



Molecular identification and habitat requirements of the hybrid *Ranunculus circinatus* × *R. fluitans* and its parental taxa *R. circinatus* and *R. fluitans* in running waters

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Abstract *Ranunculus* species grouped in the section *Batrachium* (water crowfoots) indicate the most valuable fluvial habitat in Europe under the EU Habitats Directive, and recognition of their environmental preferences plays a crucial role in efficient conservation. Moreover, water crowfoots are used in various monitoring systems throughout Europe. The aim of this study was to identify the distribution patterns of two common water crowfoot species (*Ranunculus fluitans*, *R. circinatus*) and their hybrid (*R. circinatus* × *R. fluitans*) in relation to environmental variables in rivers. Various chemical and hydromorphological parameters were estimated for 54 river sites where 58 different *Batrachium* populations were recorded. Our study revealed the most distinct positive reaction of *R. fluitans* to rhithral conditions characterized by rapid currents and coarse substrate. *R. circinatus* × *R.*

fluitans preferred larger channels, although this taxon also flourished in rhithral rivers. The preferences of *R. circinatus* were not so evident confirming its occurrence under a wide range of habitat conditions. The ecological reaction of the hybrid was compared with its parental species, revealing their ecological specificity, which is useful in designing appropriate conservation programmes.

Keywords *Batrachium* · Water crowfoots · Rivers · Hydromorphology · Water quality

Introduction

The water crowfoots, a monophyletic group of aquatic plants classified as the section *Batrachium* within the cosmopolitan, morphologically and ecologically diverse genus *Ranunculus* L. (hereafter *Batrachium*; Hörandl et al., 2005), are very valuable elements of aquatic flora, playing an important role in the fluvial systems, and their presence significantly supports stream biodiversity. These plants efficiently modify channel flow as well as nutrient and sediment dynamics, which subsequently creates habitats for other flora and fauna (Mony et al., 2006; Wiegand et al., 2017; Koutecký et al., 2021). *Batrachium* riverine habitats were classified as important by the European Community (Council Directive 92/43/EEC, 1992, known as the Habitat Directive) and are protected in the UE as *Water courses of plain to montane levels*

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with the *Ranunculon fluitantis* and *Callitricho-Batrachion* vegetation (Code 3260) (Hatton-Ellis et al., 2003). Therefore, the precise identification of habitat requirements of this type of vegetation is essential for the conservation efficiency of valuable rivers in Europe. The ecology of *Batrachium* is blurred by complicated taxonomy and unclear species delimitation (Preston & Croft, 2001; Lansdown, 2009). *Batrachium* taxa express extreme phenotypic plasticity (e.g. Cook, 1966; Wiegler & Herr, 1983; Webster, 1988) combined with allopolyploidization and autopolyploidization (e.g. Turała, 1970; Dahlgren, 1995), frequent hybridization (e.g. Cook, 1966; Lansdown, 2009; Zalewska-Gałosz et al., 2015) and the persistence of genetically independent, sterile lineages (Zander & Wiegler, 1987; Lansdown, 2007).

Recently applied molecular tools provided new possibilities for *Batrachium* taxa identification (e.g. Telford et al., 2011; Bobrov et al., 2015; Zalewska-Gałosz et al., 2015; Prančl et al., 2018; Koutecký et al., 2021). Moreover, molecular studies shed a new light on the role of hybridization in *Batrachium* diversity. According to recent reports, approximately 15% of the studied samples from Central Europe represented different hybrid generations (Prančl et al., 2018; Koutecký et al., 2021). Some of the well-known species are confirmed to be allopolyploids, e.g. *Ranunculus schmalhauseni* Luferov, *Ranunculus aquatilis* L. (Bobrov et al., 2015; Zalewska-Gałosz et al., 2015; Koutecký et al., 2021) and have particular ecological preferences that differ essentially from those of the parental species. In this study, we turn our attention to the little known hybrid *Ranunculus circinatus* × *Ranunculus fluitans* and its ecological characterization, as well as ecological differences between the hybrid and the parental species.

Despite the reasonably detailed characteristics of the habitats with *Batrachium* vegetation themselves (e.g. due to protection under the Habitat Directive), the information available about individual species' ecological requirements is rather general and often not recent (Cook, 1966; Haslam, 1987; Dawson & Szoszkiewicz, 1999; Mony et al., 2006). Moreover, some of the recently completed ecological studies are not convincing, since the taxonomy and identification have not been adequately clarified (Lansdown & Pankhurst, 2010; Steffen et al., 2014). In the whole group, both species *R. circinatus* and *R. fluitans*, as mentioned above, are unique and easy to identify.

Even so, there is a need for more recent and detailed studies for running waters for both species. It has already been revealed that *R. circinatus* is frequent in standing waters and slow-flowing rivers but only rarely found in rivers with a fast current velocity. Communities with this species create an independent vegetation type, different to that of other *Batrachium* species (Wiegler & Herr, 1985). A previous study showed that *R. fluitans* communities differ clearly from other *Batrachium* communities with *Ranunculus peltatus* Schrank and *R. penicillatus* complex in terms of botanical metrics, such as richness and evenness. However, it was not possible to identify clear relationships with most of the environmental parameters (Mony et al., 2006). Other works showed that *R. fluitans* was noted in fast-flowing rivers with a medium-coarse substrate and with the absence of fine sediment (Wiegler & Herr, 1985; Garbe et al., 2016).

The requirements of individual *Batrachium* species concerning water quality have been described more often, as they are widely applied in ecological status assessment with nutrients. Moreover, *Batrachium* species as a stenotopic are used in various monitoring systems throughout Europe. In many of them, both *R. fluitans* and *R. circinatus* are specific indicators of mesotrophic (Haury et al., 2006; Szoszkiewicz et al., 2020) and eutrophic conditions (Willby et al., 2012). Therefore, as, for example, Schneider (2007) showed, *R. circinatus* prefers eutrophic lakes, while its ecological tolerance in rivers is much broader. Mony et al. (2006) concluded that *Batrachium* species, due to their occurrence in a broad range of nutrient levels, cannot be indicators of specific trophic conditions. A similar situation applies to any hybrid taxa, not only *Batrachium*, which are not included in most river assessment systems (Haury et al., 2006; Schneider, 2007; Willby et al., 2012; Szoszkiewicz et al., 2020).

