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Urtica kioviensis, a rare species of stinging nettle threatened by hybridization

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Abstract: Hybridization is a widespread and important force in plant evolution. It can either hinder speciation or result in the formation of new species. Repeated hybridization with backcrossing with one or both of two hybridizing species is referred to as introgressive hybridization and leads to the introgression of genetic variation from one taxon to another or to other taxa. Hybridization can have consequences for rare species if they are in contact with a more abundant relative with incomplete genetic barriers, as it can lead to genetic erosion, population decline and even the extinction of species. Hybridization between two closely related species of the genus *Urtica* were studied where the rare diploid species *U. kioviensis* and the widespread species complex *U. dioica* with two cytotypes (2x and 4x) occurred at six sites in central Europe. Flow cytometric relative genome size estimation, morphometrics and analyses of nuclear and chloroplast DNA markers were used to confirm the hybrid origin of intermediate plants. The results provide proof of both homoploid (2x × 2x) and heteroploid (2x × 4x) hybridization. The detected continuous variation in relative genome size and morphology at the diploid level indicate homoploid hybridization between *U. kioviensis* and the diploid cytotype of *U. dioica*; subsequent introgression is possible but not proved with certainty and needs further study. Triploid individuals were also detected, showing differences in relative genome sizes and different positions in morphometric analyses compared to the parental taxa. They also have lower fertility (pollen viability, 68.9%) compared to their parents and diploid hybrids and no introgressive hybridization (back crosses) involving triploids was recorded. Based on the results, it is not possible to unequivocally determine their origin. They may be triploid hybrids between *U. kioviensis* and tetraploid *U. dioica*, between diploid and tetraploid cytotypes of *U. dioica* and between diploid hybrids (*U. kioviensis* × *U. dioica* 2x) and tetraploid *U. dioica*. The frequency of hybridization differs between sites; the highest risk of genetic erosion was recorded at Plačkův les in southern Moravia (Czech Republic), where it might be a threat to *U. kioviensis* via both genetic and demographic swamping. The results also demonstrate a case where hybridization could pose a risk to small populations of a rare species and highlights the increasing need to protect endangered species of plants.

Keywords: central Europe, cpDNA, hybridization, introgression, ITS, polyploidy, relative genome size, *Urtica dioica*, *Urtica kioviensis*

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Introduction

Small populations of rare species face a multitude of pressures and threats, such as habitat loss and fragmentation, loss of genetic diversity and demographic and environmental stochasticity (Frankham et al. 2010). In the last few decades, the threat posed by introgressive hybridization between rare and more common species, often caused or influenced by human activities, has also emerged as an increasingly important topic in plant species protection (Rhymer & Simberloff 1996, Quilodrán et al. 2020).

Hybridization is common in vascular plants (interspecific hybridization is reported in at least 25% of plant species; Mallet 2005) and has a significant effect on their diversity (Soltis & Soltis 2009, Abbott et al. 2013). It can be viewed as a creative evolutionary force leading to genotypic and phenotypic novelties and is often coupled with polyploidization, resulting in the establishment of new genotypes (Rieseberg et al. 2003, Stelkens & Seehausen 2009). On the other hand, hybridization can lead to the extinction or serious decline in rare species surrounded by more abundant related species with incomplete genetic barriers (Todesco et al. 2016). This can occur either through genetic swamping or demographic swamping. In genetic swamping a rare species is gradually replaced by hybrids (both primary hybrids and products of backcrosses) with higher fitness in a given environment and time. Demographic swamping, by contrast, occurs where a population of a rare species is reduced further as a result of the production of maladapted hybrids (Wolf et al. 2001, Todesco et al. 2016). If gene flow is limited in a rare species threatened by inbreeding depression, this can be partly counterbalanced by a resulting gain in fitness (a phenomenon referred to as genetic rescue; Todesco et al. 2016).

Despite the growing threat of hybridization for small and fragmented populations of rare species, there are very few well documented cases and theoretical studies on this topic. Most studies are on crop-to-wild gene flow, for example in *Prunus* L. (Delplancke et al. 2012, Macková et al. 2017, 2018), *Malus* Mill. (Coart et al. 2006, Cornille et al. 2015, Bitz et al. 2019) and *Aegilops* L. (Arrigo et al. 2011). Repeatedly documented is the extinction via hybridization of insular species, as islands are generally more susceptible to invasions by non-indigenous species than continents (Levin et al. 1996). Detailed studies that focus on hybridizing populations of rare and more common native species from both the perspective of threatened species and the risk of newly established hybrids, are, however, very scarce (e.g. in the genera *Viola* L., Krahulcová et al. 1996; *Senecio* L., Prentis et al. 2007; *Rhododendron* L., Ma et al. 2010; *Cerastium* L., Vít et al. 2014; *Onopordum* L., Balao et al. 2015; *Dianthus* L., Vítová et al. 2015; *Prunus*, Macková et al. 2017, 2018 and *Elymus* L., Urfusová et al. 2021), although interspecific hybridization is more or less common in many genera. Here, hybridization between two closely related species of the genus *Urtica* L., the rare species *U. kioviensis* Rogow. and the common species *U. dioica* L., in central Europe, is studied.

Urtica kioviensis is a perennial subcontinental, Pontic-Pannonian species. Its geographic range is disjunct and extends across floodplains from the valleys of the rivers Khopyor and Don in European Russia, via Ukraine, Moldova and Romania southwards to Bulgaria and westwards into the Pannonian Basin in Hungary, northern Croatia, southern and eastern Slovakia, easternmost Austria, and southern Moravia (Czech Republic). It also occurs in north-eastern Germany, mainly in the Berlin-Potsdam area and in the

Havel valley between Berlin and the river Elbe, reaching its northern limit in Denmark. It has also been reported from Israel (Konczak et al. 1968, Wolters et al. 2005). In central Europe, this species occurs in flooded basins, in oxbow lakes in alder forests and willow carr and reed beds that are partially permanently or intermittently flooded (Danihelka & Lepší 2004, Haszonits et al. 2021). *Urtica dioica* is a widespread perennial species native to the temperate zone of Eurasia and north Africa that has been introduced into many other parts of the world (Meusel et al. 1965, Taylor 2009). In central Europe, two major cytotypes, namely diploid and tetraploid, can be distinguished. The former has a disjunct distribution and occurs in partly relict and less human-affected habitats with a shallow water table, such as alluvial forests, willow growing along riverbanks, wet lowland alluvial meadows and reed beds; in contrast, the latter occupies a wide range of habitats, especially those with an increased degree of human influence (Henning et al. 2014, Grosse-Veldmann & Weigend 2015, Grosse-Veldmann et al. 2016, Rejlová et al. 2019). In Europe diploids are traditionally classified into three subspecies, i.e. subsp. *pubescens* (Ledeb.) Domin, subsp. *sondenii* (Simmons) Hyl. and subsp. *subinermis* (R. Uechtr.) Weigend, while tetraploids are treated as subsp. *dioica*. However, because the morphological delimitation of the subspecies and their geographic distribution are not yet fully resolved, only the cytotypes are referred to in this paper. Despite partly contrasting ecological demands, both species as well as both cytotypes of *U. dioica* grow often in close proximity in alluvial forests (Urfus et al. 2021). Both species are wind-pollinated, but their seed can be dispersed by wind (anemochory), water (hydrochory) and animals (zoochory). Hybridization between *U. dioica* and *U. kioviensis* is occasionally reported in Moravia (Rejlová & Urfus 2018). Due to differences in monoploid genome size, at least primary F1 hybrids can be determined by flow cytometry, which makes these species an excellent model system for studying hybridization at the population level.

