Cytotype variation, cryptic diversity and hybridization in *Ranunculus* sect. *Batrachium* revealed by flow cytometry and chromosome numbers

Cytotypová variabilita, kryptická diverzita a hybridizace u lakušníků (*Ranunculus* sect. *Batrachium*) odhalená pomocí cytometrických a karyologických analýz

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Prančl J., Koutecký P., Trávníček P., Jarolímová V., Lučanová M., Koutecká E. & Kaplan Z. (2018): Cytotype variation, cryptic diversity and hybridization in *Ranunculus* sect. *Batrachium* revealed by flow cytometry and chromosome numbers. – Preslia 90: 195–223.

Water-crowfoots (*Ranunculus* sect. *Batrachium*) are one of the taxonomically most intricate groups of aquatic plants. Investigation of its species is complicated by morphological reduction and phenotypic plasticity, as well as by the frequent occurrence of polyploidy and hybridization, giving rise to numerous intergrading or morphologically undetectable taxa. We used, for the first time in this group, flow cytometry to gain an insight into evolutionary processes and genome size differentiation in *Ranunculus* sect. *Batrachium*. Flow cytometry complemented by confirmation of chromosome counts was applied to an extensive dataset of 3354 individuals from 612 localities in 13 mainly central-European countries, of which the genome size was estimated for 1032 individuals. In total, 34 *Batrachium* cytotypes of five ploidy levels ranging from diploids to hexaploids were detected. The results indicate that the genome size is a reliable marker for distinguishing most of the traditionally recognized species, including those with identical chromosome numbers. Although variation in chromosome numbers is reported for six of eight central-European species, we detected only two taxa variable at the ploidy level (*R. fluitans* 2x + 3x, *R. penicillatus* 4x + 6x). However, the situation is much more challenging due to the presence of numerous hybrids and cryptic lineages. Cryptic variation was revealed within *R. trichophyllus*, in which three distinct tetraploid cytotypes were detected, which differ in their ecology and distribution. The allopolyploid complex of running-water taxa referred to as *R. penicillatus* is even more complex, including six different cytotypes. We also revealed 16 cytotypes of hybrid origin, which accounted for ca 15% of the individuals studied. Seven of the hybrid cytotypes were identified as F_1 hybrids and the rest are of unknown or uncertain origin. In the *R. penicillatus* group, the occurrence of a large hybrid swarm was documented. Hybrids with variable genome sizes frequently arise also from hybridization of the tetraploids *R. peltatus* and *R. trichophyllus* in which the gene flow tends to be largely unidirectional towards *R. peltatus*.

K e y w o r d s: aquatic plants, chromosome number, cryptic variation, determination, flow cytometry, genome size, hybridization, karyology, ploidy level, polyploidy, *Ranunculus* sect. *Batrachium*

doi: 10.23855/preslia.2018.195

Introduction

Beyond doubt, aquatic plants are one of the taxonomically most intricate angiosperms. Although phylogenetically unrelated, many aquatics share similar environmental adaptations, which altogether predetermine the formation of hardly detectable diversity (Sculthorpe 1967, Barrett et al. 1993, Kaplan 2002). Their simplified morphology combined with high phenotypic plasticity results in species, which although well separated reproductively, geographically or ecologically, may be indistinguishable morphologically, i.e. they are cryptic (Crawford et al. 2001, Thum et al. 2011, Kaplan & Marhold 2012). The same phenomena may also result in the parallel evolution of similar traits in unrelated taxa (Jobson et al. 2003, Kaplan & Štěpánek 2003, Kaplan & Fehrer 2011). On that account, traditional morphologically-based classification of numerous aquatic plant groups is rather misleading and unable to reflect the actual evolutionary relationships. Furthermore, additional microevolutionary processes can substantially shape the diversification of aquatic plants. In 80% of the aquatic genera, for which chromosome numbers are available, there is intrageneric variation in chromosome number. In the majority of them (68% of the total) this can be attributed to polyploidy (Les & Philbrick 1993; comparable with 61% estimated for all vascular plant genera; Wood et al. 2009). The great importance of hybridization became obvious with the advent of molecular techniques (e.g. Les & Philbrick 1993, Kaplan et al. 2009, Zuellig & Thum 2012). In certain genera, the number of hybrids may even exceed the number of species, as documented in *Potamogeton* (Kaplan et al. 2013). Newly established polyploids and hybrids often persist due to prolific clonal growth and fast vegetative spread (Grace 1993).

Water-crowfoots (*Ranunculus* sect. *Batrachium* according to Hörandl & Emadzade 2012, *Ranunculaceae*; hereafter referred to as *Batrachium*) is evolutionarily one of the most complex groups of aquatic plants. The section consists of about 30 species, occurring predominantly in the Northern Hemisphere (Wiegleb et al. 2017). The world diversity centre is believed to be in Europe, where 14–18 species are reported (Cook 1966, Wiegleb et al. 2017), of which eight occur in central Europe (Tables 1, 2). *Batrachium* taxa occupy a wide range of wetland habitats from eutrophic lowland lakes and fishponds to high alpine oligotrophic lakes and from standing to fast running water, which is reflected in their extensive phenotypic plasticity. The group is well known for the occurrence of heterophylly, i.e. presence of two completely different types of leaves: laminar leaves with a flat, more or less lobate and dentate lamina, which usually float on water and capillary leaves with lamina consisting of branched capillary segments, which are mostly submerged. Presence or absence of heterophylly closely correspond to different life strategies of particular taxa (e.g. Cook 1966, 1969, Hong 1991). Flower size differs among species of *Batrachium* and is associated with the mode of reproduction: small-flowered taxa are considered largely autogamous or even cleistogamous whereas big-flowered species exhibit several adaptations for outbreeding and particular taxa may even be self-incompatible (Cook 1966, Turała-Szybowska 1978, Hong 1991).

Polyploidy is frequent within *Batrachium*, covering five ploidy levels ranging from diploids (2n = 16) to hexaploids (2n = 48) (Table 1). Variation in ploidy level is reported for about a half of the European species (e.g. Cook 1966, Wiegleb et al. 2017). Aneuploid chromosome numbers are also reported but are rare (Cook 1966, Dahlgren 1991, Diosdado et al. 1993). Interspecific hybridization occurs frequently and probably all taxa

are potentially capable of crossing. Many hybrids are at least partially fertile (Cook 1966, 1970, Dahlgren 1991). Clonal growth and autogamy are considered to be important factors in the persistence of hybrids and establishment of new polyploids (Cook 1966, Wiegleb & Herr 1983). The combination of multiple polyploidization and hybridization events may even result in the formation of extremely complex allopolyploid conglomerates. An example of such a group is *Ranunculus penicillatus*. This obligatory running-water "species" actually consists of various crosses and allopolyploids arising from hybridization of *R. fluitans* with several other species (namely *R. peltatus*, *R. aquatilis*, *R. trichophyllus* and perhaps *R. baudotii*), apparently including backcrossing and introgressive hybridization among particular taxa (Wiegleb & Herr 1983, Webster 1988, Dahlgren 1993, Zalewska-Gałosz et al. 2014). Ploidy level is probably one of the crucial factors influencing the direction of gene flow and consequently even the identity of taxa. This is particularly evident in the case of *R. trichophyllus* (Table 1). In northern Europe, *R. trichophyllus* is reported to be represented by an hexaploid cytotype that freely hybridizes with hexaploid *R. aquatilis* and the subsequent introgressive hybridization blurs the boundaries between these species (Hong 1991). In contrast, central-European *R. trichophyllus* populations are predominantly tetraploid, which raises questions about their potential to hybridize with *R. aquatilis.*

Despite this group showing notable morphological and ecological diversity as a result of various evolutionary processes, only a few biosystematic studies have included *Batrachium* since Cook's comprehensive monograph (Cook 1966). Recent molecular studies of European water-crowfoots are mostly confined to small regions and only three studies employed molecular markers to analyse genetic variation of more than two species (British plants by Telford et al. 2011, Polish plants by Zalewska-Gałosz et al. 2014 and north-European plants by Bobrov et al. 2015, all of them using a combination of cpDNA and the ITS region). Despite their limited scope, all three studies reveal substantial genetic variation in the morphologically strongly reduced species *R. trichophyllus*. Phylogenetic studies that focus on the entire genus *Ranunculus* include only a few samples of *Batrachium* (e.g. Hörandl et al. 2005, Emadzade et al. 2010, Hörandl & Emadzade 2012). The reticulate evolution of *Batrachium*, however, makes research on its phylogeny very difficult (Hörandl & Emadzade 2012, Bobrov et al. 2015).

Because of a high degree of phenotypic plasticity and probably underestimated occurrence of hybrids, various taxonomic concepts are adopted across Europe (e.g. Cook 1966, Cook et al. 1986, Husák et al. 1988, Pizzaro 1995, Dahlgren & Jonsell 2001, Englmaier 2016, Wiegleb et al. 2017). In an attempt to change this disappointing state of knowledge, we decided to analyse the variation in ploidy level and genome size in an extensive set of *Batrachium* populations using flow cytometry (FCM), which (i) is an efficient tool for analysing a large number of individuals rapidly, allowing to reveal the structure of populations and to detect rare cytotypes and hybrids, (ii) is frequently used in studies of evolutionarily and taxonomically intricate groups of plants, including polyploid and hybridogenous complexes (e.g. Suda et al. 2010, Trávníček et al. 2011a, b, 2012, Chumová et al. 2015, Flatscher et al. 2015, Lepší et al. 2015, Kobrlová et al. 2016, Vít et al. 2016, Bressler et al. 2017, Feulner et al. 2017) and homoploid plants (Loureiro et al. 2010, Prančl et al. 2014), (iii) has been successfully used as a basic method for taxonomic determination and delimitation of other complex aquatic taxa, such as *Callitriche* (Prančl et al. 2014) and *Nymphaea* (Volkova et al. 2010, Kabátová et al. 2014), and

Table 1. – List of central-European species of *Ranunculus* sect. *Batrachium* and the chromosome numbers previously published for each taxon. Note that *R. pseudofluitans* (Syme) Newbould ex Baker et Foggitt and *R. kauffmannii* Clerc are included in *R. penicillatus* while *R. confervoides* (Fr.) Fr. is classified as *R. trichophyllus* subsp. *eradicatus*, following Cook (1966).

