Preslia 98: 105–125, 2026
doi: 10.23855/preslia.2026.105

Preslia

Hidden diversity of *Ranunculus* sect. *Batrachium* in the Pannonian Basin: A Mediterranean trace in central Europe?

Vojtěch Dolejšek[1], Attila Mesterházy[2], Magdalena Lučanová[1,3],
Viktorie Brožová[1], Zdeněk Kaplan[3,4], Jiří Košnar[1], Jan Prančl[3] & Petr Koutecký[1,*]

[1]Faculty of Science, University of South Bohemia, Branišovská 1760, CZ-37005 České Budějovice, Czech Republic; [2]Hunyadi utca 55, H-9500 Celldömölk, Hungary; [3]Czech Academy of Sciences, Institute of Botany, Zámek 1, CZ-25243 Průhonice, Czech Republic; [4]Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-12801 Prague, Czech Republic
*corresponding author: kouta@prf.jcu.cz

Abstract: *Ranunculus* sect. *Batrachium* is one of the most taxonomically challenging groups of aquatic plants. The main factors contributing to this complexity include morphological reduction, phenotypic plasticity, polyploidy and hybridization. In this study, *Batrachium* diversity in the Pannonian Basin was investigated for the first time using molecular methods combined with morphological evaluation, genome size measurements and chromosome counting. Across 51 sampled populations, we identified seven traditionally recognized species known from other parts of central Europe, along with several hybrids, some of which have a complex origin involving backcrossing and introgression. Remarkably, we identified four previously unrecognized non-hybrid cytotypes, two of them belonging to the *Ranunculus peltatus* complex and two to the *Ranunculus trichophyllus* complex. DNA sequences indicate that these novel cytotypes are closely related to specific Mediterranean lineages of the European *Batrachium* species, either as their direct descendants or through unsampled or unknown ancestors closely related to them. The Pannonian Basin hosts a more diverse array of *Batrachium* lineages than previously described, including elements highly similar to Mediterranean lineages that are unique within central Europe, suggesting long-distance dispersal or relict persistence.

Keywords: aquatic plants, flow cytometry, DNA sequencing, chromosome counting, genome size, hybridization, polyploidy, reticulate evolution, origin of taxa

Introduction

Aquatic plants are an excellent model for studying patterns of cryptic diversity (Ito et al. 2020). They often have simplified bodies (Sculthorpe 1967, Cook 1999), frequently undergo hybridization (Cook 1966, Les & Philbrick 1993, Ito et al. 2020) and tend to exhibit a high degree of phenotypic plasticity (Sculthorpe 1967, Kaplan 2002, Santamaria 2002). These factors make morphological identification difficult and contribute to high levels of unrecognized diversity in aquatic plants, as evidenced by several recent studies (e.g. Li et al. 2020, Fehrer et al. 2022).

Water-crowfoots (*Ranunculus* section *Batrachium*, hereafter referred to as *Batrachium*) are among the most complex of all aquatic plants. Frequent hybridization, polyploidization

Received: 26 Aug 2025; Revised: 14 Feb 2026; Accepted: 17 Feb 2026; Published: 25 Mar 2026

and high morphological plasticity combined with overall simple morphology and inconspicuous nature of numerous morphological features often result in overlooked species and confusion in identification (Pizarro 1995, Englmaier 2016, Wiegleb et al. 2017, Koutecký et al. 2022). Their variable morphology depends on environmental factors as well as the phenological stage within the season (Cook 1966, Zander & Wiegleb 1987, Hong 1991, Garbey et al. 2004). From a practical perspective, unresolved taxonomy and the inability to distinguish rare forms from common species can hinder conservation efforts (Wiegleb et al. 2017).

Batrachium is a monophyletic lineage within the genus *Ranunculus* (Emadzade et al. 2010, Hörandl & Emadzade 2012). It is distributed worldwide, with a centre of diversity in western Europe (Cook 1966, Pizarro 1995, Wiegleb et al. 2017). Recent investigations have revealed high and taxonomically undescribed diversity in the Mediterranean area (Jopek et al. 2023, Prančl et al. 2025). *Batrachium* is found in a wide range of freshwater habitats, including both running and still waters. Some species exhibit specific habitat preferences and can tolerate elevated salinity or brackish water, such as *R. baudotii* and *R. rionii* (Cook 1966, Wiegleb et al. 2017). The ability to produce morphologically distinct types of leaves (heterophylly) is common in the section. Some species can form both leaves with flat lamina floating on the water surface and submerged capillary leaves consisting of hair-like segments (heterophyllous species), while others produce only one leaf type (homophyllous species, in central Europe represented merely by those forming only capillary leaves) (Cook 1966, Englmaier 2016, Wiegleb et al. 2017). Some species capable of both often form only capillary leaves (e.g. *R. aquatilis*). Many *Batrachium* species have the potential to produce terrestrial forms with reduced morphology on exposed wet substrate when the water level drops. These forms have shortened internodes and leaves, with capillary leaves outside water being denser and more rigid than submerged ones (Cook 1966). Identification of such forms based solely on morphology is often impossible.

Until recently, five ploidy levels have been repeatedly reported in *Batrachium*: from diploids (2n = 16) to hexaploids (2n = 48) (Wiegleb et al. 2017, Prančl et al. 2018). In Europe, three species have been reported to be exclusively diploid, including *R. circinatus*, *R. rionii* and *R. sphaerospermus*. Other species include both diploids and polyploids (*R. fluitans*, *R. hederaceus*, *R. ololeucos*, *R. omiophyllus*, *R. peltatus*, *R. saniculifolius*, *R. trichophyllus*). The remaining European species are polyploid, usually with multiple, mainly even-numbered ploidy levels. Triploids are rare and among the distinguished species, they are regularly present only in *R. fluitans* (in addition to diploids). Apart from that, uncommon triploid hybrids are recorded, which may, however, form extensive stands via clonal propagation (Zalewska-Gałosz et al. 2023). Pentaploids are rare and of hybrid origin (Cook 1966, Wiegleb et al. 2017, Prančl et al. 2018, 2025, Koutecký et al. 2022, 2025). Only recently, several new high ploidy levels (heptaploids, decaploids and dodecaploids) were discovered in plants from the Iberian Peninsula, resulting from allopolyploidy (Prančl et al. 2025).

Prančl et al. (2018) demonstrated that genome size obtained by flow cytometry is a useful tool for delimitation of taxa in *Batrachium*. They showed that almost all central-European taxa are characterized by unique, non-overlapping genome size ranges. Their study also revealed cryptic diversity in *R. trichophyllus*, detecting two distinct tetraploid cytotypes (provisionally referred to as *R. trichophyllus* A and *R. trichophyllus* B) differing slightly in morphology and ecology, and in *R. penicillatus*, where several tetraploid and one hexaploid cytotype were found.

The global phylogeny of *Batrachium* remains unresolved. There are only four molecular studies that include a broader sampling of European taxa (Bobrov et al. 2015, Koutecký et al. 2022, Jopek et al. 2023, Prančl et al. 2025). In these studies, plastid DNA and the ITS markers were used for phylogenetic reconstructions. Based on plastid DNA, several consistent haplotype groups can be distinguished in central Europe, namely those of *R. peltatus*, *R. trichophyllus* and *R. circinatus*. The ITS genotypes show reticulate patterns that are inconsistent with plastid haplotype groups, likely due to hybridization and allopolyploidy, resulting in reticulate evolution. Some currently recognized species are polyphyletic, heterogeneous or include cryptic taxa, with *R. trichophyllus* being the most evident example.