This article examines the extent of interspecific hybridization in the rare species *Urtica kioviensis* with diploid and tetraploid cytotypes of *U. dioica* at six localities in central Europe using flow cytometry, multivariate morphometrics and molecular markers (ITS, trnH-psbA). Specifically, the questions addressed are: (i) how does genome size correlate with the pattern of morphological variation, (ii) do mixed populations differ from each other in the extent of hybridization/introgression, (iii) what is the extent of homoploid hybridization (*U. dioica* 2x × *U. kioviensis*) on the one hand and heteroploid hybridization (*U. dioica* 4x × *U. kioviensis*, or alternatively, *U. dioica* 2x × *U. dioica* 4x) on the other, and (iv) what is the effect of homoploid and heteroploid hybridization on the genetic integrity of *U. kioviensis*.

Material and methods

Plant material

Six sites were selected in central Europe, where there are populations of *U. dioica* and *U. kioviensis*, and are the main subareas in the distribution of *U. kioviensis* in this region (Table 1, Fig. 1, Supplementary Table S1). At each site, a plot of 25–100 m^2 was established in which hybridizing populations (based on an initial evaluation of phenotypic variation) of the parental species were present. The sampling was designed to include the majority of the plants/clones in the plot. As the first estimations of genome size showed

Table 1. Overview of populations and plants used in this study, assigned to five categories: *Urtica kioviensis*, diploid *U. dioica*, tetraploid *U. dioica*, diploid hybrids and triploid hybrids. GS – relative genome size, morpho – morphometric analyses, DNA – plants for molecular analyses, pollen – pollen viability tests. Plants of *U. kioviensis* from Potsdam served as reference plants, as homoploid hybridization at the diploid level was not observed at this locality.

Locality	*U. kioviensis*				*U. dioica* 2x				*U. dioica* 4x				Hybrids 2x				Hybrids 3x			
	GS	morpho	DNA	pollen	GS	morpho	DNA	pollen	GS	morpho	DNA	pollen	GS	morpho	DNA	pollen	GS	morpho	DNA	pollen
D: Potsdam	56								35								8			
CZ: Plačkův les	74	30	5		47	15	11		28	10		2	31	7	5	1	58	31		3
CZ: Ranšpurk	12	11	1		6	1			20	1			7	6						
SK: Šúr	96	46	1	10	74	39	2	5	32	20		2	3	1	1	1	2	2		
H: Fehér-tó	50				2				4				0				1			
H: Kóny	3				31				2				6				2			
CZ: Myslivna (ref.)					19															
CZ: Jiřice (ref.)					7															
CZ: Křivé jezero (ref.)					78	5	3		5	5										
SK: Šúr (ref.)	65	10	3																	

Fig. 1. (A) *Urtica dioica* (locality Ranšpurk, southern Moravia, Czech Republic) and (B) *U. kioviensis* (locality Šúr, western Slovakia).

a continuum, especially between *U. kioviensis* and the diploid cytotype of *U. dioica*, it was necessary to include reference plants in order to delimit the boundaries between the groups. Reference plants of *U. kioviensis* were collected in the central part of the Šúr alluvial woodland in south-western Slovakia, where *U. dioica* does not occur (both cytotypes of *U. dioica* grow together with *U. kioviensis* only at the edge of this alluvial woodland), and at the Potsdam site. Reference plants of *U. dioica* (2x) came from Křivé jezero in southern Moravia, and from two localities in central (Jiřice) and north-western (Myslivna) Bohemia, respectively, where *U. kioviensis* has never been reported. The ranges in variation are presented in the Results section. Similarly, reference plants were previously used, for example, in studies that focused on hybridization in the genera *Prunus* (Macková et al. 2017) and *Diphasiastrum* Holub (Hanušová et al. 2014).

First, at each site one mature leaf per plant/clone was collected for estimating genome size using flow cytometry (864 in total). In a subset of well-developed plants at Plačkův les, Ranšpurk and Šúr and at the reference localities Křivé jezero and Šúr, representative parts of their stems (non-destructive sampling) were collected for morphological analyses (240 plants in total). Furthermore, to confirm the hybrid origin of plants deemed to be hybrids and to detect the direction of crossing, a subset of plants (32 in total) was assigned to *U. kioviensis* (10 plants), diploid *U. dioica* (16 plants) and to their putative hybrids (six plants), for molecular analyses (ITS and chloroplast DNA analyses). Pollen stainability was recorded for 24 plants (*U. dioica* 2x – five individuals; homoploid 2x hybrid – two individuals; *U. kioviensis* – 10 individuals; 3x heteroploid hybrid – three individuals; and 4x *U. dioica* – four individuals (Table 1). The classification was based on relative genome size (see Results) and additional morphological characters were not used in the present analyses.

Flow cytometry

To obtain information on ploidy level and relative genome size, the relative fluorescence of nuclei obtained from fresh intact leaf petiole tissue (no later than 48 hours after being collected) was determined using flow cytometry, following a simplified two-step protocol (Doležel et al. 2007). The cytometer used was a Partec CyFlow ML instrument equipped with a 365-nm UV LED light source. Petioles were used in this analysis because of the high incidence of reduplicated (endopolyploid) tissues in leaf blades in members of the genus *Urtica*. The petioles were chopped together with the internal reference standard *Bellis perennis* L. (2C = 3.16 pg; Temsch et al. 2021) with a razor blade in a plastic Petri dish containing 500 µl of ice-cold Otto I buffer (0.1M monohydrate citric acid and 0.5% Tween 20). The suspension was then filtered through a 42-µm nylon mesh, and the isolated nuclei stained for at least 5 minutes with 1 ml of the buffer Otto II (0.4M $Na_2HPO_4 \cdot 12H_2O$) supplemented with fluorochrome 4',6-diamidino-2-phenylindole (DAPI; final concentration 4 $\mu g \cdot ml^{-1}$) and ß-mercaptoethanol (final concentration 2 $\mu l \cdot ml^{-1}$). The optimization of flow cytometric analyses was based on previous cytogeographic studies (Rejlová et al. 2019, Urfus et al. 2021).