Taxon	Distribution	Ecology	2n	Selected references
R. aquatilis L.	most of Europe, Asia (rarely), N Africa, W of North and South America	predominantly standing water	32	Dahlgren 1993 (Denmark)
			48	Cook 1966 (Denmark, England, Germany, Japan), Hong 1991 (Sweden), Májovský 1978 (Czechoslovakia), Turała 1969 (Poland)
R. baudotii Godr.	coastal parts of Europe and N Africa, rarely inland	brackish and mineral-rich water	16	Diosdado et al. 1993 (Spain)
			32	Cook 1966 (Austria, Denmark, England, Finland), Dahlgren 1991 (Aegean Islands), Dahlgren 1993 (Denmark), Dvořák & Dadáková 1984 (Czechoslovakia), Hong 1991 (Sweden), Turała 1969 (Poland)
R. circinatus Sibth.	temperate Europe, temperate and boreal(?) Asia	predominantly standing water	16	Cook 1966 (England, Germany, Poland), Dahlgren 1993 (Denmark), Hong 1991 (Sweden), Měsíček & Jarolímová 1992 (Czechoslovakia), Turała 1969 (Poland)
R. fluitans Lam.	NW and central Europe	rivers, big streams	16	Cook 1966 (England, Germany, Sweden), Hong 1991 (Sweden), Měsíček & Jarolímová 1992 (Czechoslovakia), Turała 1969 (Poland), Turała-Szybowska 1977 (Germany)
			24	Cook 1962 (Germany), Měsíček & Jarolímová 1992 (Czechoslovakia), Turała-Szybowska 1977 (Germany)
			32	Cook 1962 (England), Turała-Szybowska 1977 (Czechoslovakia, Germany, Lithuania)
R. peltatus Schrank	most of Europe, N Africa	both standing and running water	16	Cook 1966 (Portugal, Spain), Dahlgren 1991 (Aegean Islands), Ferarella et al. 1981 (Italy), Fernandez Bernaldo de Quirós 1987 (Spain)
			32	Cook 1966 (Denmark, Germany), Dahlgren 1991 (Aegean Islands), Diosdado et al. 1993 (Spain), Fernandez Bernaldo de Quirós 1987 (Spain), Hong 1991 (Sweden), Murín et Májovsky 1978 (Czechoslovakia), Turała 1969 (Poland)

Taxon	Distribution	Ecology	2n	Selected references
			48	Cook 1966 (England)
R. penicillatus (Dum.) Bab.	most of Europe	rivers, big streams	16	Diosdado et al. 1993 (Spain), Dahlgren 1991 (Aegean Islands – var. *pseudofluitans*)
			24	Dahlgren 1993 (Denmark)
			32	Cook 1966 (Germany – both var. *penicillatus* and *pseudofluitans*), Dahlgren 1993 (Denmark, both var. *penicillatus* and *pseudofluitans*), Diosdado et al. 1993 (Spain), Turała 1969 (Poland)
			48	Cook 1966 (England, var. *pseudofluitans*, *vertumnus*; Germany – var. *pseudofluitans*; Ireland – var. *penicillatus*)
R. rionii Lagger	central and E Europe, Middle East, Tibet	mineral-rich standing water	16	Cook 1962 (Austria, Iraq), Dahlgren 1991 (Aegean Islands), Gadnidze et al. 1998 (Georgia), Podlech & Dieterle 1969 (Afghanistan)
R. trichophyllus subsp. *eradicatus* (Laest) C. D. K. Cook	arctic and alpine regions of Europe, Asia and North America	oligotrophic lakes	32	Cook 1966 (Germany, Greenland, Iceland), Löve & Kjellqvist 1974 (Spain)
R. trichophyllus Chaix subsp. *trichophyllus*	Europe, Asia (except of S parts and Japan), N and S Africa, N and S America, SE Australia, New Zealand	both standing and running water	16	Diosdado et al. 1993 (Spain), Löve & Solbrig 1964 (Canada), Roberts 1976 (USA)
			32	Cook 1966 (Algeria, Canada, England, Germany), Dahlgren 1991 (Aegean Islands, Turkey), Favarger et al. 1979 (Morocco), Larsen & Laegaard 1971 (Sicily), Murín & Záborský 1976 (Czechoslovakia), Turała 1969 (Poland)
			40	Dahlgren 1991 (Aegean Islands)
			48	Dahlgren 1991 (Aegean Islands), Hong 1991 (Sweden), Löve & Kjellqvist 1974 (Spain), Měsíček & Jarolímová 1992 (Czechoslovakia), Turała 1969 (Poland)

Table 2. – Diagnostic characters of central-European species of *Ranunculus* sect. *Batrachium*, based on a combination of the most relevant taxonomic studies (e.g. Cook 1966, Englmaier 2016, Wiegleb et al. 2017) and our observations. The most useful diagnostic characters are in bold. The length of capillary leaves refers to leaves in the middle part of generative shoots. To determine the hairiness of fruits it is necessary to examine young achenes and the occurrence of laminar leaves has to be studied in adult (flowering or fruiting) plants.

Taxon	Capillary leaves	Laminar leaves	Peduncles	Corolla length	Nectar pits	Receptacles	Achenes	Other characters
R. aquatilis	mostly flaccid, densely branched, 2–6 cm long, usually shorter than the adjacent internode	sometimes present; with 3–5 primary lobes, dissected up to 2/3 of the lamina or deeper (or even divided into leaflets), **secondary lobes 17–26,** margin dentate, **basal sinus of the lamina 0–90°**	1–6 cm, straight to recurved	4–10 mm	circular	hairy	~20–50, hairy	
R. baudotii	somewhat rigid, densely branched, 1–10 cm long, shorter or as long as the adjacent internode	sometimes present; **mostly with 3 primary lobes**, dissected up to 2/3 of the lamina or deeper (or even divided into leaflets), secondary lobes 8–14, margin acutely crenate to dentate, basal sinus of the lamina 90–180°	3–18 cm, **strongly recurved in fruit**	4–10 mm	variable, lunate to pyriform, often cup-shaped	sometimes elongating in fruit, hairy	~25–60, glabrous to sparsely hairy, **often narrowly winged when dry**	plants pale green with whitish, often fleshy stems
R. circinatus	**rigid,** markedly shorter than the adjacent internode, circular to semicircular, 1–3 cm long, **segments lying in one plane**	absent	2–10 cm, straight to slightly recurved	6–10 mm	lunate	hairy	~20–50, hairy	
R. fluitans (both 2x and 3x)	**rigid, sparsely branched (only 2–4 divisions), 8–40 cm long, usually longer than the adjacent internode**	absent	4–10 cm, straight to slightly curved	7–15 mm	pyriform	**glabrous to sparsely hairy**	**mostly undeveloped**	**stems very long (up to 6 m); petals often more than 5 (up to 13)**
R. peltatus	mostly flaccid, densely branched, 3–8 cm long, usually shorter than the adjacent internode	usually present; with 3–5 primary lobes, dissected up to 1/3 to 2/3 of the lamina, secondary lobes 7–15, **margin most often obtusely crenate**, basal sinus of the lamina 45–180°	3.5–10 cm, straight to slightly curved	8–15 mm	pyriform	hairy	~20–50, hairy	

Taxon	Capillary leaves	Laminar leaves	Peduncles	Corolla length	Nectar pits	Receptacles	Achenes	Other characters
R. penicillatus (cytotypes A–F)	mostly flaccid, densely branched (4–8 divisions), **5–20 cm long, usually longer than the adjacent internode**	usually absent, present only in some populations of cytotype A; very variable, with 3–5 primary lobes, dissected up to 1/2 of the lamina or deeper, secondary lobes 5–11, margin almost entire to dentate, basal sinus of the lamina 90–180°	5–20 cm, straight to slightly curved	8–15 mm	variable, most often pyriform	hairy	~20–50, hairy	**stems very long (up to 5 m)**
R. rionii	mostly flaccid, densely branched, 1–3 cm long, shorter than the adjacent internode	absent	1–3 cm, recurved	2–5 mm	lunate, rarely pyriform	hairy	**~30–80, glabrous**	
R. trichophyllus A	flaccid, sparsely to densely branched, 1–3 cm long, shorter than the adjacent internode, segments fine	absent	1–3 cm, recurved	2–4 mm, rarely cleisto-gamous	lunate	hairy	~10–30, hairy	often flowering underwater
R. trichophyllus B	mostly flaccid, sparsely to densely branched, 1–5 cm long, shorter than the adjacent internode	absent	1.5–5 cm, recurved	3.5–7 mm, rarely cleisto-gamous	lunate	hairy	~15–40, hairy	often flowering underwater
R. trichophyllus subsp. *eradicatus*	flaccid, sparsely branched, 1–3 cm long, shorter than the adjacent internode, segments extremely fine	absent	1–3 cm, recurved	2–4, but **flowers mostly cleisto-gamous**	lunate	hairy	~5–15, hairy	delicate plants with **creeping shoots rooting at nodes**, mostly flowering underwater

(iv) has never been used to investigate *Batrachium*, with the exception of a single *R. trichophyllus* population (Hidalgo et al. 2015).

We used flow cytometry combined with chromosome counting to improve our understanding and identification of water-crowfoots in central Europe. Specifically, we addressed the following questions: (i) Is the genome size a suitable marker for reliable determination of *Batrachium* taxa? (ii) What are the patterns in cytotype and ploidy level variation in the area studied? Does the variation in genome size correlate with current taxonomic concepts? (iii) What is the frequency of interspecific hybridization? (iv) Is there any evidence of cryptic variation within morphologically recognized species?

Materials and methods

Field sampling

Plant material was collected in Austria (38 localities), Czech Republic (495 localities), Denmark (18 localities), Germany (16 localities), Hungary (13 localities) and Slovakia (17 localities), additional individual samples were obtained from Lithuania, Moldova, Poland, Romania, Slovenia, Switzerland and United Kingdom (in total 15 localities). The sampling was carried out to include all eight central-European species from the widest possible range of aquatic habitats and covering the whole range of morphological variation. If possible, multiple individuals were collected from each population. The number of individuals per population depended on population size and the extent of observed phenotypic variation. In total, 3354 individuals from 612 localities were obtained (for locality details, see Electronic Appendix 1). Voucher specimens are preserved in the herbaria of Charles University in Prague (acronym PRC), Faculty of Science of the University of South Bohemia in České Budějovice (CBFS) and National Museum in Prague (PR).

Morphological identification

We included only well-developed plants that were identified based on a combination of the most relevant taxonomic studies and our field experience (see Table 2 for diagnostic characters). The accepted taxonomic concept mostly follows Cook (1966). In the case of *Ranunculus penicillatus*, we did not adopt any intraspecific units because the observed variation is too complex and did not fully correspond to any of the currently used names (see below). Our tentative determinations were then compared with the genome sizes obtained from the FCM analysis. Plants that could not be identified unambiguously were cultivated in garden tanks in order to obtain the optimal morphological stage of development for their reliable determination.

Flow cytometry

Genome size was estimated using flow cytometry. If multiple individuals were collected from a population, they were first screened for genome size homogeneity using bulked samples of up to five individuals in a single run. Subsequently, selected individuals (usually three individuals per each morphological species and cytotype within a population, and all individuals that had divergent genome sizes; in total 1707 individuals) were

analysed individually. The number of individuals measured from each population is summarized in Electronic Appendix 1.

