahelka et al. (2005) report that the percentage of hybrids within their collection of 238 *Elymus* plants from 55 localities is 16%, but their sampling was intended to cover the morphological variation at each locality, so it is likely to be biased in favour of hybrids. In addition to hexaploid hybrids, they found six nonaploids (2.52%). Although natural hybridization between *E. hispidus* and *E. repens* has been studied by others (e.g. Szczepaniak et al. 2007), we are not aware of any other study in which the frequency of hybridization is estimated in natural populations.

Another peculiar phenomenon recorded in the Čertoryje population is the pattern in the morphological and flow cytometric data, which indicates a marked introgressive hybridization towards *E. hispidus* (Figs 2 and 5, Supplementary Figs S3 and S4). This pattern is mirrored by a greater AGS of hybrids at Čertoryje where the average AGS

values of parental species are very close to values reported from across central Europe, whereas the genome size of the hybrids in the Čertoryje population is larger by more than 1 pg (27.74 pg vs 26.45 pg; Urfusová et al. 2021a). The introgressive pattern is consistent with the findings of Szczepaniak et al. (2007), who report that hybridization between *E. hispidus* and *E. repens* is introgressive towards *E. hispidus* in populations from Poland. Interestingly, Mahelka et al. (2007) show that the direction of hybridization is highly asymmetrical in hybrids from the Czech populations, as cpDNA identified *E. hispidus* as the maternal parent in 61 out of 63 hybrids (Mahelka et al. 2007). The potential propensity of hybrids to backcross primarily with *E. hispidus*, could be the underlying factor for introgressive hybridization towards *E. hispidus* observed by Szczepaniak et al. (2007) and Mahelka et al. (2007). The *E. x mucronatus* hybrid is in fact a self-sustaining entity at some localities, namely thanks to its long creeping rhizomes and fertility (Mahelka et al. 2007, Szczepaniak et al. 2007). However, this does not explain the abnormally high percentage of hybrids found exclusively in the Čertoryje nature reserve. One potential explanation is the biotic and abiotic specificity of the locality.

The formation of the hybrid swarm may be linked with the (a)biotic uniqueness of the locality

The grassland in the Čertoryje nature reserve, like some others in the Bílé Karpaty Mts, are famous as holders of records in terms of species richness at different scales (Merunková et al. 2012, Wilson et al. 2012, Chytrý et al. 2015, Fajmonová et al. 2020). The reason for this species richness has been studied repeatedly (Merunková et al. 2012, Hettenbergerová et al. 2013, Mudrák et al. 2013, Michalcová et al. 2014), but the major contributing factors were only identified recently (Fajmonová et al. 2020). Fajmonová et al. (2020) report that the major drivers of the extraordinary species richness are a combination of abiotic factors (intermediate soil moisture and base rich soils) favourable for many ecological groups of species and biotic interactions resulting from the type and duration of management practices. Another factor maintaining the species diversity is the high diversity of edaphic parameters at the locality (pH, humidity connected to the deep flysch soils; Klimeš 2008, Wilson et al. 2012, Chytrý et al. 2015). In the Bílé Karpaty Mts, species of *Elymus* inhabit a mosaic of habitats seemingly atypical for *E. hispidus*, including nearly mesophytic meadows, dry meadows with *Carex montana*, dry grasslands with *Brachypodium pinnatum* on base-rich soils, less often dry meadows and pastures with *B. pinnatum* and *Agrostis capillaris*, oligotrophic meadows and pastures, and mesic meadows with *Arrhenatherum elatius* (Škodová et al. 2008). Because similar localities occur elsewhere in the south eastern part of the Bílé Karpaty Mts, the findings reported for *Elymus* growing at Čertoryje could be extended to this whole area, where *E. hispidus* occurs in the same plant associations and differ from those in mostly dry habitats in other central-European regions.

Under certain circumstances, the specificity of conditions could be the reason behind the long-term co-occurrence of species of *Elymus* and their continuous interbreeding. For example, the fine-scale patchy habitats at Čertoryje, created especially by landslides disturbing the soil surface, may have enabled the germination and easy establishment of the products of hybridization between the ecologically specialized *E. hispidus* and the generalist *E. repens*. Hybrids survive in wet conditions better than *E. hispidus* (Mahelka 2006). The frequency of hybrids would therefore increase with occasional backcrossing to *E. hispidus*. Alleles of *E. repens* and of hybrid origin, providing the recipient species with

a greater potential to adapt to mesic conditions, would become fixed in the *E. hispidus* genome. For this reason, introgressive hybridization towards *E. hispidus* might have led to the genetic swamping of pure xerophyte *E. hispidus*, and its replacement by introgressed genotypes adapted to mesic conditions. Such a scenario is consistent with many examples of introgressive hybridization (Todesco et al. 2016). In this respect, a study on species traits underlying plant adaptation to the specific conditions at the Čertoryje reserve is needed to verify this hypothesis.