Classification of diploid specimens

The relative fluorescence of nuclei from specimens from the five reference populations was studied using exploratory data analysis techniques (Supplementary Data S1). Within

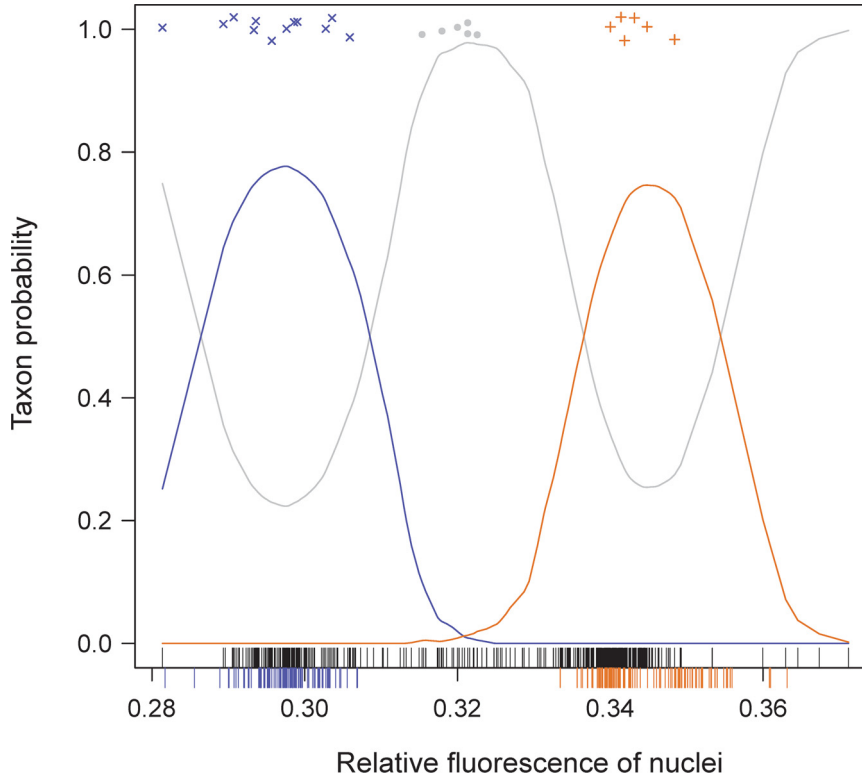

Fig. 2. Classification of diploid individuals based on the relative fluorescence of nuclei. The lines depict estimated probability of an individual belonging to a particular taxon for the given relative fluorescence of its nuclei (*Urtica kioviensis* – orange, *U. dioica* – blue, hybrids – gray). Rugplots on abscissa show relative fluorescence of nuclei for all of the individuals sampled, with the individuals from the reference populations outside the frame of the plot (*U. kioviensis* reference populations – orange, *U. dioica* reference populations – blue, individuals from the non-reference populations – black). Marks in the upper part of the plot denote relative fluorescence of the nuclei of the individuals, for which the taxon identity was checked by ITS sequencing (*U. kioviensis* – orange +, *U. dioica* – blue ×, hybrids – gray dot).

populations, the data were not distributed normally, and the variance differed among populations. The normal distribution hypothesis was tested using Shapiro-Wilk test, the equality of variances hypothesis by the Fligner-Killeen test.

Based on the results, it was decided to build the classifier using a resampling (bootstrap) approach. A linear mixed-effect model of the data was developed, in which the relative fluorescence is the response variable and the taxon (*U. kioviensis*, *U. dioica*) the fixed-effect predictor (Supplementary Data S2). Population identity was included as a random effect, affecting the intercept. The model was fitted using the lmer function in the lme4 package (ver. 1.1-31; Bates et al. 2015). In this way, an estimate of the standard deviation of the population identity effect was obtained. Next, 99 replicates for each of the populations were obtained after subtracting the population-level effect and adding the random population-level effect to the data. The random values for the population effects were generated from a normal distribution with zero mean and the standard deviation as estimated by the mixed-effects model. Probability density functions of the relative fluorescence

Table 2. Characters and their acronyms used in morphometric analyses. See also Supplementary Fig. S2.

Character acronym	Character definition and comments
UTR	density of unicellular trichomes on the underside of a leaf (trichome count per cm^2 in the middle part of a leaf blade)
UTU	density of unicellular trichomes on the upper side of a leaf (trichome count per cm^2 in the middle part of a leaf blade)
STR	stinging trichomes count on the underside of a leaf
STU	stinging trichomes count on the upper side of a leaf
UTS	density of unicellular trichomes on the stem (1 cm of stem)
STS	density of stinging trichomes on the stem (5 cm of stem)
LLB	length of leaf blade (mm)
LLP	length of leaf petiole (mm)
LLB/LLP	length of leaf blade over length of the leaf petiole
WLB	width of leaf blade (mm)
LLB/WLB	length over width of leaf blade
LBB	shape of leaf blade base
LTH	tooth height at widest point of leaf (mean of three teeth; mm)
LTW	tooth base width at widest point of leaf (mean of three teeth; mm)
LTH/LTW	tooth height over width of tooth base
STI	stipules fused / not fused (binary)
LST	length of stipules (mm)
LUS	length of unfused part of stipules (mm)
WST	width of stipules (at base)

were estimated for each of the species separately, using function density with the Epanechnikov's kernel and default bandwidth. The probability density function for each species was built using 200 populations (two real ones and 2×99 simulated ones) (Fig. 2).

In the putatively mixed populations, the value of the two probability density functions (one for each of the pure taxa) for the local individuals were estimated using linear approximation, and combined with the flat one, that represented the probability of not being any of the pure taxa (Fig. 3). For the purpose of the pure taxon/hybrid discrimination in the current study, individuals were considered to be putative hybrids if the probability of being a hybrid was 0.85 or higher according to this criterion. The threshold value is arbitrary, but is in accord with the classification based on ITS sequences and is further supported by the classification based on morphological characters (see below). All of the relative genome size data was used to determine the effect of a particular value of the criterion (Supplementary Fig. S1). Four plants among the diploids with the highest RGS from Plačkův les were classified as hybrids, which is an artefact of the statistical model. These plants were ex post reclassified as *U. kioviensis.*

Morphology

To determine phenotypic variation, 19 characters (including three ratios; Table 2, Supplementary Fig. S2) were selected based on the literature (Tutin et al. 1993, Weigend 2005, 2006, Grosse-Veldmann & Weigend 2015, Rejlová et al. 2021). Most characters were measured or scored directly on herbarium specimens of plants collected in the field. Trichomes on the stem, leaves and petals were observed under a stereomicroscope. To avoid the distortion of multivariate analyses, Spearman's rank correlation coefficients