The sample preparation followed the simplified two-step procedure described by Doležel et al. (2007). About 0.25 cm^2 of leaf tissue (one individual or a mixture from several individuals) was chopped together with an appropriate volume of the internal standard using a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). *Bellis perennis* L. was selected as a primary reference standard, as it has a similar but non-overlapping genome size with all the samples studied. The crude suspension was filtered through a 42-μm nylon mesh and incubated for about 5 min at room temperature. After incubation, isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented with 2-mercaptoethanol (2 μg/ml) and either 4,6-diamidino-2-phenylindole (DAPI) (4 μg/ml) or propidium iodide (PI) and RNase IIA (both 50 μg/ml). Samples were run on the flow cytometer after about one minute of staining. The DAPI-stained samples were analysed using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany, now Sysmex) equipped with a mercury arc lamp as the UV light excitation source. The fluorescence intensity of 3000–5000 particles was recorded. The PI-stained samples were analysed using a Partec CyFlow instrument equipped with a green diode-pumped solid-state laser (Cobolt Samba, 532 nm, 150 mW output power) and the fluorescence intensity of 5000 particles was recorded. Selected samples for precise genome size measurement were analysed with propidium iodide and the analyses repeated 2–3 times on different days to account for random measurement error; if the range of variation of the repeated measurements exceeded the 2% threshold, the outlying value was discarded and the sample reanalysed. Histograms were evaluated using FloMax software, ver. 2.4d (Partec GmbH) or FlowJo 10 (TreeStar Inc.). In total, exact genome size was estimated for 1032 individuals, for which repeated analyses of appropriate quality were available.

The genome size was expressed as the ratio of the mean fluorescence of the sample and the internal standard. For PI-stained samples, the genome size in absolute units was calculated based on the genome size of *Bellis perennis* (2C = 3.38 pg, Schönswetter et al. 2007). Basic statistics (mean, standard error, standard deviation and variation range) were calculated. The extent of the total variation in intraspecific genome size was calculated as a percentage of the difference between the highest and lowest genome size value and expressed as % of the minimum. Subsequently, 1C values, and for taxa with known chromosome counts, also 1Cx-values were derived from the mean 2C values. For the taxa having overlapping genome sizes, these were compared using analysis of variance; genome sizes were log10-transformed and TukeyHSD test was used for multiple comparisons if more than 2 groups were present. All these statistical analyses were performed in R, version 3.4.3 (R Development Core Team 2017).

Chromosome counts

Attempts were made to count the number of chromosomes for at least one sample of each cytotype detected using flow cytometry. Selected plants were cultivated in a garden tank until they formed adventive roots on their stems, which were used for counting chromosomes. Alternatively, when suitable adventive roots were not available, chromosomes were counted using roots of seedlings. First, the ripe achenes were obtained from plants

analysed using flow cytometry. The achenes were then placed on moistened filter paper in Petri dishes at room temperature to induce germination. If they germinated, the youngest primary roots about 2 mm long were collected (usually 6 days after germination).

The roots were pre-treated in a saturated water solution of p-dichlorobenzene at room temperature for approximately three hours, then fixed in a freshly prepared 3:1 mixture of 96% ethanol and acetic acid and stored at –20°C until further processing. Before chromosome preparation, the material was macerated in a 1:1 mixture of ethanol and hydrochloric acid for 10 s, then transferred onto a microscope slide, non-meristematic tissues were removed, and the meristem stained in a drop of lacto-propionic orcein, covered with a coverslip and squashed. The preparations were examined under a Carl Zeiss Jena NU microscope equipped with an Olympus E – 510 Digital SRL Camera or an Olympus BX 51 microscope equipped with DP-71 Olympus digital camera with the DP Controller imaging software 3.1 (Olympus Corp.). Only the slides, on which at least 5 mitoses were found, were considered. Altogether, chromosome numbers were determined for 37 individuals of 21 cytotypes. For the remaining cytotypes recognized using flow cytometry, the chromosome counting was unsuccessful or we did not have usable material (living plants or seeds).

Niche differentiation

Environmental parameters based on climatic data from the WorldClim database (Hijmans et al. 2005; http://www.worldclim.org) were assessed to elucidate the degree of ecological divergence among morphologically similar but ecologically different couples *R. aquatilis* – *R. peltatus* and *R. trichophyllus* A – *R. trichophyllus* B. Spatial stratification, conducted via spThin R script (Aiello-Lammens et al. 2014), was used prior to the analysis to eliminate unequal sampling across the area. Minimum spatial threshold for sample elimination was set to 20 km and consequently 67 georeferenced locations of *R. peltatus*, 20 of *R. aquatilis*, 32 of *R. trichophyllus* A and 29 of *R. trichophyllus* B were included in the analysis. For these locations we extracted elevation (see Electronic Appendix 1) and data from a database of 19 bioclimatic variables representing annual trends, seasonality, and extreme and limiting environmental factors (Electronic Appendix 2). Data from raster layers for each of the 19 bioclimatic variables were extracted using the *extract* function in the raster package in R (Hijmans et al. 2016).

First, principal trends in the variation in bioclimatic variables were detected by principal component analysis. Next, all variables were used for linear discriminant analysis. The analyses were performed separately for each of above-mentioned pairs of taxa. All analyses were done using the MorphoTools R scripts for multivariate data handling (Koutecký 2015).

Results

Cytotype diversity and chromosome counts of traditionally recognized species

Most of the traditionally recognized central-European species are well-defined in terms of mean genome size and ploidy level (Tables 3, 4, Figs 1, 2). About 85% of the individuals can be attributed to dominant cytotypes of the morphological species and the remaining ~15% to hybrids and minority cytotypes (note that we use the term “cytotype” for each

Table 3. – Summary of flow cytometric genome size estimations (propidium iodide staining). N – number of individuals; note that for precision only individuals measured repeatedly on different days are included. Ratio – mean ratio with the internal standard *Bellis perennis*. 2C – mean genome size (2C value) in pg of DNA. SE – standard error of mean (pg of DNA). Min, Max, % var – minimum and maximum 2C values and the difference between them expressed as % of the minimum. Ploidy – ploidy level; in some hybrids the ploidy level is not supported by chromosome counts and is only inferred from ploidy levels of the parental species (marked by *). 1Cx – monoploid genome size in pg of DNA calculated from the mean 2C value and the ploidy level.

Taxon / cytotype	N	Ratio	2C	SE	Min	Max	% var	Ploidy	1Cx
R. aquatilis	57	3.88	13.10	0.03	12.65	13.63	7.8	6x	2.18
R. baudotii	35	2.59	8.75	0.02	8.49	9.02	6.3	4x	2.19
R. circinatus	61	1.68	5.67	0.01	5.46	5.78	6.0	2x	2.83
R. fluitans (2x)	56	1.14	3.85	0.01	3.76	3.97	5.6	2x	1.92
(3x)	8	1.69	5.71	0.01	5.63	5.76	2.3	3x	1.90
R. peltatus	307	2.01	6.76	0.01	6.50	7.03	8.2	4x	1.69
(putative 5x)	3	2.51	8.47	0.08	8.31	8.54	2.8	?	
(putative 6x)	1	3.00	10.13					?	
(aneuploid)	2	1.92	6.48	0.11	6.38	6.59	3.3	?	
R. penicillatus A	18	2.15	7.27	0.03	7.09	7.50	5.8	4x	1.82
B	7	2.45	8.29	0.02	8.18	8.35	2.0	4x	2.07
C	1	2.68	9.06					?	
D	1	2.85	9.63					4x	2.41
E	1	3.21	10.86					?	
F	4	4.10	13.84	0.06	13.68	13.94	1.9	6x	2.31
R. rionii	86	1.60	5.39	0.01	5.18	5.56	7.4	2x	2.69
R. trichophyllus A	115	2.66	8.97	0.01	8.73	9.26	6.1	4x	2.24
B	47	2.95	9.96	0.02	9.70	10.28	5.9	4x	2.49
subsp. *eradicatus*	18	2.63	8.88	0.02	8.78	9.03	2.9	4x	2.22
Hybrids									
R. aquatilis × *R. peltatus* (F_1)	10	2.96	9.98	0.04	9.85	10.30	4.5	5x	2.01
R. aquatilis × *R. peltatus* (hybrid 1)	1	2.15	7.27					?	
(hybrid 2)	1	3.21	10.83					?	
R. aquatilis × *R. trichophyllus* B (F_1)	2	3.39	11.47	0.04	11.43	11.50	0.6	5x	2.29
R. circinatus × *R. rionii* (F_1)	1	1.63	5.52					2x*	2.76
R. circinatus × *R. trichophyllus* A (F_1)	8	2.17	7.34	0.04	7.26	7.57	4.3	3x	2.45
R. peltatus hybr.	54	2.01	6.79	0.01	6.62	7.18	8.4	?	
R. peltatus hybrid (Váh river)	4	2.68	9.07	0.02	9.02	9.10	0.9	?	
R. peltatus hybrid (Vltava river)	8	2.89	9.78	0.05	9.60	9.99	4.1	6x	1.63
R. peltatus-penicillatus A	56	2.05	6.93	0.02	6.66	7.16	7.5	4x	1.73
R. peltatus × *R. trichophyllus* A (F_1)	31	2.33	7.88	0.01	7.71	8.05	4.5	4x	1.97
R. peltatus × *R. trichophyllus* A (other hybrids)	23	2.25	7.60	0.10	7.02	8.52	21.4	4x*	1.90
R. penicillatus F × *R. trichophyllus* B (F_1)	1	3.54	11.98					5x*	2.40
R. penicillatus F (F_1 hybrid?)	1	2.06	6.97					3x*	2.32
R. rionii × *R. trichophyllus* A (F_1)	1	2.14	7.23					3x*	2.41
R. trichophyllus hybr.?	2	2.90	9.79	0.25	9.54	10.04	5.2	?	

taxonomic entity defined by a specific range of genome sizes, which reflect differences in chromosome number, structure or size, as well as for each entity defined unequivocally by the combination of specific genome size and morphology). The detected 2C-values varied 3.59-fold from 3.85 pg in diploid *R. fluitans* up to 13.84 pg in hexaploid *R. penicillatus* F (Fig 1A). Monoploid genome sizes (1Cx-values) were also highly variable,

Table 4. – Chromosome numbers of 21 water-crowfoot cytotypes recorded in this study, sorted according to ploidy level. For locality details, see Electronic Appendix 1.