Concerning other physical and chemical parameters, different *Batrachium* taxa can use bicarbonates in the photosynthetic process as compensation for CO₂ deficiency (Iversen et al., 2019). As Bodner (1994) pointed out, this mechanism enables *R. fluitans* to carry out photosynthesis, e.g. under high pH and alkaline conditions, when, for hydrophytes that do not use bicarbonate, the photosynthetic rate can decrease to zero. Mony et al. (2006) also noted significantly high alkalinity (in connection with pH) in habitats with *R. fluitans*, revealing differences compared with other *Ranunculus* species. For

R. circinatus × *R. fluitans* there are no current data on relationships with water quality parameters and other habitat conditions.

The aim of this study was to identify the geographical distribution and ecological patterns of two common water crowfoots (*R. fluitans* and *R. circinatus*) and their hybrid (*R. circinatus* × *R. fluitans*) in rivers to formulate appropriate measures for their conservation. In addition to traditional morphological identification, we used objective molecular markers that proved to be decisive in *Batrachium* determination (Bobrov et al., 2015; Zalewska-Gałosz et al., 2015). The potential application of the hybrid into river monitoring and assessment was considered. It was hypothesized that the examined *Batrachium* taxa developing in flowing waters would show different tolerances to hydromorphological and water quality elements.

Materials and methods

Study sites

The field survey was carried at 54 sites selected throughout Poland (Fig. 1) in rivers representing four eco-regions, namely lowland river types (central plains and eastern plains) and highland and mountain watercourses (central highlands). The sites with two taxa (four sites) were taken for analysis once as single sites and have not been subdivided into separate positions with one taxon. A list of the studied localities together with the data on molecularly studied taxa are presented in Table A2—Supplementary Material. Field surveys were carried out between 2014 and 2020 during the vegetative growth seasons, between mid-June and mid-September. Physicochemical studies were conducted during the summer period, in comparable hydrological conditions.

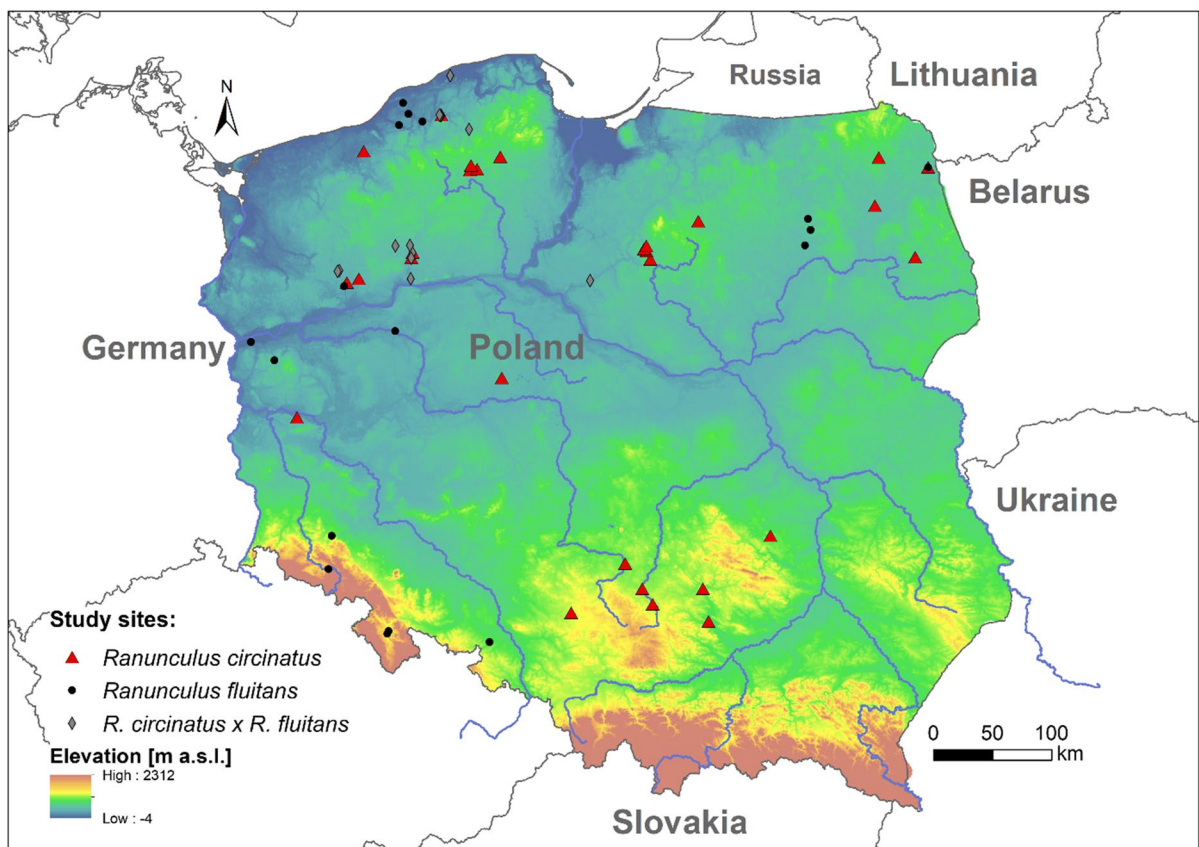


Fig. 1 Location of the study sites

Macrophyte survey

The aquatic plants were surveyed along river reaches 100 m in length. Only river macrophytes growing in the water or at least rooted in the water were recorded. The presence of each species was scored along with their percentage cover using the following nine-grade scale: <0.1%, 0.1–1%, 1–2.5%, 2.5–5%, 5–10%, 10–25%, 25–50%, 50–75% and >75%. All species were identified on the basis of key morphological characters (Preston & Croft, 2001; Jäger et al., 2017; Wiegler et al., 2017). *Batrachium* taxa, apart from traditional morphological identification, were additionally molecularly identified based on polymorphisms detected in the sequences obtained by direct sequencing of selected DNA regions (ITS, *rpl32-trnL* and *psbE-petL*) except some samples of *R. circinatus*, because morphological identification of this species is straightforward and unambiguous, even in a vegetative stage (Cook, 1966; Wiegler et al., 2017). Morphological character values for molecularly identified *R. circinatus* × *R. fluitans* are based on measurements of 20 well-preserved specimens collected during the study.