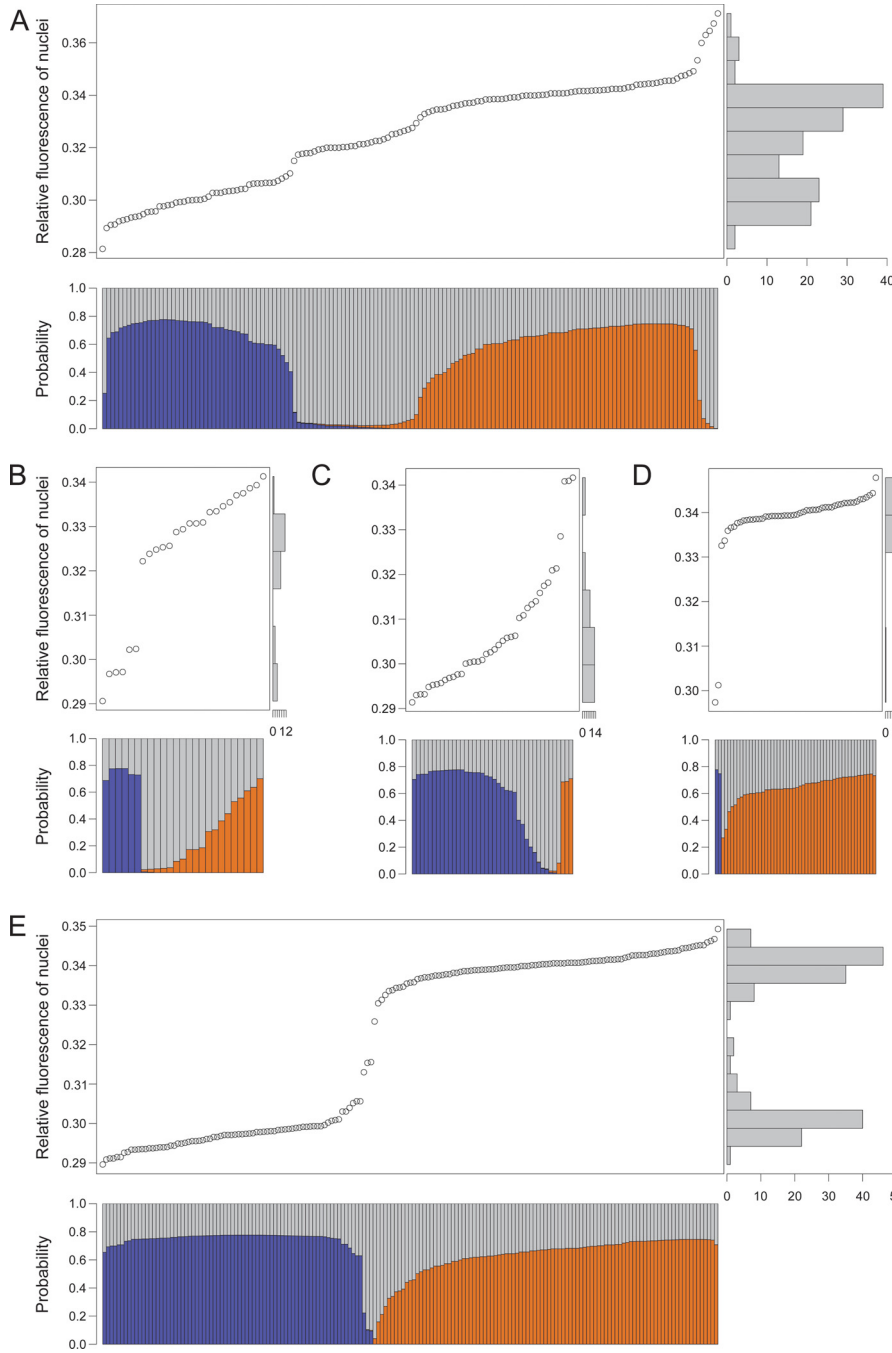

Fig. 3. Relative fluorescence of nuclei of the diploid individuals sampled. For each of the populations, the values are shown in top-left panel, distribution of the values is shown in top-right panel (histogram) and classification criteria per individual are depicted in the bottom panel. In the bottom panel, the percentage of a bar for an individual coloured corresponds to the probability of it belonging to a group or taxon (*U. kioviensis* – orange, *U. dioica* – blue, hybrids – gray). A – Plačkův les, B – Soutok, C – Kóny, D – Fehér-tó, E – Šúr.

were used to determine the extent to which the characters are correlated with one another. PCA of individual plants based on a correlation matrix was used to obtain an insight into the structure of the group studied. In addition, a canonical discriminant analysis (CDA), with individual plants assigned to groups (*U. kioviensis*, diploid *U. dioica*, tetraploid *U. dioica*, diploid hybrids and triploid hybrids; hybrids were either projected as passive samples or analysed as separate groups) based on relative genome size, was carried out. Unlike in the PCA, the character STI was not included. Redundancy analysis (RDA), with a Monte Carlo permutation test (999 permutations) was used to determine the association between morphological variation (response variables) and genome size (predictor) in diploid accessions. Differences in three characters (UTS, UTR, LTH) in *U. kioviensis*, diploid *U. dioica*, tetraploid *U. dioica*, diploid hybrids and tetraploid hybrids were determined using a one-way ANOVA followed by post-hoc comparisons (Tukey HSD method). The analyses were done in R language and a statistical environment (R Core Team 2018) with the help of the functions and scripts in the package MorphoTools v.1.01 (Koutecký 2015).

DNA analysis

DNA was isolated from herbarium material using a sorbitol extraction procedure (Štorchová et al. 2000). The ITS region was amplified using the primers ITS A and ITS B (Blattner 1999), and the chloroplast psbA-trnH intergenic spacer was amplified using the primers psbAF and trnHR (Sang et al. 1997). The amplification was done using a Mastercycler ep (Eppendorf AG, Germany). The 25-μL reaction mixture contained 1 μl of genomic DNA (10–35 ng), 2.5 μl μL of 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, and 0.5 U of Taq DNA polymerase (Top-Bio, Czechia). The cycling profile included an initial denaturation step at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min to amplify the ITS region or 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min to amplify the psbA-trnH region, followed by a final step at 72 °C for 10 min and cooling to 4 °C. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced by Eurofins Genomics (Ebersberg, Germany). Sequence electropherograms were edited manually using Chromas version 2.6.6. (Technelysium Pty Ltd, Australia) and aligned in Bioedit version 7.2.5. (Hall 1999).

Pollen viability

The viability of pollen grains from individuals in each group included in the study was compared using a modified Alexander's staining protocol (Peterson et al. 2010). Because of shortage of male plants, especially of the hybrid plants, only the pollen of 24 plants was analysed. Anthers were extracted from open flowers. They were then fixed in Carnoy's fixative (96% ethanol:chloroform:acetic acid at a ratio 6:3:1) for at least two hours, transferred onto a microscope slide and dissected with a needle in a drop of the staining solution (54.5 ml distilled water, 25 ml glycerol, 10 ml 96% alcohol, 4 ml glacial acetic acid, 5 ml acid fuchsin in a 1% water solution, 1 ml malachite green in a 1% solution in 96% alcohol and 0.5 ml orange G in a 1% water solution. Pollen grains (100–400 grains per sample) were observed under a microscope to assess its stainability. Stained grains were considered viable.

Results

Flow cytometry

The ploidy levels and relative genome sizes of 864 *Urtica* accessions were determined using flow cytometry (both hybridizing and reference populations). Three DNA ploidy levels were detected: 667 plants were diploid (including diploid *U. dioica*, *U. kioviensis* and their putative hybrid), 71 were triploid (putative heteroploid hybrid) and 126 tetraploid (*U. dioica*) (Supplementary Table S2).

Relative genome size (RGS) of diploids formed a continuous series of partly overlapping values. Based on the selected criterium (see above; and also considering intermediate morphology), 47 plants are hybrids between *U. dioica* (2x) and *U. kioviensis* (mean±SD = 0.322±0.004, range 0.315–0.329), 357 plants were assigned to *U. kioviensis* (0.342±0.006, range 0.331–0.371) and 264 plants to diploid *U. dioica* (0.298±0.005, range 0.81–0.314). Diploid hybrids were recorded at Plačkův les, Ranšpurk, Šúr and Kóny.