This paper builds upon the study of Koutecký et al. (2022), which focused on central Europe but did not include representative sampling from the Pannonian Basin. Due to its climatic characteristics and geographical location, the Pannonian Basin constitutes a specific biogeographic area with unique species and habitats rarely found elsewhere in central Europe. It is a migratory crossroads for central-European, Sub-Mediterranean, Sub-Atlantic, Ponto-Caspian and eastern-European species (Fekete & Varga 2006, Bartha & Tiborcz 2017). One of the most remarkable phenomena is the temperate grassland biome known as ‘puszta’. It is a unique mosaic of freshwater and saline wetlands, periodically drying shallow pools and steppic vegetation scarcely paralleled elsewhere in central Europe (Szilágyi et al. 2024). Saline habitats developed under specific hydrological and climatic conditions and were once widespread, particularly in the Great Hungarian Plain (Boros 2003). Large-scale 19th-century river regulation reshaped the region’s hydrology, causing many permanent wetlands to vanish or become seasonal. Agricultural intensification has further reduced these habitats, and the puszta as a whole, to small remnants (Demeter & Veen 2001, Molnár & Borhidi 2003), making them some of Europe’s most endangered habitats.

In the Great Plain, *Batrachium* species are mainly found in the Tiszántúl region (eastern Hungary, between the Tisza River and the Romanian border), where the largest remaining natural swamps persist. In the past, extensive marshes also occurred in the Danube–Tisza interfluve, but they have mostly disappeared due to regional groundwater subsidence (Fekete & Varga 2006). In the Tiszántúl region, water-crowfoots are most abundant in the marshes developed on solonetz soils, which have moderate salinity. These habitats are replenished by autumn and winter rains and dry out during summer, resembling the dynamics of Mediterranean temporary pools (Zacharias & Zamparas 2010, Lukács et al. 2013). They also host water-associated species typical of the Mediterranean area (e.g. *Myosotis sicula*, *Trifolium ornithopodioides*, *Lythrum borysthenicum*; Király 2009, Mesterházy 2017). Recently, the Atlantic-Mediterranean *Callitriche brutia* subsp. *brutia* and the strictly Mediterranean *C. brutia* subsp. *naftolskyi* have also been recorded in the region (Prančl et al. 2020, Mesterházy et al. 2025). Preliminary climate models (Bartholy et al. 2009) predict milder winters and hotter summers in the Pannonian Basin in the future, which may favour the survival and spread of Mediterranean species. These may be introduced to the Great Plain by migratory birds, for which these marshes serve as key stopover, resting and feeding sites. The main migratory route in the region leads to the Eastern Mediterranean area (Csörgő et al. 2009), though some birds (*Himantopus himantopus*, *Vanellus vanellus*) that feed on aquatic plant seeds also reach the Iberian Peninsula (Spina et al. 2022). Seeds may be introduced into the Pannonian

Basin via endozoochory by shorebirds or wild geese capable of long-distance seed dispersal (Lovas-Kiss et al. 2018, 2023).

The aim of this study is to assess the diversity of *Batrachium* in the Pannonian Basin using the same methodological approach as in our previous studies (Koutecký et al. 2022, 2025, Prančl et al. 2025), i.e. a combination of flow cytometry, chromosome counting and Sanger sequencing of plastid and nuclear DNA regions. In Hungary, we focus primarily on the puszta areas. Given the climatic and biogeographical characteristics of the Pannonian Basin, we hypothesize the presence of a specific assemblage of *Batrachium* taxa, including some absent from other parts of central Europe. It is also possible that the widespread, morphologically defined species are represented by previously unrecognized (cryptic) lineages. Additionally, we expect the presence of hybrids, as in other thoroughly studied parts of Europe, despite their lack of mention in the literature.

Materials and methods

Field sampling

Sampling was carried out in four countries within the Pannonian Biogeographic Region as defined by the European Environment Agency (EEA 2008): Hungary, northern Croatia, southern Slovakia and eastern Austria. We mainly focused on Hungary, as it encompasses the largest portion of the Pannonian area. We also added samples from a few localities with similar habitats in northern Croatia outside the Pannonian Biogeographic Region but in the adjacent Sava River plain (Fig. 1). Since *Batrachium* mostly grows there in various types of freshwater temporary marshes or water bodies with strongly fluctuating water levels, we focused mainly on these habitats. Some sampled localities were slightly halophilic, based on surrounding vegetation, but no highly saline habitat was sampled (occurrence of *Batrachium* is not known there). Permanent water bodies were represented by a side arm of the Danube River (the only site of *R. fluitans*) and deep sand/gravel pits. For details on sampled populations and individuals, see Supplementary Table S1 and Supplementary Data S1. Part of the material comes from previous studies (Prančl et al. 2018, Koutecký et al. 2022).

Individuals were collected to represent the morphological variation within each population. In most cases, at least five individuals were sampled per population. In smaller populations, fewer individuals were collected, while in populations containing multiple taxa, up to five individuals per taxon were sampled, if available. To avoid collecting ramets of the same clone, we sampled one stem per individual, ensuring a minimum distance of three metres between sampled plants. We aimed to collect only well-developed aquatic individuals (submerged or floating). However, a small number of terrestrial plants were included from localities where the water level had dropped. For each plant, an herbarium voucher, a sample of leaf tissue for DNA analyses and a sample of living tissue for flow cytometry were collected. Leaf samples for DNA analyses were placed in small paper envelopes and quickly dried in silica gel. Samples of living leaf tissue were stored in zip-lock plastic bags in a portable refrigerator and processed as soon as possible, usually within ten days. Herbarium vouchers are deposited in the PRA and CBFS herbaria.

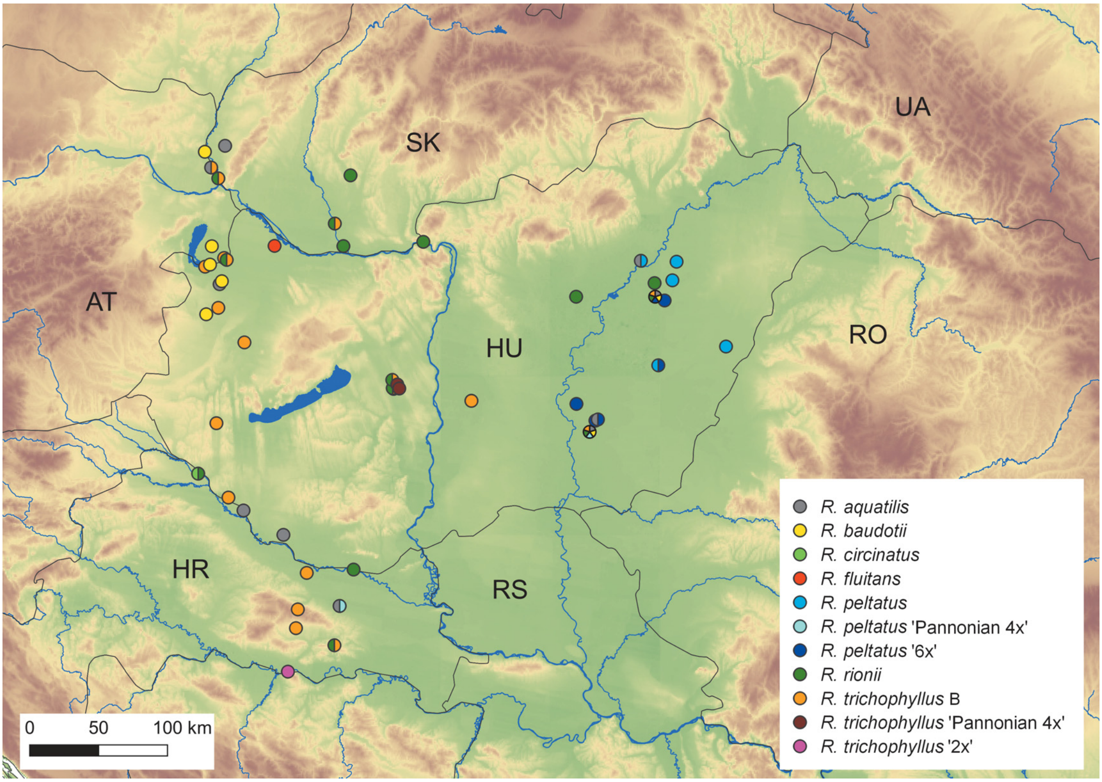

Fig. 1. Map of sampled localities.