Taxon	Ref. no.	Locality	Ploidy	2n
R. circinatus	B14-043	Czech Republic, Tvrdonice: Hnátkovská jezera pools	2x	16
R. fluitans	B14-036	Czech Republic, Lindava: Svitavka stream	2x	16
R. rionii	B11-004	Czech Republic, Chudíř: Nový fishpond	2x	16
R. rionii	B12-008	Czech Republic, Mariánské Radčice: pool in mining area	2x	16
R. circinatus × *R. trichophyllus* A (F_1)	B14-118	Austria, Hallstatt: Hallstättersee lake	3x	24
R. fluitans	B12-006	Czech Republic, Praha: Vltava river	3x	24
R. baudotii	B14-038	Austria, Sankt Andrä am Zicksee: Zicksee lake	4x	32
R. baudotii	B14-073	Czech Republic, Buškovice: fish storage ponds	4x	32
R. baudotii	B14-049	Czech Republic, Hořátev: Hliňovka fishpond	4x	32
R. baudotii	B13-010	Czech Republic: Pečky, Výrovka stream	4x	32
R. baudotii	B12-048	Denmark, Ormslev: Aarhuså stream	4x	32
R. baudotii	B12-057	Denmark, Rønge: Gudenå river	4x	32
R. peltatus	K15-19	Czech Republic, Horní Poříčí: unnamed fishpond	4x	32
R. peltatus	B12-009	Czech Republic, Řevničov: Horní Kracle fishpond	4x	32
R. peltatus	B12-045	Denmark, Ribe: artificial pool in town	4x	32
R. peltatus	K11-15	Czech Republic, Majdalena: Cep sand pit	4x	32
R. peltatus hybrid	B12-060	Denmark, Tarm: Sonderå river	4x	32
R. peltatus × *R. trichophyllus* A (F_1)	B13-037	Czech Republic, Švařec: Svratka river	4x	32
R. peltatus × *R. trichophyllus* A (F_1)	K13-23d	Czech Republic, Vlkov: Vlkovská pískovna sand pit	4x	32
R. peltatus-penicillatus A	B15-039	Czech Republic, Okounov: Ohře river	4x	32
R. penicillatus A	B14-031	Czech Republic, Mimoň: Panenský stream	4x	32
R. penicillatus B	B15-043	Czech Republic, Vysoké Mýto: Loučná river	4x	32
R. penicillatus D	B12-050	Denmark, Skivum: Sønderup Å stream	4x	32
R. trichophyllus A	B12-010	Czech Republic, Březina: Oběšenec fishpond	4x	32
R. trichophyllus A	B14-035	Czech Republic, Krásná Lípa: unnamed fishpond	4x	32
R. trichophyllus B	B14-122	Austria, Grünau im Almtal: Almsee lake	4x	32
R. trichophyllus B	K15-15	Austria, Mattighofen: Kühbach brook	4x	32
R. trichophyllus B	B12-005	Czech Republic, Litoměřice: unnamed pond	4x	32
R. trichophyllus B	K15-021	Czech Republic, Makarov: unnamed fishpond	4x	32
R. trichophyllus subsp. *eradicatus*	B14-121	Austria, Grünau im Almtal: Almsee lake	4x	32
R. aquatilis × *R. peltatus* (F_1)	B14-021	Czech Republic, Lanžhot: Bornova jama pool	5x	40
R. aquatilis × *R. trichophyllus* B (F_1)	B14-013	Czech Republic, Holasice: Ludmila pool	5x	40
R. aquatilis	B11-005	Czech Republic, Loučeň: Lutovník fishpond	6x	48
R. aquatilis	B13-034	Czech Republic, Hradec Králové: Na Plachtě pools	6x	48
R. aquatilis	B13-021	Slovakia, Malacky: Marhecké rybníky fishponds	6x	48
R. peltatus hybrid (Vltava river)	K15-17	Czech Republic, Bližní Lhota: Hamerský stream	6x	ca 48
R. penicillatus F	B15-020	Austria, Bogenhofen: unnamed stream	6x	ca 48

ranging from 1.63 pg in a hybrid cytotype from the upper Vltava river (see below) to 2.83 pg in *R. circinatus* (Fig. 1B).

No substantial variation in genome size was revealed in *R. aquatilis* (hexaploid), *R. baudotii* (tetraploid), *R. circinatus* and *R. rionii* (both diploid). In *R. fluitans*, diploid and triploid populations were detected, with diploids being more frequent in our dataset (52 and 15, respectively). Both cytotypes share almost identical monoploid genome size. In all other taxa, we revealed several cytotypes of different ploidy levels or different genome sizes within a ploidy level, or both.

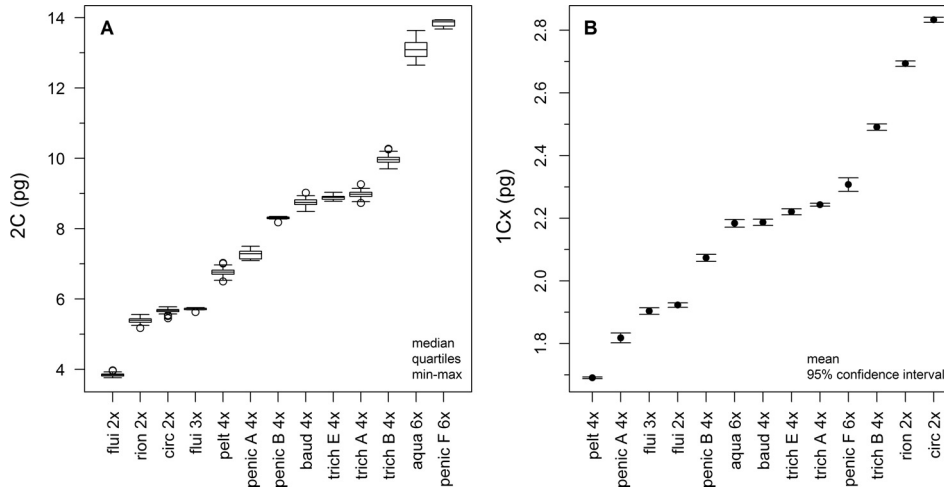

Fig. 1. – Box-and-whisker plots showing the genome size variation in 13 cytotypes of sect. *Batrachium*, for which the chromosome numbers were determined: (A) holoploid genome sizes (2C-values); (B) monoploid genome sizes (1Cx-values). Species abbreviations: aqua – *R. aquatilis*; baud – *R. baudotii*; circ – *R. circinatus*; flui – *R. fluitans*; pelt – *R. peltatus*; penic – *R. penicillatus* (cytotypes A, B and F); rion – *R. rionii*; trich – *R. trichophyllus* (cytotypes A and B; E = subsp. *eradicatus*). After cytotype abbreviations, ploidy levels are indicated.

In *R. peltatus*, all populations studied consist of one dominant cytotype, which is tetraploid. However, aberrant plants with the appearance of *R. peltatus* were rarely detected among ordinary tetraploids, which had a genome size corresponding to the putative pentaploids (three individuals in populations K10-06 and K15-19) or hexaploids (one individual in population B15-053). Unfortunately, we were not able to detect the true ploidy level by direct chromosome counting in these extremely rare individuals.

Two distinct cytotypes, both tetraploid, were recognized within *R. trichophyllus.* Their mean genome sizes differ by 11.0% (Fig. 3A) and henceforth we tentatively designate them as *R. trichophyllus* A and *R. trichophyllus* B (with smaller and bigger genome sizes, respectively). The cytotype *R. trichophyllus* B is somewhat more robust in overall morphology and appears to be ± intermediate between *R. trichophyllus* A and hexaploid *R. aquatilis* (Table 2). However, *R. trichophyllus* B is intermediate neither in ploidy nor in genome size. Both cytotypes of *R. trichophyllus* are fully fertile, with most of the achenes developing normally. The variation within *R. trichophyllus* may be even more extensive. Genome size of plants from oligotrophic alpine lakes, which correspond to the traditionally recognized *R. trichophyllus* subsp. *eradicatus* (Table 2), overlaps the lower half of the genome size range of *R. trichophyllus* A. Due to distinct ecologies and minute but constant morphological differences we treat these plants as a third group within *R. trichophyllus*. Furthermore, the *R. trichophyllus* B sample with the largest genome size (B14-119, Hallstättersee, Austria) is distinct from other samples of this cytotype (bifurcated peaks in simultaneous analyses; Fig. 3B).

Extraordinary variation was revealed within *R. penicillatus*, in which the genome size spans from 7.27 to 13.84 pg and six distinct cytotypes were detected (referred to as