Triploid individuals (0.458±0.011, range 0.426–0.499) were detected in all but one (Ranšpurk) population. Tetraploids (0.573±0.010, range 0.551–0.633) were detected in all populations, albeit at different frequencies (Table 1). Compared to diploid taxa and hybrids, RGS variation is higher in triploids and tetraploids.

The populations that hybridize differ considerably in the frequency of hybridization. The highest percentage of diploid hybrids was detected in populations at Ranšpurk (15.6%), Kóny (13.6%) and Plačkův les (13.0%). In contrast, the population in the Šúr alluvial woodland includes a markedly smaller percentage of hybrids (1.4%) and there are no diploid hybrids at Potsdam and Fehér-tó, at the former due to a lack of diploid *U. dioica*. The highest percentage of triploid hybrids was detected in the population at Plačkův les (24.4%), distinctly smaller percentages were recorded in populations at Potsdam (8.1%), Kóny (4.5%), Fehér-tó (1.8%) and Šúr (1.0%). There were no triploid hybrids in the population at Ranšpurk.

Morphometric analyses

The PCA based on the complete set of data revealed that the parental species *U. dioica* and *U. kioviensis* are well separated along the first component axis, PC1, whereas diploids and tetraploids of *U. dioica* did not form distinct groups. Both homoploid (2x) and heteroploid (3x) hybrids were scattered in ordination space with overlaps with both *U. dioica* and *U. kioviensis*, the former being closer to *U. kioviensis* and the latter to *U. dioica* (Fig. 4). The first axis accounted for 35.7% of the variation, with LTH, WLB, WST, STI, UTR and UTS being the most important characters contributing to the divisions along this axis. The second component axis accounted for 17.0% of the variation; the most important character that correlated with this axis was LLB (Table 3).

CDA1 including the parental species and their hybrids (projected into ordination space as passive samples) as predefined groups based on genome size, achieved a fairly clear separation between plants attributed a priori to *U. dioica* and *U. kioviensis* (Fig. 5). Diploids and tetraploids of *U. dioica* were partly separated from each other along the second canonical axis. Hybrids were mostly scattered in ordination space between clouds of their parental species, with overlaps with both parental species (diploid ones mainly with

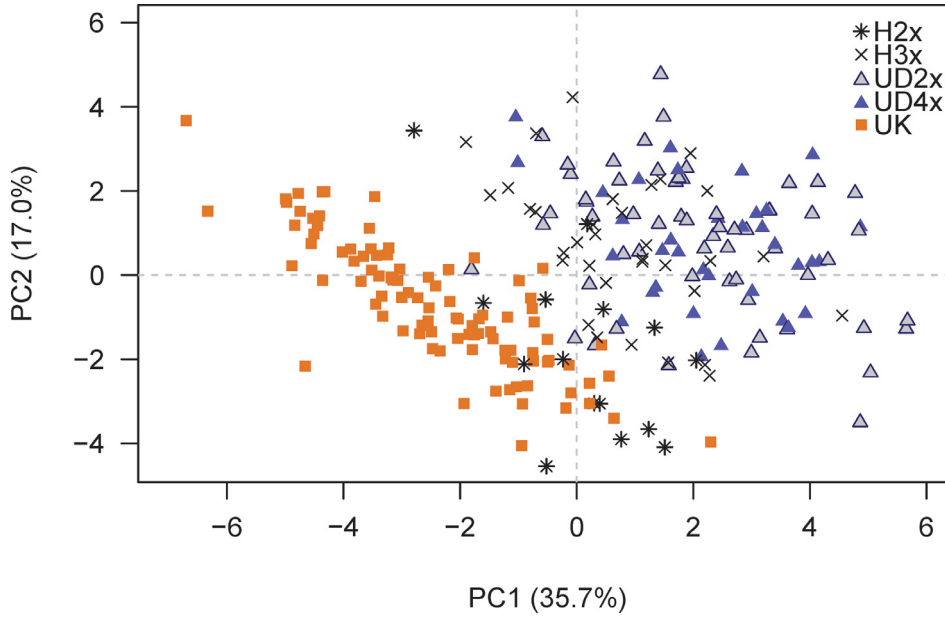

Fig. 4. Principal component analysis of individual plants of the parental species *Urtica dioica* (diploids – UD2x, tetraploids – UD4x) and *U. kioviensis* (UK, 2x), and their diploid (H2x) and triploid (H3x) hybrids, based on a set of 19 morphological characters.

Table 3. Results of principal component analysis (PCA) of *Urtica dioica* (diploids UD2x, tetraploids UD4x), *U. kioviensis* (UK, 2x), and their diploid (H2x) and triploid (H3x) hybrids. Character acronyms are explained in Table 2.

Character	PC1	PC2
UTR	0.742	0.378
UTU	0.686	0.329
STR	0.074	0.238
STU	0.000	0.138
UTS	0.720	0.510
STS	–0.420	–0.510
LLB	–0.429	0.784
LLP	–0.672	0.564
LLB/LLP	0.545	–0.072
WLB	–0.777	0.469
LLB/WLB	0.494	0.301
LBB	0.196	0.577
LTH	–0.884	0.144
LTW	–0.697	0.531
LTH/LTW	–0.584	–0.380
STI	–0.744	–0.300
LST	–0.647	0.263
LUS	–0.243	0.435
WST	–0.777	–0.104

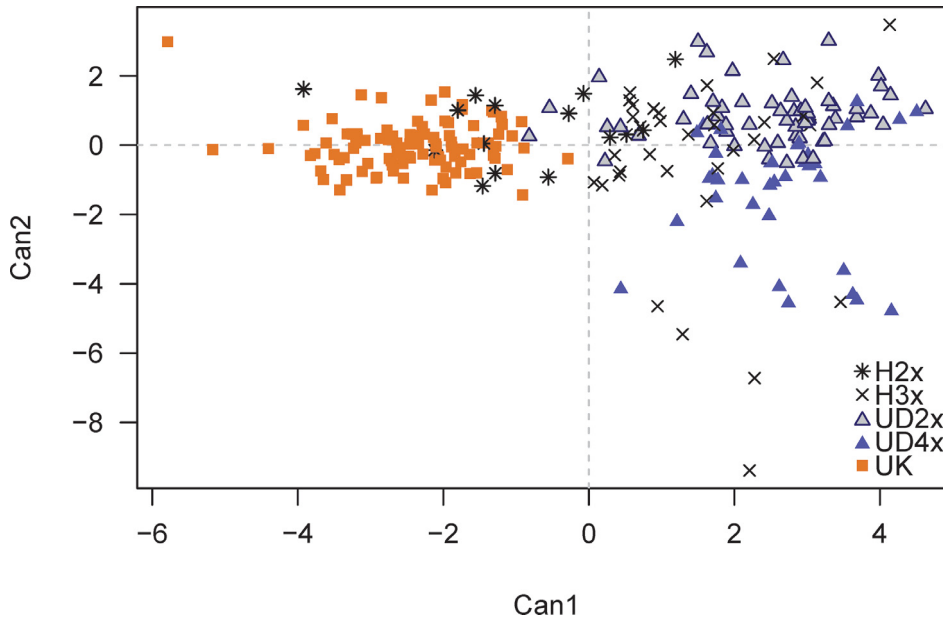

Fig. 5. Canonical discriminant analysis (CDA1) of individual plants of five groups defined based on genome size and ploidy: the parental species *Urtica dioica* (diploids – UD2x, tetraploids – UD4x) and *U. kioviensis* (UK, 2x), and their diploid (H2x) and triploid (H3x) hybrids projected onto the ordination space as passive samples, based on a set of 18 morphological characters. For the total canonical structure, see Table 4 (CDA1).