Identification of taxa

The plants were identified based on a combination of morphological features, genome size, and chromosome counts following published data (Király 2009, Prančl et al. 2018, 2025, Kaplan et al. 2019, Koutecký et al. 2022). The biological entities distinguished in this way and characterized by a specific combination of traits are hereafter referred to as 'cytotypes'; see Supplementary Data S1 for photos of the newly recognized cytotypes. Hybrids were recognized by their intermediate characters, reduced to absent fertility and intermediate genome size. Some hybrids were further confirmed using DNA sequences. Following Kaplan et al. (2025), we distinguish between allopolyploid species and interspecific hybrids. In contrast to many *Batrachium* species that are allopolyploids, having originated long ago, established large ranges and are fully fertile, we restrict the term 'hybrid' to plants presumably produced de novo at the sampled sites through recent hybridization and characterized by reduced fertility and intermediate character states. The nomenclature used for *Batrachium* species follows Wiegleb et al. (2017), while the other plant names follow Király (2009).

Flow cytometry

The genome size of each individual was estimated using flow cytometry with the propidium iodide stain and using *Bellis perennis* (2C = 3.38 pg, Schönswetter et al. 2007) as the internal standard. At least three individuals per sampled population and taxon were measured individually; the rest of the material was measured in bulked samples to check population homogeneity. If more than one cytotype per population was detected, the individuals were reanalysed individually. The sample preparation followed the protocol of Doležel et al. (2007). About 0.25 cm^2 of leaf tissue was chopped together with the equivalent amount of leaf tissue of *Bellis perennis* (internal standard) in a plastic Petri dish using a sharp razor. The tissue was chopped in 0.5 ml of ice-cold Otto I buffer (0.1M citric acid, 0.5% Tween 20). The obtained suspension was filtered through a 42-μm nylon mesh (Uhelon 130T fabric) and incubated for about five minutes at room temperature. The nuclei were then stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with 2-mercaptoethanol (2 μg/ml) and propidium iodide (PI) (50 μg/ml). Samples were then incubated for several minutes and analysed using a Partec CyFlow SL or Sysmex/Partec CyFlow Space instrument equipped with a green diode-pumped solid-state laser (Cobolt Samba, 532 nm, 100 mW output power). The fluorescence intensity of 5,000 particles was measured. Samples were measured three times on different days and if the range of variation exceeded the 2% threshold, the sample was measured one more time and the most outlying measurement was discarded. The mean of the repeated measurements was used as the individuals' genome size. The histograms were evaluated using FloMax software, ver. 2.9 (Sysmex/Partec) and flowPloidy package for R (Smith et al. 2018).

Chromosome counting

We followed a slightly modified protocol used in Prančl et al. (2018). We tried counting chromosomes from adventive roots of cultivated plants but this failed due to the absence of mitoses. We successfully counted chromosomes only from seedlings grown from collected achenes. Ripe achenes were collected from selected herbarium specimens and placed on wet filter paper in Petri dishes to induce germination. We were limited by a small number of germinating achenes but managed to improve germination rates by stratification. The non-germinated achenes were left to dry and stored at room temperature for about six months, then placed in a refrigerator for three weeks before being transferred again to wet filter paper in Petri dishes. Some additional achenes germinated after this treatment. This contrasts with the results of Carta et al. (2012), who reported lower germination rates of *R. baudotii* after achene desiccation. After germination, the youngest primary roots were collected. The roots were pre-treated in a saturated water solution of p-dichlorobenzene at room temperature for three hours, then fixed in a freshly prepared 3:1 mixture of 96% ethanol and glacial acetic acid and stored in a refrigerator. During preparation, the material was first macerated in a 1:1 mixture of ethanol and hydrochloric acid for 20–30 seconds, depending on the rigidity of the root. After that, it was washed in water and transferred onto a microscope slide. Only the meristematic tissues were kept and stained in a drop of lacto-propionic orcein. The tissue was then covered with a coverslip and firmly pressed. The preparations were examined under an Olympus BX 51 microscope equipped with a DP-71 Olympus digital camera with the DP Controller imaging software 3.1 (Olympus Corp.).

DNA extraction and amplification

DNA was extracted from leaves dried in silica gel, which had been fixed in the field. Laminar leaves were preferred for the extraction; if unavailable, young capillary leaves were used instead. The extraction followed the CTAB protocol (Doyle & Doyle 1987); the DNA extract was diluted in a ratio 1:10 with sterile water. Three loci were amplified following the study of Koutecký et al. (2022). Two noncoding plastid spacers, *rpl*32–*trn*L^{UAG} and 3'*rps*16–5'*trn*K, were amplified using primers by Shaw et al. (2007); for *rpl*32–*trn*L^{UAG} internal primers described in Koutecký et al. (2022) were also used. The ITS region of the nuclear ribosomal DNA was amplified using the primers ITS-F (King et al. 2001) and ITS4 (White et al. 1990). Each PCR was run in a reaction containing 0.5 U Taq polymerase (Top-Bio, Czech Republic) in the reaction buffer of the manufacturer, 0.3 µm of each primer, 0.2 mM dNTPs, 0.5 µl of sample DNA and sterile water to a total volume of 10 µl. The following PCR protocol was used: 94 °C for 5 min, 35–40 cycles at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, 72 °C for 10 min. If a sample showed weak amplification, the reaction was repeated using a higher amount of DNA extract. Sanger sequencing was performed by Eurofins Genomics. To confirm the variable ITS positions, at least several individuals per cytotype were sequenced in both directions, and only polymorphisms seen in both sequencing reads were used in the analyses.

Data analyses

Sequences were manually checked using Finch TV 1.4.0. The intra-individual polymorphisms in the ITS were coded using the IUPAC ambiguity codes. The sequences were then manually aligned using BioEdit 7.2.0 (Hall 1999). The two cpDNA regions were concatenated, and both alignments (cpDNA and ITS) were trimmed according to the shortest sequence. Using FaBox 1.5 (Villesen 2007), the sequences were collapsed into unique haplotypes. Plastid sequences were analysed using the TCS network (Clement et al. 2002) implemented in PopART (Leigh & Bryant 2015); gaps were coded as unique substitutions to count as one mutation step in the analyses. The ITS data were analysed with SplitsTree 4 (Huson & Bryant 2006). The NeighborNet algorithm based on uncorrected P distance (Hamming distance) was applied, and handling of ambiguous states was set to average.

For the plastid DNA markers, 71 sampled individuals, including six hybrids, were used in the analysis along with additional 27 Pannonian individuals from Koutecký et al. (2022). Sequences of haplotypes of the central-European cytotypes selected from Koutecký et al. (2022) were added for comparison (one per haplotype) and labelled with their cp-haplotype names. Namely, we included three haplotypes of *R. peltatus* (one frequent and two rare, marked as pelt, pelt-2, pelt-3), two haplotypes each for *R. circinatus* and *R. rionii* (circ, circ-2 and rio, rio-2) and one haplotype each for *R. baudotii* (baud), *R. fluitans* (flui), *R. penicillatus* A (pelt), *R. trichophyllus* A (trich-A) and *R. trichophyllus* B (trich-B). Additionally, selected haplotypes of *R. peltatus* s.l. and *R. trichophyllus* s.l. from south-western Europe distinguished in Prančl et al. (2025) were included: *R. peltatus* B (diploid, haplotype pelt), *R. peltatus* C (mostly tetraploid, pelt-c) and *R. trichophyllus* C (likely tetraploid, trich-c). We performed the analysis of the ITS region twice. First, we included 65 individuals sampled during this study, along with 27 Pannonian sequences from Koutecký et al. (2022), but excluding recent hybrids to provide a clearer structure. As with the plastid regions, we added selected ITS sequences of genotypes of the central-European cytotypes from Koutecký et al. (2022) and sequences of genotypes from cytotypes from south-western Europe from Prančl et al. (2025). The selected genotypes from Koutecký et al. (2022) are labelled with their ITS genotype numbers in the analyses. The selected genotypes of plants from south-western Europe are labelled with their ITS genotype numbers prefixed by 'I-' (standing for Iberian) in the analyses. Second, we performed the analysis in the same way as the previous one but also included the hybrids.