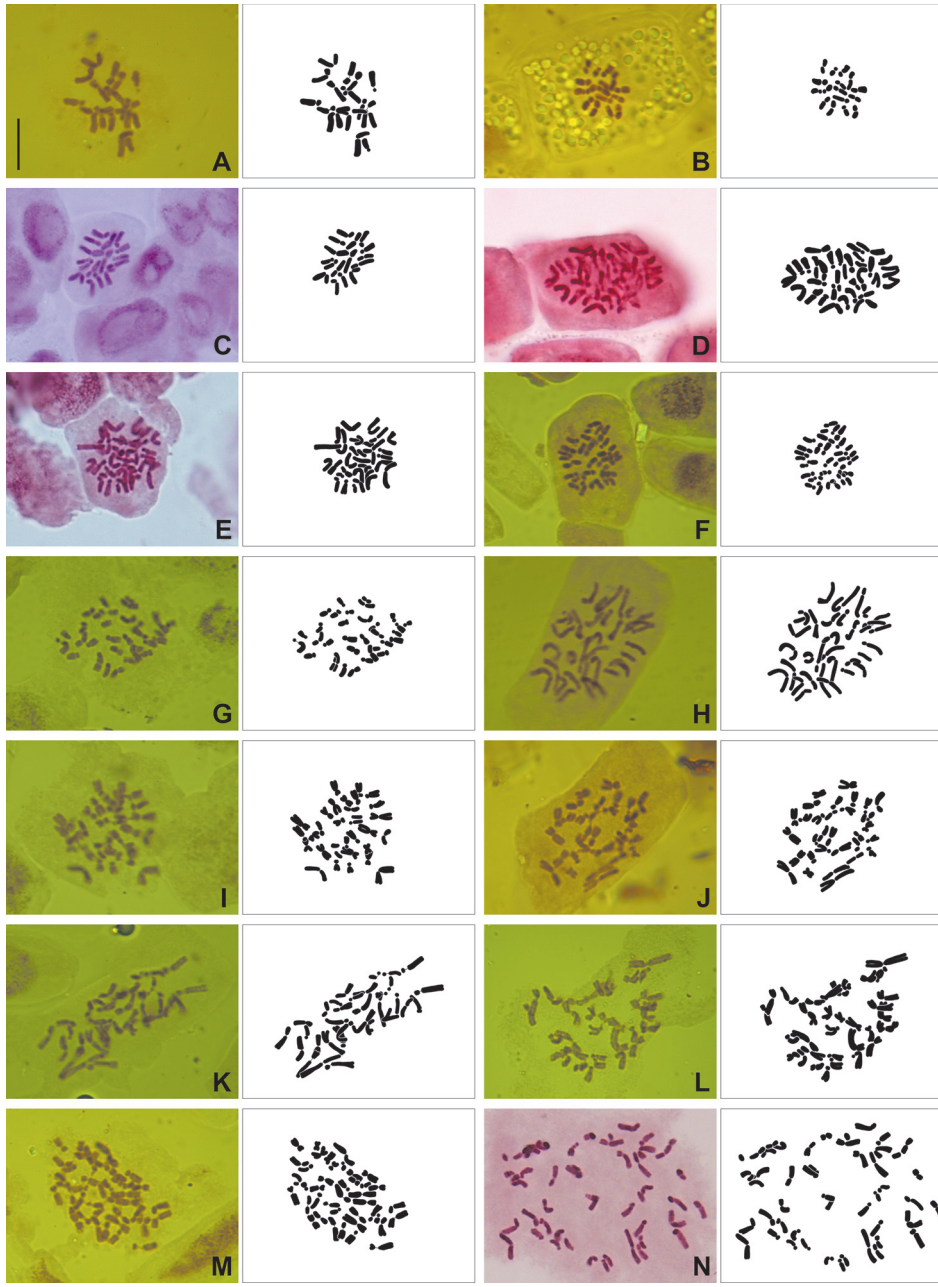

Fig. 2. – Chromosomes (photograph of cytological preparation on the left with its interpretation on the right) of selected cytotypes of *Ranunculus* sect. *Batrachium* at mitotic metaphase in somatic cells, arranged according to chromosome number: A – *Ranunculus circinatus*, sample B14-043, 2n = 16; B – *R. fluitans*, sample B14-036, 2n = 16; C – *R. rionii*, sample B12-008, 2n = 16; D – *R. baudotii*, sample B12-048, 2n = 32; E – *R. baudotii*, sample B14-073, 2n = 32; F – *R. peltatus*, sample B12-045, 2n = 32; G – *R. peltatus*, sample B12-009, 2n = 32; H – *R. penicillatus* B, sample B15-043, 2n = 32; I – *R. trichophyllus* B, sample B14-122, 2n = 32; J – *R. trichophyllus* A, sample B14-035, 2n = 32; K – *R. trichophyllus* A, sample B12-010, 2n = 32; L – *R. peltatus* × *R. trichophyllus* A, sample K13-23d, 2n = 32; M – *R. aquatilis* × *R. trichophyllus* B, sample B14-013, 2n = 40; N – *R. aquatilis*, sample B13-021, 2n = 48. Scale bar identical for all figures = 10 µm.

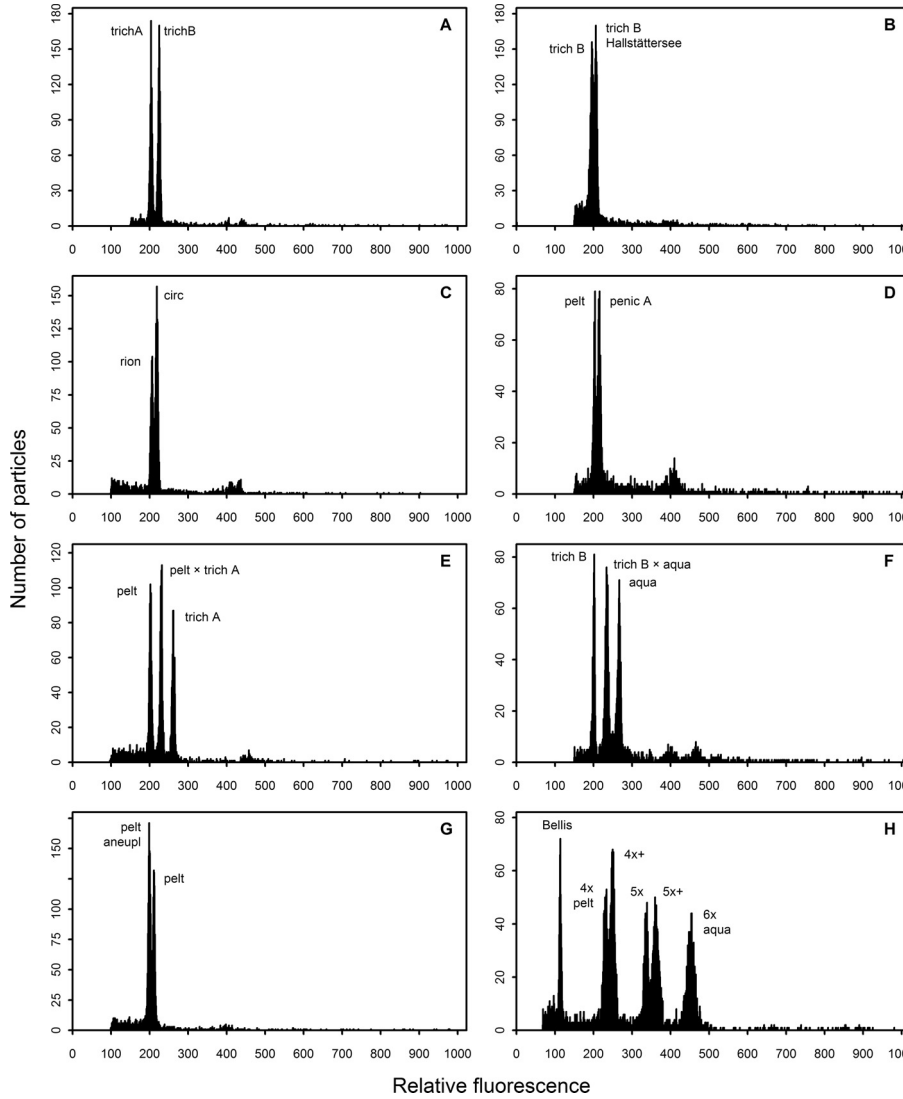

Fig. 3. – Flow cytometric histograms showing simultaneous analyses of selected sect. *Batrachium* taxa: A – simultaneous analysis of *R. trichophyllus* A (sample B14-035) and *R. trichophyllus* B (sample B12-005) documenting 11.0% divergence between these tetraploid taxa; B – simultaneous analysis of two *R. trichophyllus* B accessions (samples B14-122, Almsee, AT, and B14-119, Hallstättersee, AT) resulting in a bifurcated peak; C – simultaneous analysis of two diploid species with similar genome sizes, *R. rionii* and *R. circinatus* (samples B14-082 and B14-087); D – bifurcated peaks documenting the slight difference between the genome sizes of tetraploid taxa *R. peltatus* (sample B12-009) and *R. penicillatus* A (sample B14-031); E – simultaneous analysis of the tetraploid taxa *R. peltatus* (sample B14-075), *R. trichophyllus* A (sample B14-076) and their hybrid (sample B14-077) from the locality "Bražecké hliňáky", CZ; F – simultaneous analysis of tetraploid *R. trichophyllus* B (sample B14-011), hexaploid *R. aquatilis* (sample B14-010) and their pentaploid hybrid (sample B14-013) from the pool "Ludmila", CZ; G – slightly different genome sizes of two individuals of *R. peltatus* (Javorenský fishpond, CZ; samples B14-080 and B14-079), i.e. the putative aneuploid with the smaller genome size and "typical" *R. peltatus*; H – simultaneous analysis of several individuals from "Choryňský mokřad", CZ, resulting in five peaks, represented by *R. peltatus* (sample K13-18), putative backcross towards *R. peltatus* (sample K13-18-11), two individuals of the hybrid *R. peltatus* × *aquatilis* with slightly different genome sizes (sample K13-20) and *R. aquatilis* (sample K13-19), respectively. All analyses were performed using DAPI fluorescence dye, except for histogram H for which PI staining was used.

R. penicillatus A–F). Three of these cytotypes, including the most frequently occurring, were found to be tetraploids, whereas the single population with the largest genome size among all *Batrachium* taxa was hexaploid. Unfortunately, the chromosome counting of the remaining cytotypes failed as the preparations contained only mitotically inactive cells or the observations were inconclusive.

The majority of *Batrachium* species (including the lineages A and B of *R. trichophyllus* as separate groups and taking only the most widespread cytotype A of *R. penicillatus* into account) have distinct DNA contents (2C-values) with almost non-overlapping ranges (Figs 1A, 3C, D, Table 3). The only two exceptions showing a considerable overlap are (i) *R. circinatus* and triploid *R. fluitans* and (ii) *R. baudotii* and *R. trichophyllus* (the cytotype A and the alpine populations marked as subsp. *eradicatus*). However, even within these groups the differences are statistically significant ($P < 0.05$).

Hybridization

Besides the taxonomically pure species (some including several cytotypes), numerous putative hybrids were found based on a combination of morphology and genome size. These hybrids accounted for ~15% of the individuals analysed and were recorded in 14% of the populations analysed, making the overall pattern in the cytotype variation very complicated. At least 16 different hybrid cytotypes were revealed (Table 3). Five of these cytotypes were identified as F_1 hybrids, being morphologically ± intermediate, with genome sizes intermediate between the putative parents (Figs 3E, F) and usually being only partially fertile (only a small number of achenes develop normally, the rest of the carpels on receptacles is shrivelled). Among them, only *R. peltatus* × *R. trichophyllus* A (tetraploid, like its parental taxa) occurred relatively frequently, being found at 17 localities (at seven localities co-occurring with both parental species, four localities where *R. trichophyllus* A was absent and six localities where the hybrid occurred without any of the parental species). The remaining hybrid combinations, represented by triploid and pentaploid hybrids that resulted from heteroploid crosses, were rarely detected. We found two additional putative F_1 hybrids but do not have firm evidence for their unequivocal identification. A single plant (B16-036) with slightly reduced fertility, found within a mixed population of *R. circinatus* and *R. rionii*, was morphologically intermediate between these species. However, due to small difference in the parental 2C-values, it is not possible to distinguish the putative hybrid from individuals at the extremes of the variation of either parent. The second case was a single plant growing in a stand of *R. trichophyllus* A, which had a genome size intermediate between *R. trichophyllus* A and *R. rionii*, but the latter species was not detected at the locality. This young individual was in the initial stages of flowering and bore no fruits at that time, which prevented reliable morphological identification. In both cases, further analyses using molecular methods are needed.