Table 4. Results of canonical discriminant analyses (CDA1) of *Urtica dioica* (diploids UD2x, tetraploids UD4x), *U. kioviensis* (UK2x) and their diploid (H2x) and triploid (H3x) hybrids presenting total canonical structure values that express correlations of morphological characters with canonical axes. Character acronyms are explained in Table 2.

Character	Can1	Can2
UTR	0.462	–0.008
UTU	0.322	0.015
STR	0.094	–0.666
STU	0.048	–0.441
UTS	0.570	–0.005
STS	–0.301	0.211
LLB	–0.037	0.172
LLP	–0.145	–0.009
LLB/LLP	0.167	0.254
WLB	–0.192	–0.010
LLB/WLB	0.211	0.248
LBB	0.246	0.115
LTH	–0.340	0.007
LTW	–0.168	0.002
LTH/LTW	–0.289	0.033
LST	–0.282	0.171
LUS	–0.041	0.237
WST	–0.506	–0.013

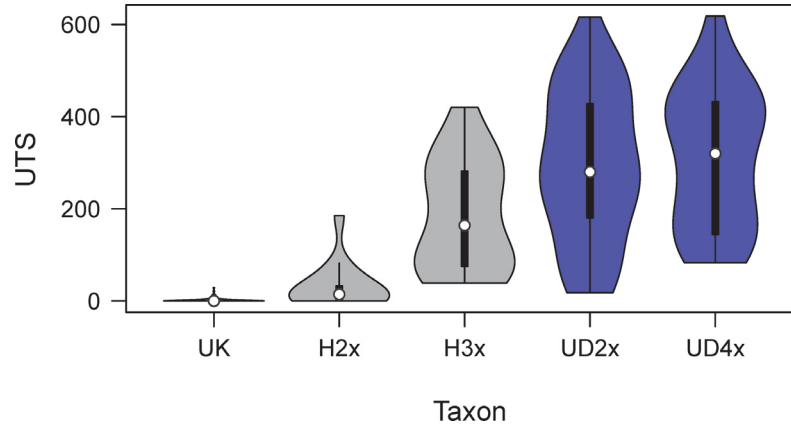

Fig. 6. Variation in the density of unicellular trichomes on the stems (UTS, trichome count per 1 cm stem length) of *Urtica kioviensis* (UK), diploid *U. dioica* (UD2x), tetraploid *U. dioica* (UD4x), and their diploid (H2x) and triploid (H3x) hybrids. The violin plots show the minimum, first quartile, median, third quartile, and maximum.

U. kioviensis, triploid ones with *U. dioica*). The first canonical axis accounted for 69.2% of the variation among the groups with the following variables contributing strongly to the separation along this axis (in descending order): UTS, WST and UTR. The second canonical axis accounted for 15.9% of the variation among the groups. The most important characters that correlated with this canonical axis were STR and STU (Table 4).

In CDA2, based on the parental species and their hybrids analysed as groups, a distinct separation of plants assigned a priori to *U. dioica* and *U. kioviensis* was achieved. Hybrids had an intermediate position between the parental species, but their separation was very weak and did not have any unique feature (Supplementary Fig. S3). The characters that were correlated most with the first canonical axis (which accounted for 53.5% of the variation) were related to UTS, WST and UTR. Those most correlated with the second axis (which accounted for 18.8% of the variation) were STS and WST (Supplementary Table S3).

A significant association between morphology and genome size in diploid accessions was confirmed by RDA (pseudo-F = 203.85, P = 0.001, 999 permutations), which accounted for 54.8% of the total variation in phenotypic traits.

The density of unicellular trichomes on the stem (UTS) differed significantly between *U. kioviensis*, diploid *U. dioica*, tetraploid *U. dioica*, diploid hybrids and tetraploid hybrids ($F_{4,235} = 95.6$, $P < 0.001$; Fig. 6). A follow-up test revealed significant differences between all but two pairs: *U. kioviensis* – diploid hybrids and diploid *U. dioica* – tetraploid *U. dioica*. The density of unicellular trichomes on the underside of a leaf (UTR) also differed significantly between the defined groups ($F_{4,235} = 65.4$, $P < 0.001$; Fig. 7). A follow-up test also revealed significant differences between all but three pairs: *U. kioviensis* – diploid hybrids, diploid hybrids – triploid hybrids and diploid *U. dioica* – tetraploid *U. dioica*. Similarly, tooth height at the widest point of a leaf (LTH) differed significantly between the defined groups ($F_{4,235} = 36.1$, $P < 0.001$; Fig. 8) and a follow-up test revealed differences between *U. kioviensis* and triploid hybrids, *U. kioviensis* and diploid *U. dioica*, and *U. kioviensis* and tetraploid *U. dioica*.

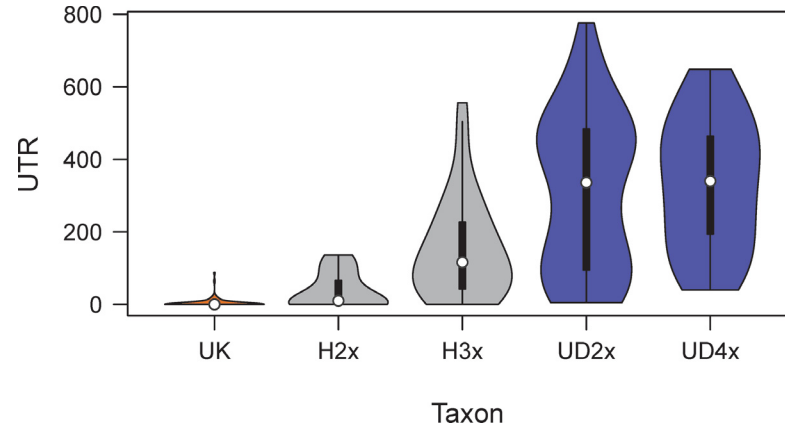

Fig. 7. Variation in the density of unicellular trichomes on the underside of the leaves (UTR, trichome count per cm^2) of *Urtica kioviensis* (UK), diploid *U. dioica* (UD2x), tetraploid *U. dioica* (UD4x), and their diploid (H2x) and triploid (H3x) hybrids. The violin plots show the minimum, first quartile, median, third quartile, and maximum.