Results

Genome size and chromosome counts

Genome size data are summarized in Table 1. In total, 242 individuals from 51 populations were collected and analysed using flow cytometry. Based on genome size, seven *Batrachium* cytotypes previously reported from central Europe (Prančl et al. 2018, Koutecký et al. 2022) were found: *Ranunculus aquatilis* (6x), *R. baudotii* (4x), *R. circinatus* (2x), *R. fluitans* (2x), *R. peltatus* (4x), *R. rionii* (2x) and *R. trichophyllus* B (4x). In addition, four previously unknown cytotypes were identified based on genome size, morphology and DNA sequences. Additionally, hybrids were found in two populations.

Table 1. List of species, cytotypes and hybrids recorded. The delimitation of 'morphological' species follows the taxonomic concept of Wiegleb et al. (2017). The cytotypes are defined by their genome size ranges, DNA markers and morphology. N_T – total number of recorded individuals with genome size measured via flow cytometry. N_S – number of sequenced individuals, from which the following statistics are computed. Ratio to standard and mean ratio are related to internal standard *Bellis perennis*, 2C – mean genome size (in pg of DNA), SD – standard deviation of the genome size; Min – minimal recorded genome size; Max – maximal recorded genome size. The ploidy is inferred based on genome size according to chromosome counts published for particular cytotypes by Prančl et al. (2018) and Koutecký et al. (2022) as well as on newly determined chromosome counts (in the case of previously unpublished cytotypes, 2n) or was not estimated (?). Individuals of *R. peltatus* 'Pannonian 4x' and *R. peltatus* '6x' cytotypes with outlying values of genome size are presented separately and marked with *. Their ploidy could not be directly determined by chromosome counting and is therefore inferred from genome size, it is indicated with a tilde. 1Cx – monoploid genome size in pg of DNA (2C / ploidy).

Morphological species	Cytotype (based on genome size, sequences and morphology)	N_T	N_S	Ratio to standard	Mean ratio	2C	SD	Min	Max	Ploidy	2n	1Cx
R. aquatilis	*aquatilis*	30	14	3.67–3.93	3.79	12.81	0.12	12.40	13.28	6x		2.14
R. baudotii	*baudotii*	15	6	2.52–2.63	2.58	8.72	0.03	8.52	8.89	4x		2.18
R. circinatus	*circinatus*	2	1	1.67		5.64				2x		2.82
R. fluitans	*fluitans*	3	1	1.14		3.85				2x		1.93
R. peltatus	*peltatus*	24	5	1.87–1.98	1.95	6.59	0.04	6.32	6.69	4x		1.65
	peltatus 'Pannonian 4x'	6	4	2.46–2.51	2.49	8.42	0.02	8.31	8.48	4x	32	2.11
	peltatus 'Pannonian 4x' *	1	1	2.23		7.54				~4x		
	peltatus '6x'	27	12	3.31–3.60	3.44	11.63	0.07	11.19	12.17	6x	48	1.94
	peltatus '6x' *	1	1	3.85		13.01				~6x		
R. rionii	*rionii*	56	16	1.54–1.61	1.59	5.37	0.02	5.21	5.44	2x		2.69
R. trichophyllus	*trichophyllus* B	72	24	2.77–3.00	2.90	9.80	0.08	9.36	10.14	4x		2.45
	trichophyllus 'Pannonian 4x'	10	5	2.73–2.82	2.78	9.40	0.03	9.23	9.53	4x	32	2.38
	trichophyllus '2x'	2	2	1.71–1.72	1.71	5.79	0.01	5.79	5.80	2x	16	2.90
Hybrids												
hybrids HR-06		4	3	1.91–1.94	1.92	6.49	0.02	6.46	6.56	?		
hybrid HR-06-7	*R. aquatilis* × *R. peltatus*	1	1	2.84		9.60				~5x		
hybrids HU-13	*R. aquatilis* × *R. peltatus*	3	2	2.58–2.80	2.69	9.09	0.45	8.72	9.46	~5x		

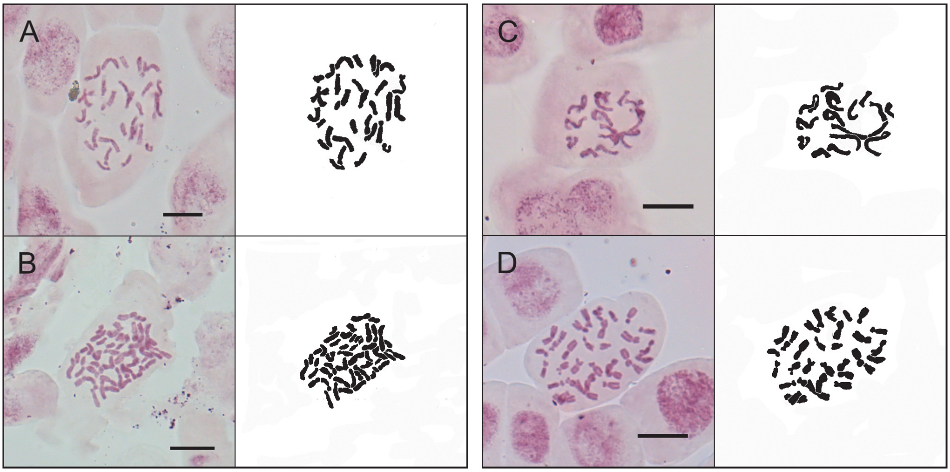

Fig. 2. Microphotographs of mitotic chromosomes and their interpretations:
(a) *Ranunculus peltatus* 'Pannonian 4x' (2n = 32), (b) *R. peltatus* '6x' (2n = 48),
(c) *R. trichophyllus* '2x' (2n = 16), (d) *R. trichophyllus* 'Pannonian 4x' (2n = 32).
The scale bar represents 10 μm.

Of the four new putatively non-hybrid cytotypes, two belong morphologically to *R. peltatus* s.l. and two belong to *R. trichophyllus* s.l. (see Supplementary Data S1). In addition to the common tetraploid cytotype of *R. peltatus* with an average genome size 2C = 6.76 pg (Prančl et al. 2018, Koutecký et al. 2022), we identified an additional tetraploid cytotype in two populations. Although morphologically indistinguishable from the common cytotype, it exhibited a larger genome size (range of 8.31–8.48 pg). One outlying individual with a genome size of 2C = 7.54 pg was found in one of these populations. We refer to these plants as *R. peltatus* 'Pannonian 4x'. This genome size range falls within previously published values for tetraploid *Batrachium* cytotypes, and the ploidy was confirmed by chromosome counting (2n = 32, Fig. 2A). The chromosome number of the outlier could not be determined. Furthermore, we also detected a hexaploid cytotype of *R. peltatus* s.l. with the genome size ranging from 11.19 to 12.17 pg; one plant (B18-005-1) had a slightly higher value of 13.01 pg. This cytotype occurred in seven sampled populations and is referred to as *R. peltatus* '6x'. *Ranunculus peltatus* '6x' is also morphologically indistinguishable from the common central-European tetraploid cytotype. Its hexaploid status was confirmed by chromosome counting (2n = 48, Fig. 2B); however, the chromosome number could not be obtained for the outlying individual.