Apart from F_1 hybrids, we recorded several plants of *R. peltatus* × *R. trichophyllus* A appearance that had various 2C-values ranging between that of *R. peltatus* and the F_1 hybrid *R. peltatus* × *R. trichophyllus* A (Fig. 4; referred to as "other hybrids" in Table 3 and Electronic Appendix 1). These plants often co-occurred with *R. peltatus* (whereas *R. trichophyllus* A was sometimes absent). Rarely morphologically hybrid populations were found with unexpected patterns of cytotype variation, such as individuals with

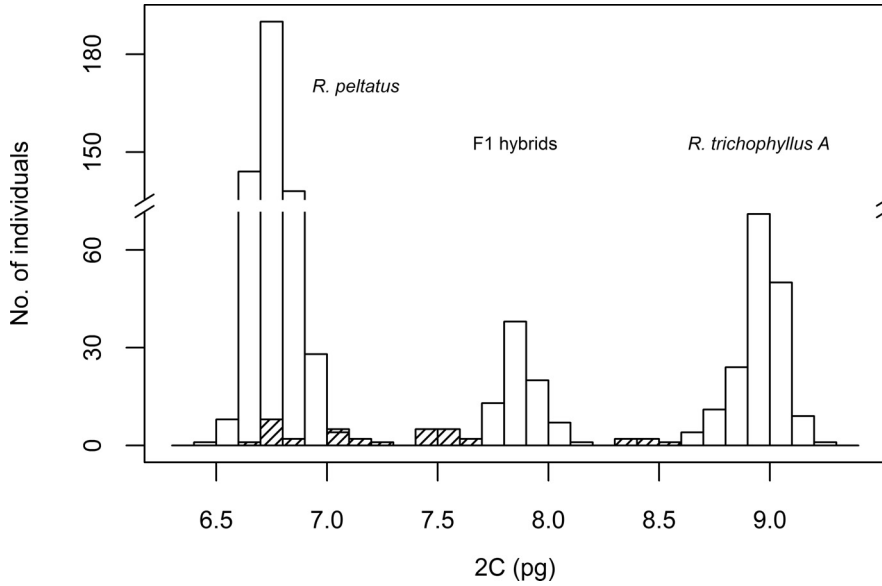

Fig 4. – Histogram of the distribution of the genome sizes (2C value) of *Ranunculus peltatus*, *R. trichophyllus* A and all products of hybridization between these taxa: empty columns – individuals classified as *R. peltatus*, F_1 hybrid *R. peltatus* × *trichophyllus* A and *R. trichophyllus* A; hatched columns – various hybrids other than F_1 determined as such based on morphology.

a genome size larger than that of *R. trichophyllus* A (referred to as "*R. trichophyllus* hybr.?") and several putative aneuploid or hybrid plants of *R. peltatus* morphology, but with a genome size slightly smaller than that of *R. peltatus* (Fig. 3G; referred to as "*R. peltatus* aneuploid").

A more complex case of hybridization was detected also in one of the two explored populations of the pentaploid hybrid *R. aquatilis* × *R. peltatus* (K13-20, Choryňský mokřad marsh, Czech Republic). Genome size data indicate the occurrence of the parental species, F_1 hybrids and two other hybrid individuals with non-intermediate genome sizes (referred to as "hybrid 1" and "hybrid 2" in Table 3), as also revealed by the simultaneous flow cytometric analysis of selected plants that resulted in five separate peaks (Fig. 3H).

In the Ohře river and its tributaries (north-western Czech Republic), numerous abundant populations were found that showed a continuous variation in morphology between *R. peltatus* and *R. penicillatus* A (hereafter referred to as *R. peltatus-penicillatus* A). Compared to "pure" *R. peltatus* and *R. penicillatus* A sampled at other sites, genome sizes of the Ohře populations are transitional, with the entire range of values between these species (Fig. 5).

Finally, populations of morphologically strange infertile or only partly fertile plants were found in several rivers. These populations were not clearly attributable to any parental species, based on either morphology or genome size, although *R. peltatus* parentage may be expected in all cases. These includes plants from several rivers in Moravia, southern Bohemia (Czech Republic) and Upper Austria (e.g. Moravice, Lužnice, Stropnice,

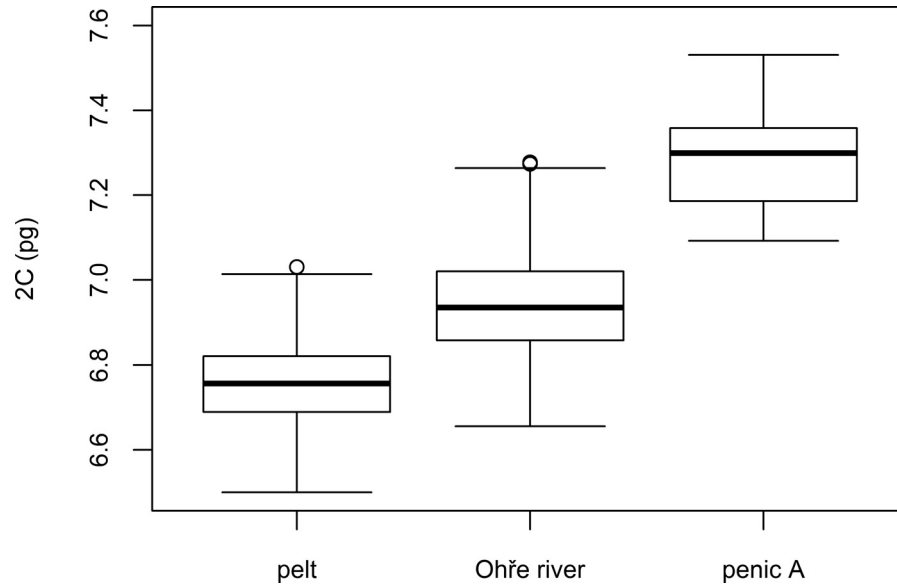

Fig. 5. – Box-and-whisker plots showing the genome size variation of *Ranunculus peltatus*, *R. penicillatus* A and a putative hybrid swarm in the Ohře river and its tributary Odrava (referred as to "*R. peltatus-penicillatus* A"). Line, box and whiskers refer to median, quartiles and non-outlier range, respectively.

Malše / Maltsch, Große Mühl) whose genome size is virtually indistinguishable from that of *R. peltatus,* but which have a set of characters typical of hybrids and very unusual for *R. peltatus* (partial absence of laminar leaves when in flower, frequent formation of intermediate types of leaves or presence of deeply dissected laminar leaves with irregularly and sharply dentate margins, unusually long peduncles, small number of developed achenes); we further refer to them as "*R. peltatus* hybr.". A unique cytotype occurs in the Váh river (Slovakia) and another one in the upper course of the Vltava river (Czech Republic). Remarkably, the plants from the Vltava were found to be hexaploid and have the smallest monoploid genome size of all the taxa analysed.

Distribution and ecology

By far the most abundant taxon in our dataset was *R. peltatus*, which was recorded at 209 of the 612 localities, followed by *R. circinatus* (77 localities) and *R. trichophyllus* A (73 localities). Only two species (*R. fluitans* and *R. penicillatus*) are confined to running water, whereas *R. peltatus* and several hybrids regularly occur both in running and still water. The remaining species were detected rather rarely in running-water habitats or are absent from them.

Regarding species with several cytotypes, the distributions of particular cytotypes do not overlap much. In *R. fluitans*, the occurrence of both (2x, 3x) cytotypes was detected in just three rivers (Berounka, Sázava and Želivka rivers, Czech Republic) and in only a single population was there a mixture of cytotypes (K14-75; however, plants at this site included some unrooted individuals caught on the bank of the river, probably originating

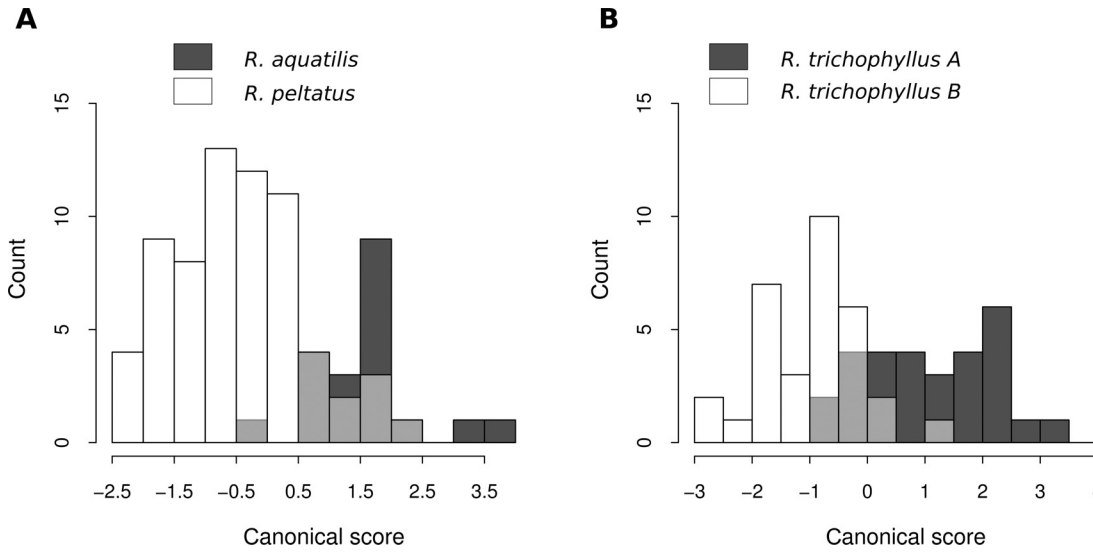

Fig. 6. – Linear discriminant analysis of range-wide climatic niche, calculated for the taxa couples *Ranunculus aquatilis – R. peltatus* (A; 87 populations) and *R. trichophyllus* A – *R. trichophyllus* B (B; 61 populations). In classes where both groups are present the less frequent one is depicted in grey ("semitransparent"). The model is based on 19 environmental variables (for details, see Electronic Appendix 2).

from upstream). Among a total of 115 *R. trichophyllus* sites, there were only two at which both cytotypes A and B co-occurred. In the Czech Republic, where the largest part of our samples was collected, both cytotypes show a distinct distribution pattern: whereas cytotype B occurs predominantly in warm areas at low elevations, cytotype A was recorded mostly at middle and rather high elevations and tended to absent in lowlands. The same pattern was observed also for the morphologically similar pair of species *R. aquatilis* and *R. peltatus* (the former at lower and the latter at higher elevations), which were found growing together at only four localities. The discriminant analyses of Bioclime data performed on these taxa indicate clear (although by far not complete) separation of both taxa couples on the basis of their fundamental bioclimatic characteristics (Fig. 6; Electronic Appendix 2).

However, several taxa often co-occurred in mixed populations. This is particularly true for the pair *R. peltatus – R. trichophyllus* A, which were found growing together at 26 localities (i.e., 35.6% of the localities of the latter).