In a previous study (Urfusová et al. 2021a), only one location in Ukraine was sampled, Kasova Hora (49.224371°N, 24.702922°E), which harbours similar vegetation to that at Čertoryje (Boychuk & Zamoroka 2017, Roleček 2019). Both *E. hispidus* and *E. repens* co-occur at this locality, but only a single hybrid plant was recorded there (out of 13 plants). It will be possible to reach a conclusion as to whether similar conditions support the formation of hybrid swarms in the *E. repens*–*E. hispidus* complex when another similar locality becomes available.

Hybrid swarm at Čertoryje: a potential source of evolutionary novelties

Hybrid swarms may be an important source of evolutionary novelties and lead to hybrid speciation (Nolte & Tautz 2010). In this respect, the hybrid swarm in the nature reserve at Čertoryje is a potential source of evolutionary novelties that is unparalleled. Moreover, a previous study (Urfusová et al. 2021b) confirmed that *Elymus* hybrids, especially octoploids, are capable of selfing if induced via the mentor effect. Thus, they are able to form hybrid lineages independent of the parental taxa.

It is evident that certain novel genotypes do occur at this locality. Especially noteworthy is the occurrence of the high polyploids, including hepta- and octoploids. Heptaploids as such are not novel cytotypes for the Čertoryje reserve, as two types of heptaploids are described by Paštová et al. (2019). In the current study two types of heptaploids were recorded, but only one type (genotypes T10P7, T14P6, T14P8) corresponds to one of those described by Paštová et al. (2019; genotype C25B therein). The other type, characterized by 14 *Das* + 14 *Aeg* + 21 *Pse* chromosomes (with a translocation from *Hordeum*), is a new one, described here for the first time. Paštová et al. (2019) proposes that the heptaploids originated from the fusion of a gamete of *E. repens* (gametes *Pse* + *Pse* + *Hor*) and a gamete (*Pse* + *Pse* + *Aeg* + *Das*) from an octoploid (probably of *E. hispidus*). While this scenario is plausible for one of the types of heptaploid described here (the T10P7 type), it does not explain the origin of the other type (T4P4). The origin of the latter type is difficult to explain, namely because of the translocation from *Hordeum*, pointing to an advanced hybrid origin of this genotype. The two heptaploids differ in AGS (Table 1), likely mirroring different sizes of chromosomes belonging to particular sub genomes. Mahelka et al. (2007) describe a similar pattern in nonaploid plants, whose AGS varied, likely depending on their origin. Similarly, it is difficult to propose the origin of the octoploid genotype, mainly because of the contribution of only four chromosomes from *Hordeum* (Fig. 8). Nevertheless, it is possible to state with a high degree of certainty that it is a hybrid of *E. hispidus* and *E. repens*. The existence of an octoploid cytotype is another new finding of this study. From the cytogenetic point of view, besides the euploid heptaploids and the octoploid, almost all the genotypes presented in Fig. 8 (other than possibly ‘pure’ hexaploid *E. hispidus* – the T3P7 type) are unique genotypes

so far unknown from other localities. Among other peculiarities there are three hexaploid plants with outlying GC content (Supplementary Fig. S1). Such abnormalities can be attributed to chromosomal rearrangements or, alternatively, to variations of the genomic composition of allopolyploid genotypes. Such rearrangements are reported in *Elymus* (Paštová et al. 2019). Different genome sizes of heptaploid plants also indicate their different genome composition and origin, as is reported for nonaploid plants (Mahelka et al. 2007).

Elymus hispidus is an important source of desirable traits for improving wheat. These include resistance to numerous biotic and abiotic stresses, which have been introgressed into wheat to increase its yield potential (e.g. Li & Wang 2009, Li et al. 2019, Zhang et al. 2021). *Elymus hispidus* is also the first perennial grain to be cultivated in North America, where it is sold under the trade name Kernza (The Land Institute, Kansas, USA). In this respect, the Čertoryje population may be of interest to wheat breeders, as it offers potentially new genotypes unavailable in other populations.

Conclusions

In this study, the structure of a peculiar population of *Elymus* plants in the Čertoryje nature reserve in the Bílé Karpaty Mts in eastern Czech Republic was recorded. The site is cohabited by the closely related species *E. hispidus* and *E. repens*, but a high percentage of the plants were hybrids based on their morphology. The flow cytometric analyses of nuclear DNA content, morphometric studies and molecular cytogenetic analyses (genomic in situ hybridization) jointly shed light on the hybridization processes taking place within this population. Despite certain inconsistencies between morphology, genome size and cytogenetic data, it is evident that the introgressive hybridization between the two *Elymus* species is skewed towards *E. hispidus*. The high percentage of hybrids, unparalleled in central-European populations, coupled with the presence of high-ploidy cytotypes (7x and 8x) and successive generations of hybrids, led to the conclusion that rather than it being just a mixed population consisting of parental species and temporary hybrids, this population is a hybrid swarm. Evidence provided indicates that interspecific hybridization is the driving force generating diverse hybrid offspring, which survive at this locality that is rich in different microhabitats and participates in ongoing introgressive hybridization producing novel genotypes that might be of potential interest to researchers working on cereal improvement.