In *R. trichophyllus* s.l., besides the widespread *R. trichophyllus* B, two additional cytotypes were found. One population (two individuals, HR-01) had a mean genome size of 2C = 5.81 pg and was diploid (2n = 16, Fig. 2C); we refer to this cytotype as *R. trichophyllus* '2x'. These diploid plants are morphologically indistinguishable from tetraploid *R. trichophyllus* B. Finally, we distinguish the cytotype *R. trichophyllus* 'Pannonian 4x', which was found in two geographically proximate populations (HU-25 and HU-27). It is tetraploid (2n = 32, Fig. 2D) with a genome size of 2C = 8.42 pg, similar to but at the lower end of the range of *R. trichophyllus* B. However, it differs morphologically (resembling *R. rionii* in its receptacle shape and achene size, see Supplementary Data S1, Supplementary Fig. S6) and genetically (see below).

Plastid regions

Except for the new haplotype belonging to *R. trichophyllus* ‘2x’, all Pannonian accessions are identical to the selected central-European haplotypes of Koutecký et al. (2022). For an overview of the variable positions, see Supplementary Tables S2 and S3. The TCS haplotype network is shown in Fig. 3. Three haplotype groups were identified. The *R. circinatus* group contains *R. circinatus*, *R. rionii* and the new haplotype specific to the diploid *R. trichophyllus* (‘trich 2x’). The *R. trichophyllus* group contains no sequences from the Pannonian samples collected for this study. The majority of the accessions are included in the *R. peltatus* group. Most individuals have the common ‘pelt’ haplotype (*R. aquatilis*, all cytotypes of *R. peltatus* s.l., *R. trichophyllus* B and *R. trichophyllus* ‘Pannonian 4x’), whereas *R. baudotii* and *R. fluitans* have specific haplotypes, each differing from the ‘pelt’ haplotype by a single mutation step. Individual taxa/cytotypes that were included in the previous study (Koutecký et al. 2022) showed the same haplotypes in the Pannonian Basin as in other parts of central Europe. The only exception is *R. trichophyllus* B, which displayed two distinct plastid haplotypes from two different haplotype groups outside the Pannonian Basin (‘pelt’ and ‘trich-B’; Koutecký et al. 2022), whereas only plants with the ‘pelt’ haplotype were found in the studied area.

The ITS region

The ITS neighbour network based on non-hybrid individuals is presented in Fig. 4; for the analysis including hybrids see Supplementary Fig. S1, and for an overview of the variable positions see Supplementary Table S4. Some of our Pannonian accessions are identical to selected genotypes of the central-European cytotypes distinguished by Koutecký et al. (2022), whereas none of our Pannonian accessions are identical to selected genotypes of taxa from south-western Europe identified by Prančl et al. (2025). The network shows the same general pattern as that reported by Koutecký et al. (2022) and Prančl et al. (2025). A group comprising *R. circinatus* and *R. rionii* is distinct. This group also includes sequences of *R. trichophyllus* ‘2x’, *R. trichophyllus* ‘Pannonian 4x’ and the south-western European *R. trichophyllus* C. The second group is formed by part of the *R. trichophyllus* B, *R. aquatilis* and *R. baudotii* accessions. Some accessions of *R. baudotii* and a single accession of *R. peltatus* ‘6x’ combine sequences of *R. aquatilis/R. trichophyllus* B with those of *R. fluitans* or *R. peltatus* ‘Pannonian 4x’, which form the third group along with south-western European *R. peltatus* B. The fourth group includes tetraploid central-European *R. peltatus* and sequences of *R. peltatus* C (south-western Europe) and *R. trichophyllus* A (central Europe), all of which are absent from the Pannonian Basin. Most samples of *R. peltatus* ‘6x’, one sample of *R. peltatus* ‘Pannonian 4x’ (HR-06-04) with an outlying genome size and central-European *R. penicillatus* show an additive pattern between the *R. peltatus/R. trichophyllus* A group and the *R. fluitans/R. peltatus* ‘Pannonian 4x’ group.

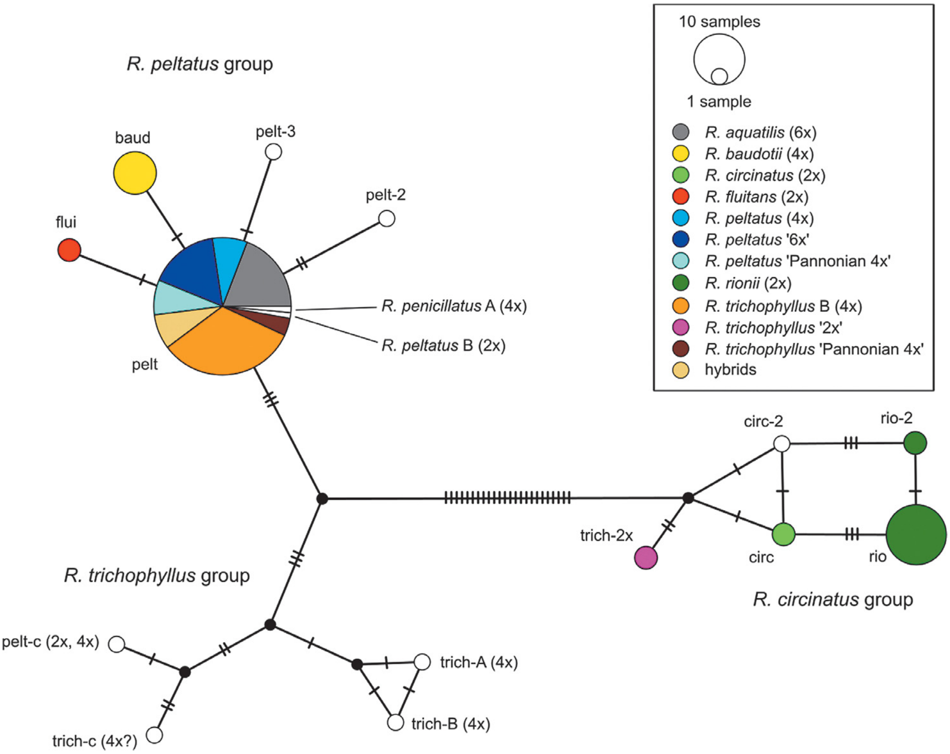

Fig. 3. Haplotype network of the concatenated plastid regions *rpl*32–*trn*L^{UAG} and 3'*rps*16–5'*trn*K, supplemented with sequences of other haplotypes (one sequence each) found in non-Pannonian central Europe (taken from Koutecký et al. 2022) and south-western Europe (adopted from Prančl et al. 2025). The size of the symbols corresponds to the number of sequences in the Pannonian dataset, cytotypes are distinguished by colours. For each taxon, the ploidy is given in brackets either in the legend or, in case of taxa not sampled in this study, in the network. The ploidy of *R. trichophyllus* C is not verified by chromosome counting. Haplotypes and taxa not sampled in the Pannonian Basin are shown in white.

Hybrids

Eight hybrid individuals, six of which were sequenced, were found in two populations. In the plastid DNA, no haplotype was specific to the hybrids; all hybrids possessed the 'pelt' haplotype. Three hybrid individuals from population HU-13 were intermediate in morphology and genome size between central-European tetraploid *R. peltatus* and *R. aquatilis*, both of which were present at the site. Their hybrid origin was also confirmed by the additive pattern of the ITS region and by their intermediate position in the ITS network (Supplementary Fig. S1). Five hybrids from population HR-06, growing along with *R. aquatilis* and *R. peltatus* 'Pannonian 4x', were more variable in terms of morphology, genome size and ITS sequences. Their origin remains unclear based on the available data, but all appear to combine features of *R. peltatus* s.l. and *R. aquatilis*/*R. trichophyllus* B in varying proportions.