Discussion

Genome size as a tool for identification of traditionally recognized species of Batrachium

We proved that flow cytometry combined with morphology is a reliable, fast, inexpensive and easy tool for identifying central-European taxa of *Batrachium* (Table 3, Figs 1, 3). Even homoploid species are recognizable on the basis of their genome size. Only a few cases of genome size overlap were detected. However, the identification of *R. circinatus* and the triploid cytotype of *R. fluitans* is easy even using vegetative characters as they are

probably the most distinctive species of *Batrachium* in Europe. Last but not least, these two species differ fundamentally ecologically and were never found co-occurring. The second group of taxa with overlapping genome sizes consists of *R. baudotii*, *R. trichophyllus* A and *R. trichophyllus* subsp. *eradicatus*. These taxa also differ ecologically and we have never found them growing together. While *R. baudotii* is a species of mineral-rich water in lowlands and is also characteristic of brackish water in coastal regions of Europe (Cook 1966), *R. trichophyllus* A seems to prefer more acidic habitats. As for *R. trichophyllus* subsp. *eradicatus*, it differs only slightly morphologically from *R. trichophyllus* A (Table 2) but is, however, distinguished also by its unusual habitat (oligotrophic alpine lakes).

Despite the large number of different chromosome numbers reported in the literature (Table 1), most of the central-European species of *Batrachium* are well-defined and uniform in ploidy level (Tables 3, 4). It seems likely that at least a part of the reported variation is due to misidentification of the counted material. Chromosome counting combined with genome size determination could eliminate most of this sort of confusion in the future.

Monoploid genome sizes and effect of polyploidy on the overall variation

Polyploidization is the main driver of sympatric speciation in plants (Otto & Whitton 2000). Differences in ploidy level often have significant effects on phenotypic and reproductive traits (Husband et al. 2013). Our study confirmed *Batrachium* as an extraordinarily remarkable group in terms of variation in ploidy levels and revealed a total of five ploidy levels within central-European representatives (2x, 3x, 4x, 5x, 6x; see Table 4, Fig. 2). Among them, pentaploid (2n = 40) counts were rarely recorded in hybrids, whereas other ploidy levels were recorded for both pure species and hybrids. The biggest part of the variation in genome size was detected at the tetraploid level, both in terms of the number of taxa and variation attributable to hybridization.

In addition to the variation in ploidy level, we also detected notable variation within individual ploidy levels, which reflects the 1.69-fold variation in monoploid genome sizes (1Cx-values) (Table 3, Fig. 1B). This prevents genome size (2C-value) being straightforwardly used for estimating ploidy level. For example, diploid *R. circinatus* (2C = 5.67 pg of DNA) has nearly the same genome size as triploid *R. fluitans* (2C = 5.71 pg), and a hexaploid hybrid from the Vltava river (2C = 9.78 pg) has a smaller genome size than the tetraploid *R. trichophyllus* B (2C = 9.96 pg) and pentaploid hybrid *R. aquatilis* × *R. peltatus* (2C = 10.06 pg). An unambiguous ploidy determination, therefore, relies on chromosome counting. Such a situation is not rare and several other examples of ecologically different congeners with genome sizes being incongruent with ploidy levels are currently known (e.g. *Chenopodium*, Mandák et al. 2012; *Callitriche*, Prančl et al. 2014; *Anthoxanthum*, Chumová et al. 2015).

The monoploid genome size reflects evolutionary relationships in many groups of plants (e.g. Hohmann et al. 2014, Mandák et al. 2016, Krahulcová et al. 2017). Among species of water-crowfoots, the largest 1Cx-values are for the pair of diploid species, *R. circinatus* and *R. rionii*, which corresponds to their isolated phylogenetic position in the sect. *Batrachium* (Bobrov et al. 2015). Monoploid genome sizes may also reflect the origin of polyploids, i.e. monoploid genome sizes of the parents and the number of copies of their chromosome complements (e.g. Kúr et al. 2012, Mandák et al. 2012, Chumová et

al. 2015). We assume an autopolyploid origin of the triploid *R. fluitans*, whose monoploid genome size is almost identical to that of the diploid cytotype of this species (1Cx = 1.92 pg and 1Cx = 1.90 pg, respectively). We also did not notice any obvious morphological differences between these cytotypes, which would be expected if the triploids were allopolyploid. The triploid cytotype may have originated by syngamy of reduced and unreduced gametes of a diploid or could be a descendant of hybridization between a diploid and hypothetical autotetraploid. Tetraploid *R. fluitans* is repeatedly reported from various regions in Europe including our area of interest (see Table 1). Nevertheless, we did not find any tetraploids during our fieldwork and the single report of tetraploid *R. fluitans* from the Czech Republic (Teplá river near the confluence with the Ohře river; Turała-Szybowska 1977) is questionable and may be an identification error because we have found only *R. peltatus* in the former river and *R. peltatus-R. penicillatus* A in the latter river.

Spontaneous formation of autopolyploids within populations of a lower ploidy level is not a rare phenomenon (e.g. Kolář et al. 2017) and is also recorded in aquatic plants (e.g. *Potamogeton*, Kaplan et al. 2013; *Callitriche*, Prančl et al. 2014). In this study, we recorded a single plant of *R. peltatus* (B15-053), growing within a population of tetraploids, whose genome size corresponded to that of a hexaploid (Table 3). This individual could have arisen by syngamy of reduced (2x) and unreduced (4x) gametes of *R. peltatus*. We recorded, however, several plants with the appearance of *R. peltatus*, whose genome size corresponded to putative pentaploids (three individuals in two populations: K10-06, K15-19; Electronic Appendix 1, Table 3) and the occasional occurrence of putative aneuploids. In these cases, there is no simple explanation of how these individuals might have arisen from tetraploid parental species. A further study employing molecular methods is in progress.

The most well-known case of allopolyploid species in *Batrachium* is the *R. penicillatus* complex, for which four ploidy levels are reported (Table 1). In this study, most populations morphologically assignable to *R. penicillatus* were identified as tetraploids (Tables 3, 4) and only a single population was hexaploid. The genome size of the most widespread cytotype A is consistent with its known allopolyploid origin from *R. fluitans* and *R. peltatus* (two chromosome sets from each; calculated 2C = 7.23 pg which is nearly identical to the recorded value of 7.27 pg). In the hexaploid *R. penicillatus* F (population K15-12, Bogenhofen, Austria), the genome size is consistent with the sum of the genome sizes of *R. fluitans* (two copies) and *R. trichophyllus* B (four copies): the calculated value is 13.81 pg, while the recorded value is 13.84 pg. The hexaploid *R. penicillatus* thus might be an allopolyploid that arose from these two species either via the union of two unreduced gametes or via a triploid F_1 hybrid that polyploidized. Indeed, *R. trichophyllus* B still occurs at the site and one individual there had a genome size intermediate between the putative parents (i.e., half of the genome size of *R. penicillatus* F), which might be a F_1 hybrid. However, all these hypotheses have to be confirmed by the ongoing molecular study.

Cryptic variation

Cryptic variation in organisms is genetic variation that is not reflected in morphology but may be an fundamental source of physiological and evolutionary potential (Bickford et al. 2007, Paaby & Rockman 2014). Water-crowfoots have a unique potential for investi-

gating patterns of cryptic diversity, which share a number of evolutionary phenomena that can blur the morphological borders among biological taxa. Indeed, we found cryptic variation in two evolutionarily distinct lineages: *R. trichophyllus* and *R. penicillatus*.

The morphologically strongly reduced species *R. trichophyllus* in our data set includes three tetraploid lineages: ecologically highly specialized alpine plants (subsp. *eradicatus*, sometimes recognized at the species level as *R. confervoides*) and two non-alpine cytotypes A and B, which differ significantly in their genome size. These two cytotypes also differ ecologically (see above) and we have not revealed any signs of hybridization between them. However, the cryptic variation in the *R. trichophyllus* group may be even more complex. Recent molecular studies revealed four haplotypes and five ribotypes within this species, some of them seemingly unrelated (Zalewska-Gałosz et al. 2014, Bobrov et al. 2015). In addition, a sample of *R. trichophyllus* B from low elevations in the Alps (B14-119) had a markedly larger genome size than other samples assigned to this cytotype, which raises doubts about their taxonomic homogeneity. The variation in *R. trichophyllus* in peri-alpine rivers and lakes, which were only marginally covered by our sampling, and elsewhere outside central Europe, thus, needs further study.

On the other hand, we have not confirmed the reported presence (see Table 1) of the hexaploid *R. trichophyllus* in central Europe. During the fieldwork, however, we recorded extensive morphological variation in several hexaploid populations (e.g. B13-085, K14-15) that contained both typical *R. aquatilis* plants with relatively big flowers and laminar leaves, small-flowered homophyllous *R. trichophyllus*-like individuals and various intermediates. We consider these plants as extremes of the variation in *R. aquatilis* instead of two putative taxa and hybrids, because (1) we never recorded *R. trichophyllus*-like hexaploids forming populations on their own, and (2) the genome size of these plants are consistent with a hexaploid derived from *R. trichophyllus* A and is much smaller than that of a hexaploid based on *R. trichophyllus* B but the habitat conditions at these sites (lowlands, mineral-rich water) match only that of *R. trichophyllus* B while *R. trichophyllus* A has never been found in such a habitat. Indeed, frequent occurrence of only homophyllous plants of *R. aquatilis* is known (e.g. Cook 1966, Englmaier 2016, Wiegleb et al. 2017). Therefore, it is possible that reports of hexaploid *R. trichophyllus* in central Europe are based on misidentification of atypical *R. aquatilis*.

Another group, in which we recorded cryptic variation, is the *R. penicillatus* complex. In most of Europe, two taxa of *R. penicillatus* s. lat. are traditionally recognized and classified either as the distinct species *R. penicillatus* s. str. and *R. pseudofluitans* (e.g. Englmaier 2016, Wiegleb et al. 2017) or at the subspecies level (e.g. Webster 1988, Dahlgren 1993). The former is defined as a heterophyllous taxon, believed to have originated by hybridization between *R. fluitans* and *R. peltatus*, while the latter is characterized by the absence of laminar leaves and *R. fluitans* along with *R. aquatilis* or *R. trichophyllus* are considered to be its putative parental species (Cook 1966). In this study we found that the actual situation is considerably more complicated. We revealed a total of six cytotypes, including at least two ploidy levels (Tables 3, 4). In central Europe, we detected two tetraploid and one hexaploid cytotype but only tetraploid cytotype A is widespread while other tetraploids are confined to two rivers (cytotype B: Loučná river, Czech Republic; Pisz river, Poland) and the hexaploid (cytotype F) is found only at a single locality (Bogenhofen, Austria). Within the widespread *R. penicillatus* A, populations with heterophyllous individuals prevailed. However, both heterophyllous

and homophyllous plants of this cytotype were often found growing together and in some rivers only homophyllous plants occurred (e.g., Opava river, Czech Republic; Freiberger Mulde and Zschopau rivers, Germany). Thus, presence/absence of laminar leaves seems to be a feature of lower taxonomic value than was previously assumed and the current taxonomic concept of *R. penicillatus* s. lat. does not correlate with the actual diversity, which remains largely unrecognized.