Molecular identification of key taxa

Fresh leaves collected from each *Batrachium* taxon detected in the studied sites were sampled and stored in small plastic tubes filled with silica gel. To strengthen the analysis, the other *Batrachium* samples collected in different parts of Poland as part of a bio-systematic study of the section *Batrachium* were also included in a molecular study. Since the aim of the present analysis was to identify molecular differences between *R. circinatus*, *R. fluitans* and their hybrid, the analysis was limited to *Batrachium* species with only exceptional inclusion of the other hybrids, which can blur the phylogenetic interpretations. Altogether, 77 *Batrachium* samples were molecularly identified. Detailed information about all specimens included in the analysis is summarized in Table A2—Supplementary Material (42 samples from the localities ecologically studied) and Table A3—Supplementary Material (35 additional samples from the other stations in Poland).

Between 10 and 18 mg of dried plant material was used for DNA isolation. The plant tissue was ground to a fine powder using an MM 400 (Retsch)

mixer mill and 3-mm tungsten beads. Total genomic DNA was extracted using the Plant Mini Kit (Qia-gen), following the manufacturer's protocol. The nuclear ribosomal Internal Transcribed Spacer region (including ITS1, 5.8S and ITS2) was amplified and directly sequenced as described by Zalewska-Gałosz & Ronikier (2010, 2012). As evidenced previously (Telford et al., 2011; Bobrov et al., 2015; Zalewska-Gałosz et al., 2015), the ITS region can serve as a valuable marker for the identification of *Batrachium* taxa. Additionally, two non-coding plastid spacers (cpDNA), *rpl32-trnL* and *psbE-petL*, were investigated to detect potential differences in the haplotypes among taxa. Amplification of cpDNA regions as well as direct sequencing was performed as described by Zalewska-Gałosz et al. (2009, 2010). All polymorphic variants of the ITS and cpDNA sequences were submitted to GenBank (Tables A2, A3—Supplementary Material). The sequences were manually verified/adjusted using ChromasPro 2.1.8. software (<https://technelysium.com.au>). Sequence alignments were conducted manually using BIOEDIT 7.0.5., based on the sequences in the forward direction, and, if the sequences showed intraindividual polymorphisms, on the consensus sequences in both (forward and reverse) sequencing directions. Additive nucleotide polymorphisms were coded using IUPAC nucleotide ambiguity codes. Ambiguous regions of the ITS alignment and incomplete data at the beginning and end of the sequences were excluded from the analysis. Accessions that yielded identical ITS or cpDNA sequences were represented in the phylogenetic analyses by the single terminals. The ITS data were visualized as a phylogenetic network using SplitsTree5 v.5.0.0 alpha software. The NeighborNet method was used to obtain a distance matrix, in which the distances were computed with the Hamming Distances Ambig States algorithm, handling ambiguous states as Average States (Hamming, 1950). A splits network was obtained using The Splits Network Algorithm (Dress & Huson, 2004).

Bayesian analysis was performed in the program MrBayes 3.2.7 (Ronquist et al., 2012). MrBayes, like most maximum likelihood programs, treats gaps and missing characters as missing data. Therefore, as we considered gaps as phylogenetically informative in this study, this information was converted into a binary matrix using FastGap 1.2. The dataset was divided into three partitions: (1) *rpl32-trnL*, (2)

psbE-petL and (3) binary-coded indels. Substitution models were estimated separately for each partition. For the first and second partitions, the MCMC (Markov Chain Monte Carlo) procedure was implemented. In this procedure, all parameter estimates are averaged across substitution models and weighted according to substitution models' posterior probability. For the third partition, a simple F81 model dedicated to restriction sites and other binary data was implemented. Bayesian analysis was performed using two independent runs with four MCMC chains running for 10^5 generations. Trees were sampled after every 100th generation. The first 25% of trees were discarded as the burn-in phase, and the remaining trees were used for the construction of a 50% majority consensus tree. The consensus tree was graphically visualized in the program FigTree v.1.4.4 (<http://tree.bio.ed.ac.uk>). In all phylogenetic analyses, *Ranunculus sceleratus* L. was used as an outgroup, with three sequences acquired from GenBank: MW430773 (ITS), KC842129 (*rpl32-trnL*) and KC842059 (*psbE-petL*)—trimmed to the required length prior to analyses.

Hydromorphological evaluation

The hydromorphological evaluation was conducted according to the River Habitat Survey (RHS) method (Raven et al., 1998). The RHS method, gathering data from 500-m stretches of rivers, delivers information on the river channel as well as on the 50-m buffer of each river valley studied. RHS data enabled calculation of two numerical metrics: Habitat Quality Assessment (HQA) and the Habitat Modification Score (HMS). High HQA values indicate an extensive presence of a number of natural river features and high landscape diversity along the river. A high HMS score indicates an extensive anthropogenic alteration, such as bank and channel resectioning, reinforcements and engineering construction. Both indices have values in the range of 0–100 and it is a result of the number of natural or anthropogenic elements of the river channel. Furthermore, the RHS protocol enabled calculation of the average grain size of the channel substrate (granulometry index) and the average flow type (flow index) using the formula of Jusik et al. (2015). Values of both indices are between 1- and 6 and are the ratio of the share of different substrate fractions or flows and numerical values

assigned to each type of substrate and each flow type (the highest values for coarse-grained, fast-flowing channels). The basis and formula for calculating both indices were provided by Jusik et al. (2015).

Water quality

Water samples were collected from each survey site immediately prior to biological sampling. Water samples were taken in the middle of the stream below the surface. Analyses of the physical and chemical parameters of the water were measured on-site using portable equipment. An additional water sample was collected for laboratory analysis. Water samples were cooled and analysed in the laboratory within a 24-h period. A part of the sample was filtered using Sartorius Cellulose filters with nominal pore size of 0.45 μm . Overall, nine physical and chemical parameters were estimated (Table 1). Electrical conductivity and pH were measured with a digital potentiometer. The dissolved oxygen was measured with a digital oxygen meter and by the Winkler method. Alkalinity and total hardness were measured by the potentiometric method—alkalinity by titrating with sulfuric acid to an end point of pH 4.5 in the presence of methyl orange and total hardness by titrating with disodium edetate. Concentrations of phosphate (molybdenum blue method), nitrate nitrogen (cadmium reduction

Table 1 Variables recorded in the environmental database

Variable	Units	Abbreviation
pH	pH scale	pH
Conductivity	$\mu\text{S cm}^{-1}$	Cond
Alkalinity	$\text{mg CaCO}_3 \text{ l}^{-1}$	Alkal
P-soluble	$\text{mg PO}_4^{3-} \text{ l}^{-1}$	P-sol
P-organic	$\text{mg PO}_4^{3-} \text{ l}^{-1}$	P-org
N-NO ₃	mg N l^{-1}	N-NO ₃
N-NH ₄	mg N l^{-1}	N-NH ₄
N-organic	mg N l^{-1}	N-org
BOD ₅	$\text{mg O}_2 \text{ l}^{-1}$	BOD ₅
Width	m	Width
Depth	m	Depth
Habitat Quality Assessment	–	HQA
Habitat Modification Score	–	HMS
Granulometry index	–	GM-ind
Flow index	–	FL-ind

method) and ammonium nitrogen (Nessler's method) were determined using a spectrophotometer.