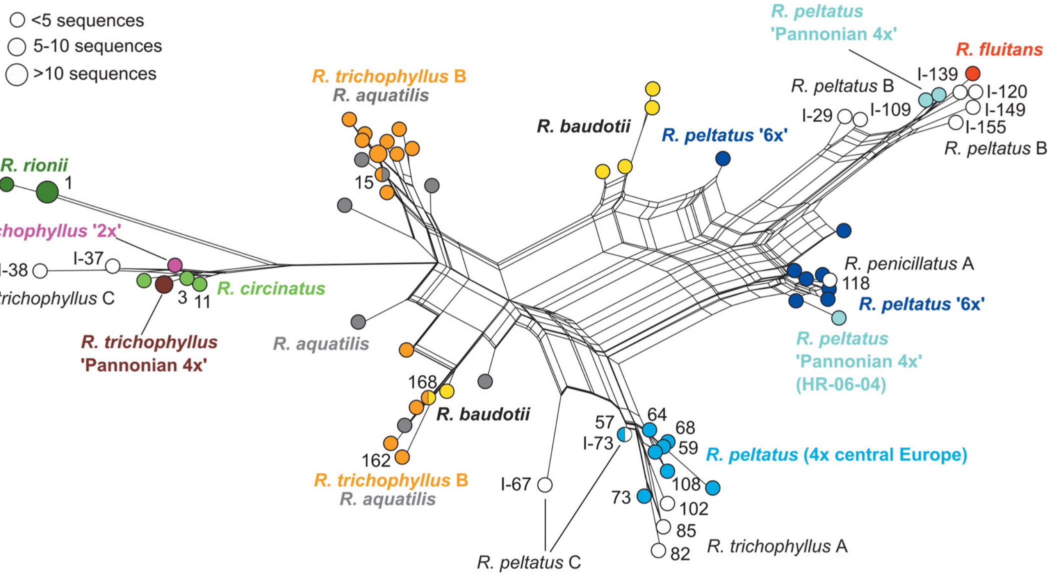

Fig. 4. Neighbour network of the ITS region. Genotypes and taxa sampled in the Pannonian Basin are distinguished by colours, those not sampled in the Pannonian Basin are shown in white. The selected genotypes of the central-European cytotypes from Koutecký et al. (2022) are labelled with their ITS genotype numbers (some of them were also found in the Pannonian Basin), the selected genotypes from south-western Europe (Prančl et al. 2025) are labelled with their ITS genotype numbers prefixed by 'I-' (indicating Iberian origin).

Discussion

Batrachium diversity in the Pannonian Basin

From the Pannonian Basin (in the territories of Hungary, southern Slovakia, northern Croatia and eastern Austria), the following species of *Ranunculus* sect. *Batrachium* have been reported so far: *R. aquatilis*, *R. baudotii*, *R. circinatus*, *R. fluitans*, *R. peltatus*, *R. rionii* and *R. trichophyllus* (Husák & Slavík 1982, Király 2009, Englmaier 2016, Nikolić 2020, Koutecký et al. 2022, Flora Croatica Database 2024). The earlier record of *R. penicillatus* (e.g. Cook 1964) is erroneous (Király 2009). We confirmed the presence of all reported species in the area. We also found *R. rionii* in northern Croatia, where it had not previously been reported. In addition, we found four previously unrecognized cytotypes and eight hybrid individuals. Contrary to the previous study from central Europe (Koutecký et al. 2022), no triploid *R. fluitans*, no *R. trichophyllus* A and none of the cytotypes of *R. penicillatus* (4x, 6x) were recorded in our samples from the Pannonian Basin. The absence of the triploid *R. fluitans* is not surprising given the overall rarity of this species in the area, where it is confined to a single branch of the Danube river (Bartha & Király 2015). We found this population to be uniformly diploid. *Ranunculus trichophyllus* A grows in rather acidic waters and does not tolerate higher mineral content of the water (Kaplan et al. 2019); thus, the predominantly alkaline waters of the Pannonian Basin are unsuitable for this cytotype. The *R. penicillatus* complex grows mainly in rivers with fast-running water (Schou et al. 2023), which are not common in the study area.

Ranunculus peltatus s.l.

Diploid (2n = 16), tetraploid (2n = 32) and hexaploid (2n = 48) populations are reported for *R. peltatus*. Diploids are mainly known from the Iberian Peninsula (Diosdado et al. 1993, Prančl et al. 2025). There are also a few reports from Germany (Zander & Wiegleb 1987), Italy (Ferarella et al. 1981) and Greece (Dahlgren 1991). Tetraploids are the most common cytotype, recorded in central and western Europe (Cook 1966, Englmaier 2016, Wiegleb et al. 2017, Prančl et al. 2018, Koutecký et al. 2022), northern Europe (Hong 1991) and the Aegean Islands, Greece (Dahlgren 1991). Hexaploids have been reported only from Great Britain (Cook 1966); however, hexaploid *R. peltatus*-like plants have also been documented from Mykonos, Greece (Dahlgren 1991). As no information on their genome sizes is available, we cannot compare their identities with our hexaploid cytotype from the Pannonian Basin. Finally, a single putative hexaploid individual was also recorded in the Czech Republic within a tetraploid population, likely arising at the site by autopolyploidization (Prančl et al. 2018, Koutecký et al. 2022).

In the Pannonian Basin, we recorded two tetraploid and one hexaploid cytotype of *R. peltatus* s.l., all having the common ‘pelt’ haplotype in plastid markers. One of the tetraploids corresponds to the widespread central-European cytotype. The other, referred to as *R. peltatus* ‘Pannonian 4x’, is a previously unrecognized tetraploid. Based on the ITS sequences, it is closely related to diploid *R. peltatus* B from south-western Europe (Prančl et al. 2025) as well as to the morphologically very different *R. fluitans*, while it appears to be unrelated to other European tetraploid cytotypes of *R. peltatus* s.l. (central-European *R. peltatus* and Iberian *R. peltatus* C). However, its mean genome size (2C = 8.42 pg) is approximately 7% higher than twice that of *R. peltatus* B (2C = 3.92 pg, Prančl et al. 2025). We therefore hypothesize that *R. peltatus* ‘Pannonian 4x’ originated through autopolyploidization from an unknown lineage closely related, but not identical, to *R. peltatus* B. Less likely, *R. peltatus* ‘Pannonian 4x’ may have originated directly through autopolyploidization of *R. peltatus* B with a shift of genome size and with changes in the ITS resulting from independent evolution since the polyploidization. Alternatively, considering the effect of concerted evolution on the ITS region, *R. peltatus* ‘Pannonian 4x’ might be an allopolyploid of *R. peltatus* B-like parent and an unknown second parent, with homogenized ITS sequences. A single individual (HR-06-4) with a slightly lower genome size (2C = 7.54 pg) and an additive ITS pattern between the two tetraploid cytotypes was originally not classified as a hybrid due to its full fertility (several normally developed achenes were dissected, containing living embryos). It may represent a product of an earlier hybridization event (see below).

The third recorded cytotype, *R. peltatus* ‘6x’, was identified as hexaploid based on its genome size and chromosome count. All individuals share the ‘pelt’ haplotype in plastid markers. In the ITS network, they cluster with *R. penicillatus* A, an allotetraploid with *R. fluitans* and central-European *R. peltatus* as parents (Prančl et al. 2018, Koutecký et al. 2022). Based on morphology and taking the sequence similarity of *R. fluitans* with the Iberian *R. peltatus* B into account, it is possible that *R. peltatus* ‘6x’ is an allopolyploid combining sequences of *R. peltatus* B and central-European *R. peltatus*. However, its genome size (2C = 11.63 pg) does not support this hypothesis, as the theoretical genome size of such an allopolyploid is about 2C = 10.5 pg. Likewise, an origin from a cross between diploid *R. peltatus* B and *R. peltatus* ‘Pannonian 4x’ is not supported, as its theoretical genome size is about 2C = 12.34 pg. By contrast, its genome size is consistent with an allopolyploid derived from *R. peltatus* ‘Pannonian 4x’ (unreduced gamete) and central-European tetraploid *R. peltatus* (reduced gamete), with an expected genome size of about 2C = 11.72 pg. We thus hypothesize that *R. peltatus* ‘6x’ is a complex allopolyploid of these two tetraploids (central-European *R. peltatus* and *R. peltatus* ‘Pannonian 4x’), themselves of potentially auto- or allopolyploid origin.