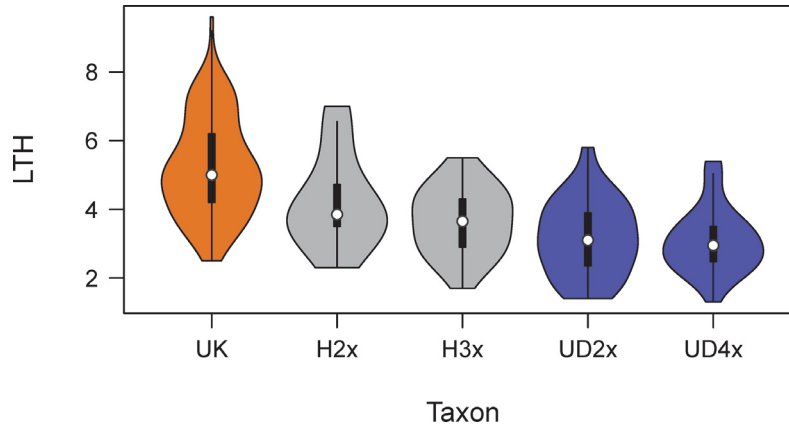

Fig. 8. Differences in tooth length (LTH, mm) of leaves of *Urtica kioviensis* (UK), diploid *U. dioica* (UD2x), tetraploid *U. dioica* (UD4x), and their diploid (H2x) and triploid (H3x) hybrids. The violin plots show the minimum, first quartile, median, third quartile, and maximum.

DNA analysis

A total of 32 samples were used for the sequencing and analysis of the ITS region. The length of the alignment after clipping high quality sequences was 569 bp and a single parsimony informative position was recorded (pos. 513, Supplementary Fig. S4A). According to the pattern in this position, the dataset could be split into three groups of accessions: (i) accessions with ribotype 'A' (nucleotide 'A' in position 513), (ii) accessions with ribotype 'G' (nucleotide 'G' in position 513) and (iii) accessions with ribotype 'R' (in position 513, both nucleotides, A and G, were detected). Ribotype 'A' was recorded in the reference population of *U. dioica* (2x) from Křivé jezero and in all other accessions assigned (based on RGS and morphology) to *U. dioica* (2x). Ribotype 'G' was recorded

in the reference population of *U. kioviensis* from Šúr centrum, and in all other accessions from hybridizing populations recognized as *U. kioviensis*. Ribotype 'R' was detected in six individuals from Plačkův les and Šúr that were recognized as hybrids.

The same 32 accessions were included in the analysis of the cpDNA marker trnH-psbA. The length of the alignment was 139 bp (after clipping the high quality sequences) with 12 variable and nine parsimony-informative positions. Although several of the variable positions were specific to accessions from a single population, the region of the alignment between position 43 and position 52 split the analysed accessions into two groups (Supplementary Fig. S4B) that were further recognized as haplotypes. Haplotype 'A' (sequence 'TTTTTTTGCT' in positions 43–52 in the alignment) was recorded in the reference population of *U. dioica* (2x) from Křivé jezero. The 2–3 bp long deletions in the variable region of haplotype A were recorded in all other accessions of *U. dioica* (2x) and in two hybrids from Plačkův les and Šúr. Haplotype 'B' (sequence 'AGCA-AAAAA' in positions 43–52 in the alignment) was recorded in all plants of *U. kioviensis* and in four hybrid plants.

Pollen viability

Whereas *Urtica kioviensis* and both cytotypes of *U. dioica* produce potentially fertile pollen (the stainability of all accessions exceeded 90%; see Supplementary Table S4), the percentage of fertile pollen produced by triploid hybrids was substantially lower. Nevertheless, even triploids (most likely interspecific *U. dioica* (4x) and *U. kioviensis* hybrids, as the genome size is in the upper part of the triploid's range) produce partially fertile pollen because significant percentages of their pollen were stained (54.9% to 81.1%). In contrast, diploid hybrids had a similar pattern to that of the parental species (over 90% potentially fertile).

Discussion

The present study revealed hybridization between the rare and endangered diploid species *Urtica kioviensis* and the widespread, mixed-ploidy (2x and 4x) *U. dioica* at six sites in central Europe. Homoploid hybridization has occurred between *U. kioviensis* and the diploid cytotype of *U. dioica* (which resembles *U. dioica* subsp. *subinermis*, but is unlikely to be mistaken for it). Heteroploid hybridization has occurred between *U. kioviensis* and the tetraploid cytotype of *U. dioica* and perhaps also between the two cytotypes of *U. dioica* and between diploid hybrids and one of the parental species.

The frequency of hybrid plants differs between localities. The cause of this may reside in the different spatial distribution of the plants of the two species/cytotypes (especially distances between them) and the frequency of plants of the particular parental species. Moreover, hybrids are considerably more frequent in populations where the diploid cytotype of *U. dioica* is more abundant compared to those where the tetraploid cytotype is predominant. The highest risk of genetic erosion was recorded at Plačkův les in southern Moravia. In contrast, it is likely that there is a much lower risk of introgression in the Šúr alluvial woodland in south-western Slovakia, where there is an extremely high frequency of *U. kioviensis* and a low anthropogenic effect (especially eutrophication), preventing the spread of *U. dioica* at this locality. Hybridization only occurs at the mar-

gins of the woodland and its frequency seems to be low. Similarly, a rather low risk of hybridization was recorded at Fehér-tó (Lake Fehér) in Hungary.

Heteroploid hybridization

The results confirm the occurrence of heteroploid hybridization at almost all sites with mixed populations of *U. kioviensis* and *U. dioica*. The fertility of triploid hybrids (68.9% of pollen viable) was lower than that of diploid hybrids (98.1% of pollen viable), because a high percentage of the pollen grains were aborted and achenes did not develop. Apparently, there was almost no backcrossing, as indicated by the discrete genome sizes (there is no continuous variation). However, variation in the genome size of triploid plants (range of RGS 0.426–0.499) might indicate different origins. There are four possible ways by which triploids may originate: (i) interspecific hybrids between *U. kioviensis* and tetraploids of *U. dioica*, (ii) hybrids between the diploid and the tetraploid cytotype of *U. dioica*, (iii) hybrids between homoploid hybrids (*U. kioviensis* × diploid cytotype of *U. dioica*) and tetraploid *U. dioica* and (iv) result of fusion of unreduced and reduced gametes of diploid plants. The results of the morphological analyses are not very clear: Triploid individuals are either in the *U. dioica* cluster or occupy an intermediate position between the clusters of *U. dioica* and *U. kioviensis*. The former could indicate crosses between the diploid and tetraploid cytotypes of this species or their autopolyploid origin from a reduced and unreduced gamete of the diploid cytotype of *U. dioica*. Alternatively, it could indicate introgressive hybridization in the direction of *U. dioica* (which is definitively not indicated by the results of flow cytometry). In contrast, triploid plants that occupy an intermediate position between the clusters of *U. dioica* and *U. kioviensis* point to their interspecific hybrid origin. The morphological traits selected also support hybrid origin of at least part of the triploid plants. Notwithstanding, one plausible argument against the hypothesis that the triploids are formed by crossing between cytotypes of *U. dioica* or by autopolyploidization of diploid *U. dioica* is the fact that triploid plants were almost never found in populations consisting only of the two cytotypes of *U. dioica* (Rejlová et al. 2019).