Statistical analysis

The environmental dataset (physical, chemical and hydromorphological parameters) was initially explored with basic statistical analysis (range, mean, median, standard deviation) calculated for each *Batrachium* taxon. Differences between mean values were verified using the Kruskal–Wallis test. Factor analysis (FA) with varimax normalized rotation was used to reveal major directions of variability. The use of FA allowed for the extraction of uncorrelated variables that were used in further analyses. Statistica 13 was used for most of the analysis including the Kruskal–Wallis test and FA. Direct ordination analysis was carried out to determine the importance of environmental factors in the development of the studied water crowfoots and other macrophytes. Factors identified by FA were used as environmental variables. Canonical

correspondence analysis (CCA) was applied (ter-Braak & Smilauer, 1998) along with a forward variable selection procedure. The statistical significance of the relationships between species data and environmental variables was evaluated using the Monte Carlo permutation test (499 permutations). CCA analysis were performed using CANOCO for Windows version 4.56.

Results

Morphological and molecular identification of *Batrachium* taxa

Comparison of morphological characters essential for identification of *R. circinatus*, *R. fluitans* and its hybrid is provided in Table 2. The description of characteristic features includes stem, leaves, petals and pits.

Table 2 Comparison of selected morphological characters of *Ranunculus circinatus* × *R. fluitans* with the parental species *R. circinatus* and *R. fluitans* based on Wiegleb et al. (2017) and own observations

Character	<i>R. circinatus</i>	<i>R. fluitans</i>	<i>R. circinatus</i> × <i>R. fluitans</i>
Flowering shoots length (cm)	Up to 300	Up to 600	Up to 250
Colour of the stem	Green	Green	Green, pale green to whitish
Floating leaves	Absent	Absent	Absent
Capillary leaves	Present	Present	Present
Length of leaves (mm)	10–30	30–600	60–160
Length of petioles (mm)	Leaves sessile or petioles up to 5 (–30)	5–220	4–20 (–50)
Character and shape of fresh leaves	Rigid, circular to semi-circular	Rigid to flaccid, fleshy, elongate-obconical	Rigid to flaccid, fleshy, obconical to suborbicular
Arrangement of leaf segments	In one plane, middle part equal to lateral ones	In different planes, middle part equal to lateral ones	In different planes, middle part shorter than lateral ones
Number of lamina divisions	3–5 (–6)	2–4	(3–) 4–5 (–6)
Length between first and second bifurcation (mm)	1–3	30–150	15–55
Number of terminal segments	80–100	< 30	30–80
Ratio of leaf length and corresponding internode	< 1	1	< 1
Number and shape of petals	5, Obovate	5–10, Broadly obovate	5, Broadly obovate
Length of petals (mm)	6–10	7–15 (–18)	6–8
Shape of nectar pits	Lunate, rarely pyriform	Pyriform	Horse-shoe like
Hairiness of receptacle	Hairy	Glabrous	Hairy

ITS network

The ITS alignment of *Batrachium* taxa sampled in this study was 573 bp long with 44 variable positions. The polymorphism detected comprised 43 substitutions (35 informative) and one, 1-bp long insertion specific for *Ranunculus baudotii* Godr. and *R. fluitans*. The polymorphic sites are summarized in the supporting information (Table A4—Supplementary Material).

The phylogenetic network showed quite a clear structure (Fig. 2). Taxa of which the sequences did not express intraindividual polymorphism are grouped into four clusters: (1) *R. circinatus*, *Ranunculus trichophyllus* Chaix ribotype r3; (2) *R. fluitans*, *R. baudotii*; (3) *R. peltatus*, *R. trichophyllus* ribotypes r1 and r2 and (4) *R. trichophyllus* ribotype r6. The rest of the taxa, the sequences of which display

intraindividual polymorphism, fell into the clusters mentioned above if the ITS sequence polymorphism was additive between species forming the same group (e.g. *R. baudotii* × *R. fluitans*, *Ranunculus kauffmannii* Clerc) and formed their own groups, if the ITS sequence polymorphism was additive between taxa from different clusters. The later stage refers to *R. circinatus* × *R. fluitans*, *R. fluitans* × *R. peltatus*, *R. aquatilis* and *R. peltatus* × *R. trichophyllus* ribotype r6.

In the phylogenetic network, the putative hybrid *R. circinatus* × *R. fluitans* forms its own cluster exactly halfway between clusters 1 and 2. Such intermediate topology evidences its origin from a hybridization event between the species belonging to both clusters; however, certain identification of the parental taxa is hardly possible, because each of these clusters comprises more than one taxon. Taxa in both clusters