Ranunculus peltatus ‘6x’ cytotype was recorded at seven localities; it is the most common *R. peltatus* s.l. cytotype at the sampled localities. We found it almost exclusively in slightly halophilic wetland habitats. *Ranunculus peltatus* ‘Pannonian 4x’ co-occurred with hexaploids at one slightly saline site, while its other locality was a freshwater seasonal wetland. There are clear disjunctions between diploid *R. peltatus* B and its presumed polyploid descendants in the Pannonian Basin (*R. peltatus* ‘Pannonian 4x’ and *R. peltatus* ‘6x’). *Ranunculus peltatus* B is only known from the Iberian Peninsula so far, while neither hexaploid *R. peltatus* nor *R. peltatus* ‘Pannonian 4x’ were recorded there (Prančl et al. 2025). Data on ploidy level/genome size and genetic variation are lacking from other parts of the Mediterranean area, except for several sequences published by Bobrov et al. (2015) and Jopek et al. (2023); no sequences of the Pannonian cytotypes are available in these studies. The available chromosome data (see references above) suggest that diploids do not occur in central and northern Europe and therefore the ancestors of the Pannonian *R. peltatus* cytotypes are likely from southern Europe. Areas such as southern France, Italy or the Balkan Peninsula might have served as potential ‘stepping stones’ for the ancestral diploids and/or as regions where descendant polyploids may have originated and may still occur. Dispersal from the Mediterranean area may be facilitated by migratory birds that are known to disperse aquatic plant seeds and sometimes also vegetative fragments across long distances (e.g. Li 2014). The Pannonian Basin lies at the intersection of the western and eastern bird migration routes, allowing potential contact with both south-western and south-eastern Europe (Spina et al. 2022). Diploid populations may exist or may have existed *en route*, giving rise to the polyploid lineages. Establishment of these polyploids in the Pannonian Basin may be facilitated by the habitat similarity of the Pannonian periodic freshwater or moderately saline wetlands and the Mediterranean periodic vernal pools, where *Batrachium* taxa occur.

Ranunculus trichophyllus s.l.

Ranunculus trichophyllus is a widespread, highly complex taxon. It has a reduced morphology and differs from some other homophyllous species (such as *R. rionii*) only by inconspicuous morphological characters, which leads to frequent misidentification (Cook 1966, Wiegleb et al. 2017). As shown by Koutecký et al. (2022), Jopek et al. (2023) and Prančl et al. (2025), *R. trichophyllus* is a polyphyletic species comprising several cytotypes and genotypes.

Diploids (2n = 16), tetraploids (2n = 32) and hexaploids (2n = 48) have been reported in *R. trichophyllus* in the literature (Cook 1966, Dahlgren 1991, Englmaier 2016, Wiegleb et al. 2017). In Europe, diploids are known from the Iberian Peninsula (Diosdado et al. 1993). The most common cytotypes are tetraploids (Cook 1966, Englmaier 2016, Wiegleb et al. 2017), which are represented by the cytotypes *R. trichophyllus* A and *R. trichophyllus* B in central Europe (outside the Alps, Prančl et al. 2018). Hexaploids have been reported from Poland (Turała 1969), Spain (Löve & Kjellqvist 1974), Scandinavia (Hong 1991), Crete and Turkey (Dahlgren 1991) and the Czech Republic (former Czechoslovakia, Měsíček & Jarolímová 1992).

The cytotype *R. trichophyllus* A was not found in the Pannonian Basin, likely due to different habitat preferences (see above). In contrast, *R. trichophyllus* B is frequent in the study area. In central Europe, this cytotype comprises two main genetic lineages: *R. trichophyllus* B1 has the plastid haplotype of the *R. trichophyllus* group ('trich-B') and specific ITS genotypes with no or a few intra-individual polymorphisms, while *R. trichophyllus* B2 has the plastid haplotype of the *R. peltatus* group ('pelt') and ITS genotypes additively combining the same specific genotype with sequences close to *R. circinatus*. It is hypothesized (Koutecký et al. 2022) that these two lineages represent reciprocal allopolyploids from the same parental species, with ITS sequences homogenized towards one parent in the former lineage. These lineages are cross-compatible, as mixed populations and all combinations of the plastid haplotypes and ITS genotypes exist, although they are uncommon (Koutecký et al. 2022). Recent broader sampling suggests that lineage B1 prevails in the Alps and their foothills (Koutecký et al. 2025), whereas only lineage B2 was found in the Pannonian Basin. This may reflect different geographical origins of the lineages, a topic requiring further study.

We report two new, previously unknown *R. trichophyllus* cytotypes. Quite surprisingly, plants from population HR-01 (*R. trichophyllus* '2x') are diploid. This is a rare ploidy in *R. trichophyllus*, previously known in Europe only from the Iberian Peninsula (Diosdado et al. 1993). This cytotype differs markedly from tetraploid *R. trichophyllus* A and B in genetic markers. In both ITS and plastid DNA, *R. trichophyllus* '2x' showed unique genotypes belonging to the *R. circinatus* group. In ITS, it is also very close to *R. circinatus*-like compound of the *R. trichophyllus* lineage B2 (see above), differing by only a few positions. Similar ITS genotypes were previously recorded by Bobrov et al. (2015) and Jopek et al. (2023), who unfortunately provide no information on the genome size/ploidy levels. In the analyses of Bobrov et al. (2015), two *R. trichophyllus* ITS ribotypes close to *R. circinatus* were found, one from Poland and the other from Croatia. Jopek et al. (2023) reported accessions with identical sequences from Croatia (coastal regions), Montenegro, Georgia and Poland. Finally, Prančl et al. (2025) report similar sequences from south-western Europe, as *R. trichophyllus* cytotype C, most likely tetraploid. It is therefore likely that *R. trichophyllus* '2x' is related to a Mediterranean taxon/taxa. However, data are still missing from many parts of southern Europe and therefore the exact origin of this taxon remains obscure.

The second new cytotype, *R. trichophyllus* 'Pannonian 4x', was found in two geographically proximate populations (HU-25 and HU-27). The population HU-25 grew in an unusual habitat: a temporarily flooded deep depression (water over 0.5 m deep) in a wheat field. The wetland forms during the wet spring season when the field is partially flooded and disappears during summer. These plants were robust with richly branched stems, but likely behaved as annuals due to the habitat conditions. This cytotype resembles robust *R. trichophyllus* B in general habitus but exhibits rather large flowers (petals up to 10 mm long and flowers up to 15 mm in diameter) and a large number of achenes (about 60 per receptacle). The achenes are rather small and borne on elongated receptacles, resembling *R. rionii* in these characters. Both achenes and receptacles are pubescent, as in *R. trichophyllus* B (Prančl et al. 2018). The genome size of these plants is within the range of *R. trichophyllus* B (although close to the lower limit) and they share the same plastid haplotype (‘pelt’); however, their ITS sequences are similar to those of diploid species *R. circinatus*, the likely tetraploid *R. trichophyllus* C, as well as the diploid *R. trichophyllus* ‘2x’. Since the plants were fully fertile, a recent hybrid origin is unlikely. We propose two hypotheses for the origin of *R. trichophyllus* ‘Pannonian 4x’. It may either be an allopolyploid arising from unknown ancestors, one of which could be *R. trichophyllus* ‘2x’ or a similar taxon, or it may fall within the variation of *R. trichophyllus* B with ITS sequences homogenized towards *R. circinatus*. However, given its morphological distinctiveness, we consider the latter less likely. Further research is needed to clarify its origin.