Supplementary materials

Fig. S1. GC content of hexaploid *Elymus* plants from Čertoryje.

Fig. S2. GC content of high *Elymus* polyploids from Čertoryje.

Fig. S3. Redundancy analysis of hexaploid *Elymus* plants from Čertoryje.

Fig. S4. Correlation of absolute genome size and morphometrics of hexaploid *Elymus* plants from Čertoryje.

Fig. S5. Boxplot comparing the morphological character, the ratio of length of lower glume to the length of 4th internode of samples from central-European populations (CE) and the Čertoryje dataset for hybrid and *Elymus hispidus*.

Fig. S6. Boxplot comparing morphological character, the shape of glume in samples from central-European populations (CE) and Čertoryje dataset for hybrids and *Elymus hispidus*.

Fig. S7. Boxplot comparing morphological character, the number of cilia on leaf sheath/1 mm in samples from central-European populations (CE) and the Čertoryje dataset for hybrids and *Elymus hispidus*.

Fig. S8. Boxplot comparing morphological character, the presence of awn on the glume in samples from central-European populations (CE) and the Čertoryje dataset for hybrids and *Elymus hispidus*.

Fig. S9. Boxplot comparing morphological character, the ratio of the length of spikelet with awn to the length of the 4th spike rachis internode in samples from central-European populations (CE) and the Čertoryje dataset for hybrids and *Elymus hispidus*.

Table S1. GPS coordinates and absolute genome sizes of the plants sampled.

Table S2. Correlation of characters with the first and second principal components axes in the dataset for the Čertoryje population.

Table S3. Correlation of characters with the first and second principal components axes in the combined dataset for central-European and Čertoryje populations.

Table S4. Correlation of characters with the first and second principal components axes in the Čertoryje dataset with 20 *Elymus repens* plants added.

Supplementary materials are available at www.preslia.cz

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Morfologické, cytometrické a cytogenetické důkazy existence hybridního roje mezi *Elymus hispidus* and *E. repens* v Bílých Karpatech

Louky v Bílých Karpatech jsou charakteristické svým mimořádným druhovým bohatstvím, kde se na malé prostorové škále kombinují lesní druhy spolu s vlhkomilnými i suchomilnými druhy. Kromě toho se zde vyskytuje řada reliktních druhů v nereliktním reliéfu. Na jedné ze zdejších lokalit, v přírodní rezervaci Čertoryje, jsme zkoumali strukturu zvláštní populace rostlin rodu *Elymus* (*Triticeae*). Přestože se na lokalitě společně vyskytují blízké příbuzné druhy *E. hispidus* a *E. repens*, podstatná část zkoumaných rostlin se vyznačovala hybridní morfologií. Použili jsme kombinaci průtokové cytometrie (analýza velikosti genomu), morfometrie (36 znaků) a molekulární cytogenetiky (genomová in situ hybridizace – GISH), abychom objasnili hybridizační procesy probíhající v této populaci. Na 14 transektech bylo nasbíráno 123 rostlin. Jejich předběžné určení na základě dvou diskriminačních morfologických znaků naznačilo, že více než 30 % analyzovaných jedinců jsou kříženci. V souboru dat převažoval hexaploidní cytotyp (85 %), ale 15 % rostlin bylo vyššího cytotypu, konkrétně heptaploidního (7x) a oktaploidního (8x). Morfometrická a cytometrická data naznačovala introgresivní hybridizaci směrem k *E. hispidus*. Genomová in situ hybridizace (GISH) byla použita k odhalení genomického složení rostlin vybraných tak, aby pokryly celou škálu hodnot velikosti genomu. Patnáct z dvaceti rostlin, včetně vyšších polyploidů, mělo hybridní složení genomu se známkami opakované hybridizace (pokročilejší než generace F1). U části analyzovaných rostlin jsme zaznamenali určitý nesoulad mezi morfologickými, cytometrickými, a cytogenetickými daty. Konkrétně, některé rostliny, určené na základě morfologie jako hybridy, měly obsah DNA a složení genomu odpovídající rodičovskému druhu *E. hispidus*. Posun v morfologii způsobil, že rozlišující znaky pro *E. repens* a *E. hispidus*, identifikované v naší předchozí studii, u této populace zcela selhaly. Abnormálně vysoký podíl hybridů, spolu s výskytem cytotypů s vyšší ploidií a pokročilými hybridními generacemi naznačují, že populace je hybridním rojem, v němž je mezidruhová hybridizace hlavní hybnou silou generující potomstvo, které na lokalitě prosperuje a podílí se na postupné introgresivní hybridizaci.

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