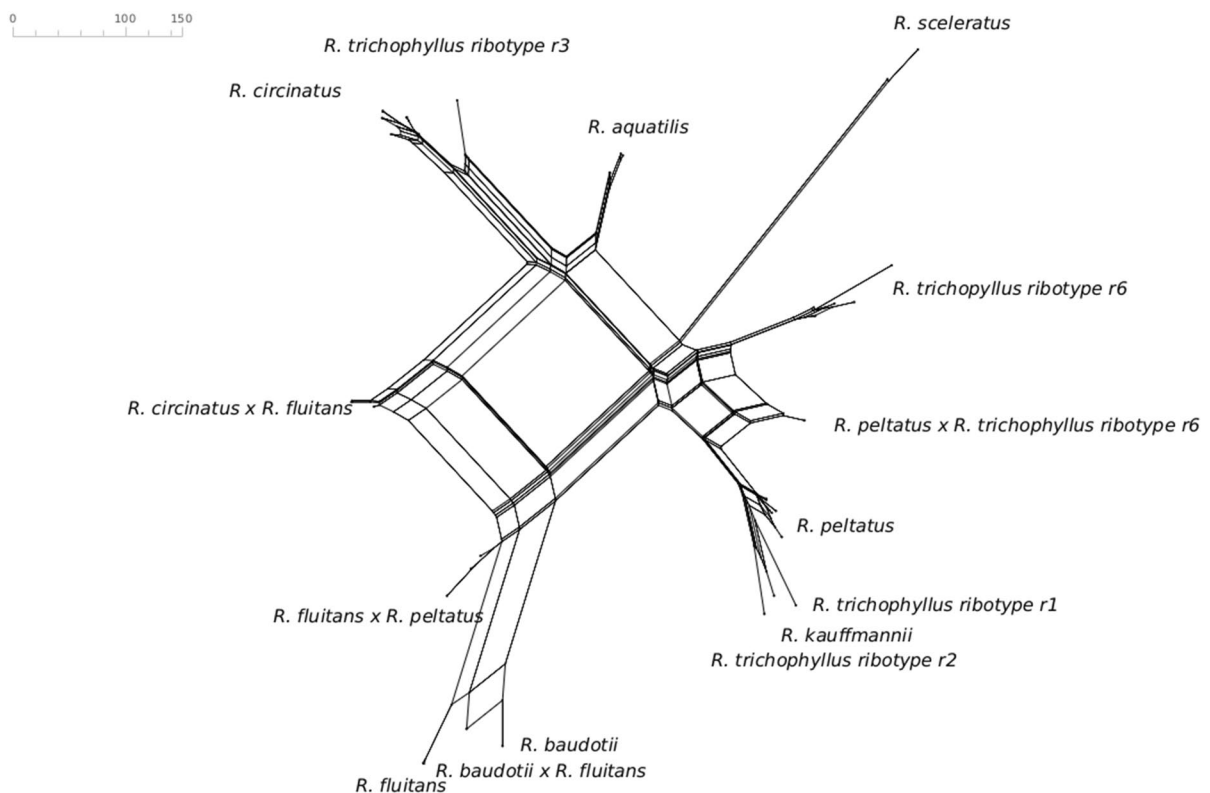


Fig. 2 NeighborNet of the ITS sequences of the *Batrachium* group sampled in Poland, indicating the topology of the hybrid *R. circinatus* × *R. fluitans*, clearly intermediate between the parental species clusters: *R. circinatus*/*R. trichophyllus* ribotype 3 and *R. fluitans*/*R. baudotii*. The origins of the

samples studied are given in Tables A1 and A2—Supplementary Material, and the details of the ribotypes are provided in Table A3—Supplementary Material. *Ranunculus sceleratus* is used as an outgroup

differed from each other by a few single-nucleotide polymorphisms (SNPs) in the ITS sequences: *R. circinatus* differs from *R. trichophyllus* ribotype r3 by five positions of the alignment (48, 86, 103, 201 and 444) and *R. baudotii* from *R. fluitans* can also be distinguished by five positions (43, 48, 76, 192, 554) (Table A4—Supplementary Material). Therefore, following the result of NeighborNet analysis indicating the topology of the hybrid between two parentage groups—*R. circinatus*/*R. trichophyllus* r3 from one side and *R. baudotii*/*R. fluitans* from another (Fig. 2)—and based on the SNP analysis, *R. baudotii* can be excluded as one parent by five positions in the alignment, namely 43, 48, 76, 192 and 554, while the parentage of *R. trichophyllus* ribotype r3 is ruled out based on the following positions: 103, 201 and 444 (Table A4—Supplementary Material). Only two species—*R. circinatus* and *R. fluitans*—can be unambiguously identified as parents for the studied hybrid.

Phylogenetic tree

The Bayesian phylogenetic tree, built based on the concatenated alignment of two cpDNA regions, i.e. *rpl32-trnL* and *psbE-petL*, was 1085-bp long, and evidenced two well-supported clades (Fig. 3). The first clade was formed only by *R. circinatus* and the second comprised the remaining taxa. Within this second clade, two subgroups were resolved: one well supported, with *R. trichophyllus* ribotypes r1 and r2 and *R. kauffmannii*. The second polytomous, with the remaining sampled taxa. All studied samples representing the hybrid *R. circinatus* × *R. fluitans* were placed in the clade with *R. fluitans*, which demonstrates that this species served as a donor of cytoplasmic DNA for all the hybrid individuals. Variable positions of both parental taxa and its hybrid, encountered in cpDNA alignment, are shown in Table A5—Supplementary Material.

Distribution of taxa

Based on the morphological (*R. circinatus*) and molecular analyses, it was possible to identify the geographical distribution of the analysed taxa. Within 54 localities (Fig. 1), 58 different *Batrachium* populations were recorded in total: 28 of *R. circinatus*, 17 of *R. fluitans* (1 joint site with *R. circinatus*) and 13 sites with the hybrid *R. circinatus* × *R. fluitans* (3 joint sites

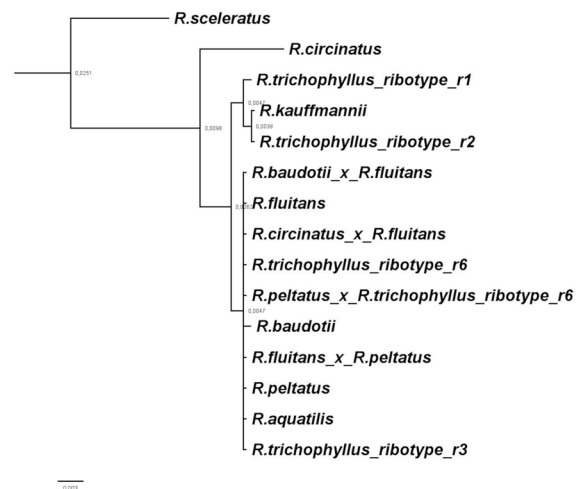


Fig. 3 Phylogenetic tree inferred by Bayesian analysis of concatenated alignment of the *rpl32-trnL* and the *psbE-petL* spacers of the plastid DNA. Posterior probabilities are indicated on branches. Details on samples studied and the GB accession numbers are provided in Tables A1 and A2—Supplementary Material. *Ranunculus sceleratus* is used as an outgroup

with *R. circinatus*). The distribution of both parental species was quite wide, covering a strip of sites across the country in the northern part. The presence of both species in southern Poland includes the central highlands (*R. circinatus*) and Sudetes (*R. fluitans*). Meanwhile, the hybrid was found in a much smaller range of longitude and latitude, limited to northern Poland.