Hybridization

Batrachium is generally known for a high frequency of hybrids (Cook 1966, Englmaier 2016, Wiegleb et al. 2017, Prančl et al. 2018). In this study, 13 of the 47 sampled populations were mixed, containing at least two species (see Supplementary Table S1). Five of the mixed populations combined *R. rionii* with one polyploid species, and one population combined *R. rionii* with *R. circinatus*. No hybrids were detected in these populations, which is consistent with the observation that diploid *Batrachium* species tend to hybridize less frequently than polyploids (Koutecký et al. 2022, Kaplan et al. 2025). Among the remaining seven mixed populations, hybrids were detected in two (HR-06, HU-13). Five hybrid individuals, of which three were sequenced, were sampled in population HR-06. These plants were homophyllous, flowering but completely sterile, and overall slender. Based on the DNA sequences, these individuals may have originated from hybridization between *R. aquatilis* and *R. peltatus*. However, only *R. aquatilis* and *R. peltatus* ‘Pannonian 4x’ were found in the population (see Supplementary Table S1). The low genome size of all but one of these hybrid individuals (around 6.52 pg, see Table 1) does not correspond to the expected value of a pentaploid *R. aquatilis* × *R. peltatus* hybrid (9.70 pg), but rather to the tetraploid central-European *R. peltatus*. Additionally, their ITS sequences are similar to one (*R. peltatus*, HR-06-8) or the other (*R. aquatilis*, HR-06-10, HR-06-11) presumed parent, without a clear additive pattern. This suggests possible backcrossing. In the same population, one hybrid individual (HR-06-7) with a larger genome size (9.60 pg) was detected. This genome size corresponds quite well to the pentaploid F_1 hybrid of *R. aquatilis* and central-European tetraploid *R. peltatus* (Prančl et al. 2018). Moreover, the ITS sequence of this individual shows an additive pattern, suggesting it is a hybrid between these two taxa. Despite the fact that central-European tetraploid *R. peltatus* was not sampled in the population, it is likely that it occurs in other parts of the extensive wetland or at some site nearby, or was dormant in the year of the survey.

In population HU-13, three hybrid individuals were found, two of which were sequenced. Given the co-occurrence of *R. aquatilis* and *R. peltatus* at the locality and the additive ITS pattern, we infer these are pentaploid hybrids of these two taxa. Their genome size is not exactly intermediate between those of the presumed parents, which may be due to backcrossing (see Prančl et al. 2018). One individual (HU13-3) has a genome size markedly lower than the average of the genome sizes of the parental values. This plant may represent an advanced-generation aneuploid hybrid or an advanced backcross towards the tetraploid *R. peltatus*. A similar situation is already known from other populations of this hybrid in southern Sweden (Hong 1991) and in Czechia (Prančl et al. 2018).

Apart from the sterile hybrids, we also recorded cases of introgression/backcrossing among fertile plants. Two individuals were initially not classified as hybrids because they were fertile, but they exhibited unique genome sizes distinct from the rest of their respective populations or cytotypes, as well as additive ITS sequence patterns. One of these individuals (HR-06-4) was tentatively included in the *R. peltatus* 'Pannonian 4x' cytotype, which occurred at the site. Its ITS sequence was additive between the *R. peltatus* 'Pannonian 4x' and the central-European tetraploid cytotype of *R. peltatus*. The genome size was also intermediate between these two cytotypes. The involvement of the central-European *R. peltatus* tetraploid is hypothesized also for other sterile hybrids found in the same population, despite the fact that this cytotype was not recorded at the site (see above). The second potentially hybrid fertile individual (B18-005-1) was assigned to the *R. peltatus* '6x' cytotype. Its genome size was larger than the usual range for this cytotype, and it differed in two ITS positions, showing a shift from *R. peltatus* '6x' towards *R. aquatilis*. It grew in a seasonally terrestrial habitat and had the general appearance of *R. peltatus*, but with laminar leaves divided into a greater number of narrower lobes than is typical for this species. This feature is characteristic of *R. aquatilis*, although it may also reflect plasticity induced by terrestrial growth (P. Koutecký et al., personal observation). We hypothesize that this individual may represent a relatively recent and fertile advanced-generation hybrid between two hexaploids, the *R. peltatus* '6x' and *R. aquatilis*.

Conclusion

Our results demonstrate that the Pannonian Basin hosts a specific and evolutionarily significant assemblage of *Batrachium* taxa. This region includes cytotypes previously known from central Europe, as well as others that have not recorded elsewhere in the region. Four previously unknown cytotypes were identified. These novel cytotypes show genetic affinities with those from the Mediterranean Basin and may therefore represent descendants of Mediterranean genetic lineages. The Pannonian Basin has apparently acted as an important contact zone and secondary centre of diversification, shaped by historical dispersal and polyploid evolution. Together, these findings highlight the biogeographical importance of the Pannonian Basin for the evolution of *Batrachium* and underscore the need for further integrative studies across southern and south-eastern Europe.

Supplementary materials

Fig. S1. Neighbour network of the ITS region based on the complete dataset, including hybrid individuals.
Table S1. Locality details, genome sizes and GenBank accession numbers.
Table S2. Variable positions in the alignment of *rpl*32–*trn*L^{UAG} sequences.
Table S3. Variable positions in the alignment of 3'*rps*16–5'*trn*K sequences.
Table S4. Variable positions in the alignment of ITS sequences.
Data S1. Photographs of the newly discovered Pannonian cytotypes and their habitats (Figures S2–S12).

Supplementary materials are available at https://www.preslia.cz

Acknowledgements

We are grateful to all colleagues who helped us in the field and contributed additional samples or helped us in the laboratory. We are also very grateful to the two anonymous reviewers who helped us improve the manuscript. The research was supported by grant no. 22-10464S from the Czech Science Foundation. ML, ZK, and JP were also supported by long-term research development project no. RVO 67985939 of the Czech Academy of Sciences.

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Skrytá diverzita lakušníků (*Ranunculus* sect. *Batrachium*) v Panonské pánvi: mediteránní stopa ve střední Evropě?

Lakušníky (*Ranunculus* sect. *Batrachium*) patří k taxonomicky nejkomplikovanějším skupinám vodních rostlin. Mezi faktory, které přispívají k jejich složité taxonomii, patří značně redukovaná morfologie, velká fenotypová plasticita, polyploidizace a hybridizace. Tato studie poskytuje první vhled do diverzity lakušníků v Panonské pánvi s využitím sekvenování DNA (chloroplastové úseky a ITS region), měření velikosti genomu, počítání chromozomů a zhodnocení morfologie. Studie navazuje na předchozí výzkum lakušníků střední a západní Evropy prováděný stejnou metodikou. Mezi 51 studovanými populacemi jsme identifikovali sedm tradičně rozlišovaných druhů známých ze střední a západní Evropy, spolu s několika různými hybridy. Někteří z nalezených hybridů vznikli pravděpodobně opakovanou hybridizací (introgresí) nebo zpětným křížením. Zároveň jsme identifikovali čtyři dosud neznámé nehybridní cytotypy lakušníků, dva náležející k okruhu lakušníku štítnatého (*Ranunculus peltatus*) a dva k okruhu lakušníku niťolistého (*Ranunculus trichophyllus*). Na základě sekvencí DNA a velikostí genomu se zdá, že tyto cytotypy jsou blízce příbuzné mediteránním druhům, a to buď jako jejich přímí potomci (autopolyploidi), nebo jako allopolyploidi vzniklí hybridizací středoevropských a mediteránních lakušníků. Jako možné vysvětlení jejich výskytu v Panonské pánvi se nabízí dálkový přenos diaspor z jižnějších oblastí nebo přežívání reliktních populací dříve široce rozšířených taxonů ve specifických biotopech periodických mokřadů, které se jinde ve střední Evropě nevyskytují. Panonská pánev hostí vyšší diverzitu lakušníků, než bylo dříve uváděno, a svým druhovým složením je v rámci střední Evropy unikátní.

How to cite: Dolejšek V., Mesterházy A., Lučanová M., Brožová V., Kaplan Z., Košnar J., Prančl J. & Koutecký P. (2026) Hidden diversity of *Ranunculus* sect. *Batrachium* in the Pannonian Basin: A Mediterranean trace in central Europe? – Preslia 98: 105–125.

Preslia, a journal of the Czech Botanical Society

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