Heteroploid hybridization is generally perceived as less of a risk to the gene pool of endangered species in terms of genetic swamping, but at higher frequencies it can cause demographic swamping. Triploid individuals in diploid–tetraploid systems usually have markedly reduced fitness and fertility compared to their mother plants, so backcrossing is rare, meaning that they tend to have a very limited evolutionary potential (a phenomenon commonly known as the triploid block; e.g. Kolář et al. 2017). The heteroploid hybridization of *U. kioviensis* with tetraploid *U. dioica* can be compared to crosses between *Dianthus carthusianorum* L. (2x) and *D. arenarius* subsp. *bohemicus* (Novák) O. Schwarz (4x) (Vítová et al. 2015), in which a triploid block is also formed. Only 1% of the individuals analysed had a hybrid origin and reduced fertility. Macková et al. (2017) also confirms sterile triploid offspring of the hybridization between the rare *Prunus fruticosa* Pallas (4x) and the common *P. avium* L. (2x). In contrast, homoploid hybridization between *P. fruticosa* (dwarf cherry) and *P. cerasus* L. (sour cherry, 4x) is much more frequent, and introgression is proven in this group.

Homoploid hybridization

Homoploid hybridization is quite common in some admixed populations of *U. kioviensis* and the diploid cytotype of *U. dioica*. In the present study, the hybrid origin of selected plants was simultaneously confirmed by molecular methods (sequencing of the ITS region and one chloroplast intergenic spacer) and supported by the intermediate morphology of hybrid plants. The cpDNA analysis also indicated that the crossing occurred bidirectionally, although in most cases the maternal parent plant was *U. kioviensis*. However, while it is likely that most of the primary (F1) hybrids are well documented here, consequent introgressive hybridization (backcrosses) and the formation of hybrid swarms still remains a moot point. Some indices support this scenario, such as fertility of diploid hybrids, proven on the basis of pollen staining and achene formation (M. Pekařová, unpublished data), and more or less continuous variation in genome size and morphological characters at some localities (e.g. Plačkův les). An alternative explanation supposes a wider range in genome sizes and morphological plasticity. The same conclusion is reached, for example, by Suda et al. (2007) and Urfus et al. (2014) for the genus *Pilosella* Hill, Hanušová et al. (2014) in *Diphasiastrum* Holub, Agudo et al. (2019) in *Anacyclus* and by Macková et al. (2018) in *Prunus*.

Conclusions

In this study, the interspecific hybridization between the rare species *Urtica kioviensis* (2x) and its common relative *U. dioica* (2x, 4x) under natural conditions was identified and quantified. The results indicate that the extent of hybridization varies between sites and is likely to depend on the abundance of the particular species and cytotypes present. Particularly concerning from a conservation point of view is the hybridization between *U. kioviensis* and the diploid cytotype of *U. dioica*, which could affect the abundance of *U. kioviensis* via both genetic and demographic swamping. Based on current knowledge, it is not possible to unequivocally distinguish putative triploid hybrids between *U. kioviensis* and tetraploid *U. dioica*, between the diploid and tetraploid cytotypes of *U. dioica*, or between diploid hybrids (*U. dioica* 2x × *U. kioviensis*) and tetraploid *U. dioica*. Future studies should therefore disentangle the origin of triploids in mixed populations of *U. kioviensis* and both cytotypes of *U. dioica*.

Supplementary materials

Data S1. Relative genome sizes of the individuals sampled at the different localities.
Data S2. R code describing the classification of the non-reference individuals into taxa.
Fig. S1. Percentage of individuals classified as putative hybrids in response to the chosen level of the probability criterion in each of the populations.
Fig. S2. Leaf with marked characters that were measured for plants included in this study.
Fig. S3. Canonical discriminant analysis of individual plants of five groups predefined based on genome size and ploidy.
Fig. S4. Fragment of ITS alignment (positions 419–560 bp) with the single parsimony informative position at 513 bp based on which three ribotypes were defined.
Table S1. List of localities.
Table S2. Relative genome size of the *Urtica* samples analysed.

Table S3. Results of canonical discriminant analyses of *Urtica dioica*, *U. kioviensis*, and their diploid and triploid hybrids.

Table S4. Pollen viability of the parental species *Urtica dioica* and *U. kioviensis*, and their diploid and triploid hybrids.

Supplementary material is available at https://www.preslia.cz.

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Urtica kioviensis – vzácný druh ohrožený hybridizací

Hybridizace náleží k významným silám rostlinné evoluce. Jejím působením mohou vznikat nové druhy, ale současně může být i zdrojem ohrožení genetické integrity vzácných druhů. Zejména v důsledku introgrese může docházet k významnému snížení početnosti původního vzácného druhu, popř. i k jeho celkovému vyhynutí. Naše pozornost byla zacílena na dvojici taxonů z rodu *Urtica. Urtica kioviensis* (kopřiva lužní) je velmi vzácný diploidní druh, který se však vyskytuje sympatricky s běžnou *U. dioica* (kopřiva dvoudomá). *Urtica dioica* je na území střední Evropy převážně tetraploidní a jen vzácně se v aluviích k tetraploidnímu cytotypu přidávají i diploidi. Na šesti středoevropských populacích *U. kioviensis* jsme studovali hybridizaci s oběma cytotypy *U. dioica* užitím analýzy relativní velikosti genomu, morfometriky a u menšího počtu vzorků také pomocí molekulárních markerů. Detekovali jsme jak homoploidní (2x × 2x), tak heteroploidní (2x × 4x) hybridizaci. Kontinuální charakter cytometrických a morfometrických dat na diploidní úrovni může naznačovat rozsáhlou introgresivní hybridizaci mezi diploidní *U. dioica* a *U. kioviensis*. Z výsledků dále vyplývá, že zpětná hybridizace probíhá spíše směrem k *U. kioviensis.* Byly zaznamenány také triploidní rostliny. Hodnoty jejich relativní velikosti genomu byly značně variabilní a podobně i jejich pozice vůči rodičovským taxonům v PCA diagramu se výrazně lišila, takže lze jen obtížně určit jejich původ. U triploidních rostlin byl zaznamenán také větší podíl abortovaného pylu, a lze tedy usuzovat na jejich celkově sníženou fertilitu. Podíl hybridů v šesti zkoumaných populacích se značně liší. Diploidní hybridi jsou nejvíce zastoupeni v moravských populacích (Ranšpurk 15,6 %, Plačkův les 13,0 %) a v jedné z maďarských populací (Kóny 13,6 %), naopak nebyli zaznamenáni v populaci u Postupimi (Potsdam). Triploidní hybridi mají největší podíl v populaci Plačkův les (24,4 %), v ostatních populacích je jejich zastoupení výrazně nižší (1,0–8,1 %), zcela chybí v jihomoravské populaci Ranšpurk. Lze předpokládat, že *U. kioviensis* by mohla být v některých územích ohrožena nejen svou celkovou vzácností, ale právě i introgresivní hybridizací. Navazující otázkou je, jak účinně detekovaným hrozbám čelit a přispět k celkově efektivní ochraně druhu.

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