Analysis of the environmental data

The comparison of the habitat conditions in three groups of rivers associated with a particular *Batrachium* taxa showed significant differences for some of the analysed environmental variables (Table A1—Supplementary Material). The largest differences were noted for reactive phosphorus concentrations, for which *R. circinatus* × *R. fluitans* showed the highest tolerance (mean and median values). A significant difference was also found for the width of the rivers. *Ranunculus circinatus* occurred in smaller watercourses compared to the other two taxa. The analysed sites also differed in terms of organic phosphorus and hydromorphological quality. For the remaining variables, results of univariate analysis were not significant.

The FA analysis of the environmental matrix (Table 3) distinguished five major factors explaining

Table 3 Factor loadings (varimax normalized rotation) of habitat variables determined by factor analysis for the analysed study sites

Variable	Factor 1 mineral pollution	Factor 2 rhithral conditions	Factor 3 organic matter	Factor 4 habitat quality	Factor 5 chan- nel dimensions
pH	−0.08	0.00	−0.16	0.75	−0.22
Conductivity	0.70	−0.24	−0.31	0.04	−0.36
Alkalinity	0.58	−0.02	−0.26	−0.12	0.05
P-soluble	0.90	−0.01	0.11	−0.13	−0.09
P-organic	0.09	0.14	0.79	−0.03	0.12
N-NO ₃	0.04	−0.23	0.16	0.31	−0.42
N-NH ₄	0.88	0.01	0.17	−0.04	0.18
N-organic	0.05	−0.04	0.79	−0.17	0.11
BOD ₅	−0.03	−0.25	0.65	0.05	−0.29
Width	−0.04	−0.06	−0.04	0.22	0.80
Depth	−0.02	−0.50	0.18	−0.13	0.76
HQA	0.01	0.29	−0.06	0.75	0.14
HMS	0.46	0.13	−0.02	− 0.66	−0.32
FT index	−0.09	0.81	−0.09	0.32	−0.09
GM index	−0.02	0.83	0.11	−0.17	−0.16
Eigenvalues	2.67	1.88	1.98	1.92	1.88
Explained variation	0.18	0.13	0.13	0.13	0.13

Loadings with absolute value greater than 0.6 are shown in bold

60% of the total variance. The identified factors were associated with distinct elements of the fluvial environment. The first factor was associated with dissolved minerals, including soluble nutrients such as phosphates (P-soluble) and ammonium nitrogen (N-NH₄), as well as alkalinity and conductivity. The second factor corresponded to rhithral conditions associated with coarse substrate and fast flows. The third factor was correlated with organic matter content indicated by organic P and N enrichment and BOD level. The fourth factor was positively related to the abundance of river natural features related to fluvial processes (HQA) and it was in opposition to HMS representing hydromorphological modifications. The fifth factor was related to channel dimensions (width and depth).

Ecological requirements of the studied macrophytes

The CCA determined the relative importance of environmental variables (FA-derived factors) in explaining the ecological preference of analysed macrophytes based on the sample of 54 river sites with *Batrachium* taxa (Table 4). Three environmental factors were revealed as influential for the development of the river vegetation: rhithral conditions, channel dimensions

Table 4 Ranking environmental variables in importance by their effects on the analysed macrophyte data, as obtained by forward selection

Environmental variables	<i>P</i>	Variance explained
Rhithral conditions	0.002	0.12
Chanel dimensions	0.002	0.10
Hydromorphological quality	0.002	0.09
Mineral pollution	0.290	0.04
Organic matter	0.342	0.04

P significance level of the effect, as obtained with a Monte Carlo permutation test under the null model with 499 random permutations. Environmental variables are explained in Table 3

and hydromorphological quality, explaining 12%, 10% and 9% of variance, respectively ($P=0.002$ in each case). The mineral pollution level, as well as the content of organic matter, was not found to be significant ($P>0.05$). The variance explained was very low in both cases (4%).

The results of CCA with the three *Batrachium* taxa analysed together with other macrophytes (45 taxa with a minimum 15% frequency) are presented graphically as ordination diagrams in Fig. 4 (1st and 2nd axes) and Fig. 5 (1st and 3rd axes). The first two axes

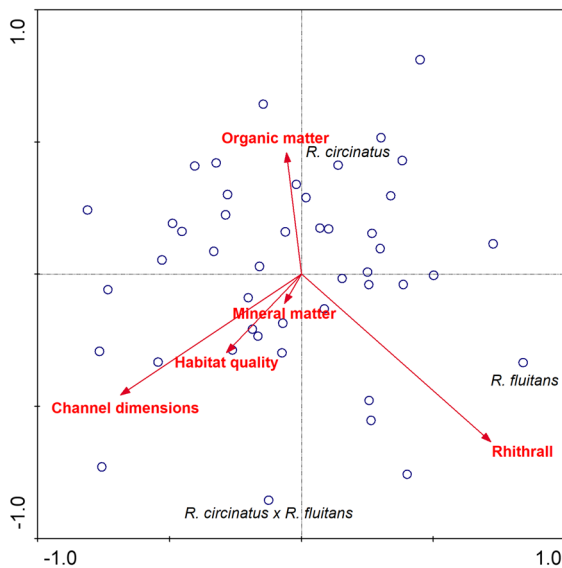


Fig. 4 CCA ordination diagram of macrophyte data and environmental factors—1st and 2nd axis. Only frequent macrophyte species were analysed (over 15% frequency). Vector meaning is provided in Table 2

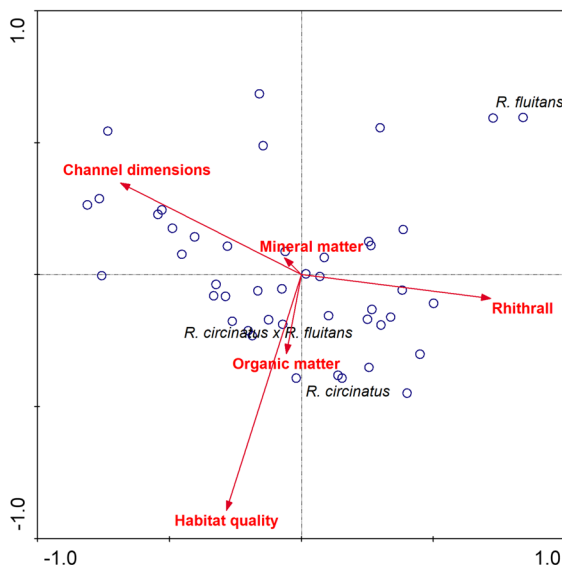


Fig. 5 CCA ordination diagram of macrophyte data and environmental factors—1st and 3rd axes. Only frequent macrophyte species were analysed (over 15% frequency). Vector meaning is provided in Table 2

in the CCA ordination between studied *Batrachium* taxa and environmental factors (Fig. 4) explained 62.2% of species–environment relation variance,

and when the third axis was considered (Fig. 5) the explained variance reached 81.5%.