As previously reported, it is often difficult to distinguish stabilized hybridogenous taxa of *R. penicillatus* s. lat. from recent hybrids of *R. fluitans* with other species (Cook 1966, Zalewska-Gałosz et al. 2014). During our study, we did not record any populations with the appearance of *R. penicillatus* with markedly reduced fertility. Therefore, we include all these populations under *R. penicillatus*.

Interspecific hybridization

In aquatic plants, newly formed interspecific hybrids often persist by vegetative reproduction for a long time (even after one or both parental species have disappeared from a site) and may also spread to other sites and become an important part of the overall biodiversity (e.g. Moody & Les 2007, Kaplan et al. 2009, Prančl et al. 2014). Morphological reduction of many aquatic plants combined with extensive phenotypic plasticity result in (i) aquatic plant hybrids often being neglected and (ii) extreme morphotypes of "pure" species erroneously being considered to be hybrids. For *Batrachium*, an extraordinary number of hybrids is reported in the literature (e.g. Cook 1966, 1970, Dahlgren 1991, 1995, Hong 1991, Wiegleb et al. 2017). Our study confirmed the large effect of hybridization in this group. We detected hybrids in almost 14% of the populations studied and revealed at least 16 cytotypes of putative hybrid origin, which makes the overall variation much more complicated (Table 3, Figs 3E, F, H, Figs 4, 5). Seven of them we consider to be F_1 hybrids based on both their intermediate morphology and genome sizes.

Intensity of hybridization in the *Batrachium* group seems to be species-specific. The diploid species appear to rarely form hybrids. For the polyploid taxa, the frequency of hybridization is uneven. The ecologically specialized species *R. baudotii* grows alone without the presence of other species at most of the localities where it occurs and no hybrids of *R. baudotii* were recorded. The same holds true for the alpine *R. trichophyllus* subsp. *eradicatus*. The ecologically similar taxa *R. peltatus* and *R. trichophyllus* A, however, often grow in mixed populations and the F_1 hybrids of them are relatively frequent, being found at 17 localities, sometimes occurring even without one or both parents (four and six localities, respectively). This quite frequent and morphologically conspicuous hybrid is reported only sporadically (cf. Wiegleb et al. 2017). This may be due to frequent confusion with the morphologically similar but not closely related *R. aquatilis*. In *R. penicillatus* A (the only widespread cytotype of this species) the pattern of hybridization is rather unexpected. Although it co-occurs with *R. peltatus* and sometimes also with *R. fluitans* in many of the rivers sampled we found *R. penicillatus* hybrids only in the Ohře river and its tributary Odrava in the Czech Republic. In this river many plants show transitional appearance between *R. peltatus* and *R. penicillatus*, occurring in a 150 km stretch of the river. These plants most likely represent a fertile hybrid swarm, which may include F_1 hybrids, subsequent filial generations and backcrosses. Other species of *Batrachium* hybridize only rarely because of their rare co-occurrence. However, the

breeding barriers might be week, as exemplified by the occurrence hybrids at two out of the four sites sampled where *R. aquatilis* and *R. peltatus* co-occur, and at two out of the five sites where *R. aquatilis* and *R. trichophyllus* B co-occurred.

Most of F_1 hybrids were found to be at least partially fertile. We revealed marked genome size variation in *R. peltatus* × *R. penicillatus* A (Fig. 5, see above), *R. peltatus* × *R. trichophyllus* A (Fig. 4) and *R. aquatilis* × *R. peltatus* (Fig. 3H). Hybrid individuals with genome sizes not halfway between the parents may be either backcrosses or F_2 or later-generation hybrids, which may be variable due to the segregation of homeologous chromosomes of different sizes even in homoploid crosses (e.g. Hutchinson et al. 1979; analogous is also the within-species variation in *Festuca pallens* as documented by Šmarda et al. 2008). In progeny of triploid and pentaploid hybrids (if not sterile), additional variation may be introduced by the unbalanced number of chromosomes in gametes. In the most frequent hybrid combination at the tetraploid level, *R. peltatus* × *R. trichophyllus*, the genome size of hybrids is biased towards *R. peltatus* (Fig. 4). We hypothesize that this is more likely the result of backcrossing than variation in F_2 and later generation hybrids. The rather unidirectional gene flow might be driven by a large difference in flower size: the big-flowered *R. peltatus* is more likely to be a pollen donor than the small-flowered *R. trichophyllus*. We have even repeatedly encountered populations of plants of definite hybrid appearance (see above; named as "*R. peltatus* hybr." here), which have, however, a genome size within the range of "pure" *R. peltatus*. We hypothesize that these plants might be multiple backcrosses towards *R. peltatus*. It is possible that such multiple backcrosses are quite frequent and over time become indistinguishable from *R. peltatus* in terms of morphology, karyology and genome size. In any case, a molecular analyses or in situ hybridization techniques could bring more clarity to this issue in the future.

Rivers and streams may be an "evolutionary incubator" for newly arising hybrids and polyploids, because even completely sterile individuals can spread effectively downstream via clonal propagation. Due to the heterosis effect, hybrids can survive for a very long time in streams even after the disappearances there of their parents, as frequently documented for aquatic plants (e.g. Preston et al. 1998, Kaplan & Fehrer 2011, Prančl et al. 2014, Kaplan et al. 2018). Except for putative multiple backcrosses of *R. peltatus* we recorded two unique cytotypes of unknown origin (upper Vltava river, Czech Republic; Váh river, Slovakia; Table 3), which are both heterophyllous and apparently derived from *R. peltatus*, forming plentiful populations without the presence of any other *Batrachium* taxa. The upper Vltava hybrid is especially remarkable, because this hexaploid cytotype has the lowest monoploid genome size so far recorded in *Batrachium* and it is lower than in any of its potential parental species (Table 3). This completely sterile hybrid occupies an approximately 25 km long section of this submontane river above the Lipno I dam; however, downstream we surprisingly recorded only *R. peltatus*.

Ecological differences among Batrachium taxa

Our study revealed clear ecological difference between the morphologically similar and often confused species *R. aquatilis* and *R. peltatus* (Fig. 6A). This finding is in accordance with the observations of the monographer C. D. K. Cook, who states that he has never seen these two species growing together at the same site (Cook 1966). Comparison of bioclimatic characteristic showed that the temperature-related variables are the most

informative characters, i.e. *R. aquatilis* prefers warmer areas than *R. peltatus*. The same pattern was observed also for the pair of cryptic *R. trichophyllus* cytotypes A and B, with the latter being more thermophilous (Fig. 6B). However, other factors than temperature may be involved or even the determining ones. In general, the distribution of species of aquatic plants is less dependent on temperature than that of terrestrial plants (Sculthorpe 1967). In the area where most samples were collected, temperature is clearly correlated with elevation and the bedrock: the warm lowlands are composed mainly of mineral-rich sediments while in the colder highlands more acidic (both sedimentary and igneous) bedrock dominates. Nevertheless, there are also several cases of central-European species of aquatic plants with climatically limited distributions regardless of the substrate pH, such as *Hottonia palustris* in lowlands or *Eleocharis mamillata* subsp. *austriaca* in mountainous areas (Kaplan et al. 2015, 2016).

We also noticed a difference in the distribution of the cytotypes of *R. fluitans*. While we found triploids predominantly in large rivers, diploids occur in all types of habitats including small and mid-sized water-courses. If both cytotypes occur in the same river (a total of three cases, see Electronic Appendix 1), diploids were found in the upper and triploids in the lower sections of the river. A detailed study focusing on rivers with common occurrence of both cytotypes would be useful to confirm this pattern.

Acknowledgements

We are grateful to Klára Nunvářová Kabátová, Jaroslav Rydlo, Jan Rydlo, Johana Hanzlíčková, Magdalena Hrdinová, Soňa Píšová, Bjarne Moeslund, Jens Christian Schou, Attila Mesterházy, Dietmar Jäger, Michael Hohla, Milan Štech, Kateřina Šumberová, Richard Hrivnák, Peter Englmaier, Clemens Pachschwöll, Olga Lepšová and many others for their help during fieldwork and/or providing samples of *Batrachium*. Tony Dixon kindly improved our English. This work was supported by the Czech Science Foundation (project no. 17-06825S) and by Charles University (GAUK project no. 744213) with additional support provided by The Czech Academy of Sciences (long-term research development project no. RVO 67985939).

Souhrn

Lakušníky (*Ranunculus* sect. *Batrachium*) jsou často považovány za taxonomicky nejsložitější skupinu vodních rostlin v Evropě. Jejich výzkum je komplikován zejména celkovou redukcí tělní stavby, vysokou měrou fenotypové plasticity, výskytem vnitrodruhové ploidní variability a častou hybridizací. Pomocí průtokové cytometrie doplněné o počítání chromozomů jsme hodnotili rozsáhlý soubor 3354 jedinců z 612 lokalit, shromážděných na území 13 převážně středoevropských zemí, absolutní velikost genomu byla určena u 1032 jedinců. Celkem jsme zaznamenali 34 cytotypů o pěti různých ploidiích, od diploidů po hexaploidy. Velikost jaderného genomu se ukázala být spolehlivým znakem pro rozlišení většiny tradičně uznávaných druhů, včetně taxonů o stejné ploidní úrovni. Ačkoli byla variabilita v ploidním stupni udávána pro většinu evropských i středoevropských druhů, naše studie odhalila větší počet ploidních úrovní pouze u dvou druhů, konkrétně u *R. fluitans* (2x, 3x) a *R. penicillatus* (4x, 6x). Situace ve střední Evropě je nicméně značně komplikovaná kvůli četnému výskytu hybridních a kryptických taxonů. Kryptická variabilita byla objevena u druhu *R. trichophyllus*, u kterého byly zaznamenány tři signifikantně odlišné tetraploidní cytotypy, lišící se i ekologicky. Ještě složitější je situace u allopolyploidního komplexu *R. penicillatus*. U tohoto říčního taxonu bylo zaznamenáno celkem šest cytotypů. Dále jsme objevili 16 různých cytotypů hybridního původu, ke kterým patří asi 15 % analyzovaných jedinců. Sedm z těchto cytotypů bylo identifikováno jako F_1 kříženci díky intermediární velikosti genomu, zbylé cytotypy jsou neznámého nebo nejistého původu. V řece Ohři se *R. penicillatus* podílí na vzniku rozsáhlého hybridního roje. Četní kříženci s variabilní velikostí genomu byli odhaleni také ve společných populacích druhů *R. peltatus* a *R. trichophyllus*, přičemž křížení pravděpodobně probíhá převážně jednosměrně směrem k *R. peltatus*. Průtoková cytometrie je ideální metodou pro základní výzkum diverzity lakušníků, a rovněž je schopna odhalit mnohé evoluční procesy, které v této skupině probíhají.

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Received 11 April 2018
Revision received 19 June 2018
Accepted 28 June 2018