The most distinct positive reaction to rhithral conditions was shown by *R. fluitans*, revealing the preferences of this taxon for rhithral rivers. The substrate of most of the rivers with *R. fluitans* was dominated by gravel pebbles or rivers with gravel and sand, and several sandy stretches were also found. Moreover, *R. circinatus* × *R. fluitans* also indicated preferences for rhithral rivers, although not as strong as those of *R. fluitans*. It was revealed that *R. circinatus* × *R. fluitans* prefers larger rivers, and this taxon was only found in lowland rivers with a distinctly lower share of pebbles in the bottom material. *R. circinatus* × *R. fluitans* also showed clear geographical patterns, occurring only in northern and north-western Poland. The preferences of *R. circinatus* were not so evident, and it occurred under a wide range of habitat conditions. This species occurred in rivers with various dominating substrates, significantly different in terms of flow types and water quality. CCA, however, revealed a dominant preference for smaller and slow-flowing channels impacted by organic content. The geographical distribution of *R. circinatus* was wide—from central to northern Poland.

Analyses based on the 1st and 3rd axes revealed the tolerance of *R. fluitans* to hydromorphological degradation (Fig. 5). The reaction of other taxa to this gradient was not evident. The analysis showed that the ecological tolerance of the three considered *Batrachium* taxa was quite distinct compared with that of other macrophyte species. These taxa showed clear preferences for environmental factors, especially *R. fluitans* and *R. circinatus* × *R. fluitans*. Also, there was a group of species located in the graph further away from the centre, indicating their stronger response to the abiotic factors of the river habitat.

Discussion

Ranunculus circinatus and *R. fluitans* are essentially morphologically different and their identification is straightforward (Wiegand et al., 2017). Their hybrid, *R. circinatus* × *R. fluitans*, expresses some intermediate characters, e.g. character and shape of capillary leaves, the number of lamina divisions and the shape of nectar pits (see Table 2). *Ranunculus circinatus* × *R. fluitans* resembles *R. baudotii*

or homophyllous individuals from *R. penicillatus* complex. Due to morphological variation, accurate morphological identification of the hybrid, especially in a vegetative stage, is hardly possible. Therefore, in this work, we applied an objective, molecular survey to identify the *Batrachium* taxa. Based on direct sequencing of selected DNA regions (ITS and cpDNA) the parentage of the studied hybrid was proved. Recently, due to molecular survey, some *Batrachium* taxa have been successfully molecularly characterized and general phylogenetic clades have been resolved (Bobrov et al., 2015; Zalewska-Gałosz et al., 2015; Koutecký et al., 2021). Molecular studies of the *Batrachium* taxa were enriched by flow cytometry (FCM) analysis of genome size, the results of which to a large extent were congruent with the molecular characteristics revealed by direct sequencing of the chosen DNA regions (Prančl et al., 2018; Koutecký et al., 2021). Our study, beside definite molecular identification of taxa, also revealed significant intraspecific genetic variation of *R. trichophyllus* (marked as ribotypes 1–3 and 6), not directly related to morphological characters, which has been reported before and indicated the existence of cryptic taxa under *R. trichophyllus* complex (Rott et al., 2004; Bobrov et al., 2015; Koutecký et al., 2021).

Parental species of this hybrid, namely *R. circinatus* and *R. fluitans* Lam., are widely distributed in Europe; however, their ranges differ in detail. *Ranunculus circinatus* occurs in most parts of meridional and boreal Europe eastwards to the Ural Mountains and Kazakhstan. It also occurs in the meridional regions of North Africa and West Asia, while *R. fluitans* is restricted to temperate and meridional regions of Europe, from Ireland to Belorussia (Wiegler et al., 2017). Although *R. circinatus* and *R. fluitans* are widely distributed, and their ranges partly overlap, the hybrid *R. circinatus* × *R. fluitans* is recorded only sporadically. At first, the hybrid was mentioned from Germany (Vollrath & Kohler, 1972). It was also reported from the UK (Lansdown, 2009, 2015) and Poland (Nobis et al., 2016 and this study). Recently, the new station of *R. circinatus* × *R. fluitans* was documented from Bavaria (Koutecký et al., 2021). Our study in Poland showed the presence of the hybrid in 13 rivers in the northern part of Poland (Fig. 1).

It is possible that due to morphological similarities between *R. circinatus* × *R. fluitans* and different generations of *R. fluitans* and *R. baudotii* hybrids

this taxon is more abundant in Europe but not recognized properly. The medium-sized, homophyllous macrophyte with rigid, submerged capillary leaves is recognized, depending on the treatment, as a species, subspecies or a variety of *R. penicillatus* (Dumort.) Bab. (e.g. Cook, 1966; Webster, 1988; Wiegler et al., 2017). In the latest worldwide taxonomic account of *Batrachium*, the authors assumed that *R. pseudofluitans* is an allopolyploid taxon derived from the hybrid *R. circinatus* × *R. fluitans* (Wiegler et al., 2017). The possibility cannot be excluded that this hybrid is more widespread than reported in the literature, and the ecological requirements of the hybrid and *R. pseudofluitans* are somewhat similar.

To identify the major factors influencing the distribution of the analysed water crowfoots, the habitat parameters were measured in localities of properly identified taxa. As outlined in the “Introduction” section, the identification of parental species is easy and straightforward, even when based on vegetative morphological characters, because of the presence of very distinctive features (Cook, 1966; Wiegler et al., 2017). In some stages, however, it is more difficult to distinguish between the hybrid *R. circinatus* × *R. fluitans* and *R. fluitans*, or between one *R. fluitans* hybrid and another. Therefore, in this study, we applied molecular identification. As is clear from the obtained results (Fig. 2), both parental species *R. circinatus* and *R. fluitans* are unequivocally differentiated based on the unique ITS polymorphism, and their hybrid is characterized by the presence of an additive ITS polymorphism pattern of the parental species. Plastid DNA, unlike most nuclear chromosomes, is typically uniparentally inherited, and in the case of angiosperms, predominately within a maternal line (Zhang & Sodmergen, 2010). All hybrid plants of *R. circinatus* × *R. fluitans* from Poland inherited plastid DNA from *R. fluitans*, which is presumably its maternal parent.

Rivers, due to considerable diversification of ecological parameters, are suitable habitats for a number of different kinds of newly created macrophyte hybrids (e.g. Kaplan & Fehrer, 2011; Zalewska-Gałosz, 2011; Prančl et al., 2018). River currents facilitate clonal propagation, even of sterile individuals, which can spread vegetatively downstream and persist within the river system for a long time (e.g. Preston et al., 1998; Zalewska-Gałosz, 2010).

Three analysed water crowfoot species, especially *R. circinatus* and *R. fluitans*, were reported to be relatively widespread throughout Poland (Fig. 1) and worldwide (Wiegleb et al., 2017), showing a wide biogeographical gradient. Despite this fact, they cannot be considered as common species in Poland; the search for the location was based on almost 2,000 river sites collected in the database of the University of Life Sciences in Poznan, which indicates that they are relatively rare in Polish rivers. Differences in the presence of various *Batrachium* species were also described for different types of waters and different European countries (Wiegleb & Herr, 1985).

Among the distinguished hydromorphological factors, the extent of **rhithral conditions** was particularly important for the development of macrophytes; this factor was found to explain the greatest amount of species variability of the analysed macrophyte matrix. Rhithral habitats associated with fast-flowing and coarse-grained rivers are essential for the development of macrophytes representing many bryophytes (Hrivnák et al., 2010), benthic algae (Stevenson, 1996) and vascular rheophilic species (Wiegleb & Herr, 1985; Wiegleb et al., 2014, 2015). The *R. fluitans* reaction to this gradient was apparently positive, confirming previous findings of other authors (Wiegleb & Herr, 1985; Spink et al., 1997; Tremp, 2007). Analysis proved that *R. fluitans* is a rheophilic species (Spink et al., 1997; Tremp, 2007) developing even in distinct rhithral river sections (Wiegleb & Herr, 1985). The reaction of *R. circinatus* to this rhithral condition was negative, as this species has already been recognized as characteristic of slow-flowing or even standing waters (Wiegleb & Herr, 1985; Tremp, 2007; Lumbreras et al., 2011; Baattrup-Pedersen et al., 2015). Although the preferences of *R. circinatus* × *R. fluitans* have not been reported before, we observed preferences of this taxon for larger water-courses and also a positive reaction to rhithral conditions, but the reaction to this factor is weaker than that of *R. fluitans*.

A strong relationship has been demonstrated between the **natural attributes** of a river habitat and the development of macrophytes, including the studied taxa of *Batrachium*. This gradient was revealed by two principal hydromorphological multimetrics: HQA (indicating the abundance and diversity of natural features) and HMS (artificial modification extent). Therefore, this factor may represent a

gradient between modified river sites and natural river stretches. The tolerance of *R. fluitans* for hydromorphological degradation was confirmed by Mony et al. (2006). We could observe its abundant development in moderately altered channels, whereas the preferences of other taxa for this factor were not evident.

The third hydromorphological factor analysed was related to **channel dimensions**, mainly channel width (FA eigenvalues 0.80), and also quite strongly to water depth (0.76, respectively). This environmental gradient strongly influenced macrophyte differentiation, but among the studied water crowfoots, the apparent reaction was demonstrated by *R. circinatus* × *R. fluitans* developing in larger rivers. In the analysed research stretches, this hybrid was also present in smaller streams, but all of them were estuary sections connected to large rivers. It can be assumed that, during the flood events of a large river, this taxon dispersed to these habitats. The other two water crowfoots were generally neutral to this gradient, although *R. fluitans* is generally recognized as associated with larger rivers (Spink et al., 1997). This preference of *R. fluitans* was also confirmed by the analysis of the differences, which indicated greater width of rivers with this taxon.

Analysis of the surveyed river sites overgrown by the studied water crowfoots proved that the influence of **organic and mineral content** on macrophyte differentiation is limited. It was previously known that both *R. fluitans* and *R. circinatus* are tolerant to water quality, developing in a broad range of nutrient levels, including mesotrophic as well as eutrophic conditions (Tremp & Kohler, 1995; Haury et al., 2006; Mony et al., 2006; Schneider, 2007; Tremp, 2007; Willby et al., 2012; Szoszkiewicz et al., 2020), and tolerant to variable alkalinity and pH (Bodner, 1994; Mony et al., 2006). Knowledge on *R. circinatus* × *R. fluitans* ecology has been scant so far, and we have revealed that, like its parental species, this hybrid is not sensitive to the chemical properties of water.

The morphological similarity of *R. fluitans* and *R. circinatus* × *R. fluitans* and complexity of diagnostic features cause problems with their accurate identification, and the use of molecular identification methods is required (Telford et al., 2011; Wiegleb et al., 2017). Such problems also concern other species of the section *Batrachium*, as well as their hybrids (Wiegleb & Herr, 1985; Telford et al., 2011; Lumbreras et al., 2014). Among water crowfoots found in Europe, only

the clearly morphologically distinguished *R. circinatus* is not affected by this issue. Recently, Wiegleb et al. (2017) presented a key for morphological identification of almost 30 species which were verified using molecular methods. Our analysis, as well as those of other investigators (Wiegleb & Herr, 1985; Mony et al., 2006; Lumbreras et al., 2011), showed that various species have different habitat preferences and can grow in contrasting habitats. Moreover, the whole *Batrachium* group is the botanical determinant of habitat 3260, which occurs in a variety of abiotic conditions (rivers of plain to montane levels) (Council Directive 92/43/EEC). Based on our present findings, as well as those of previous research (Wiegleb & Herr, 1985; Mony et al., 2006; Lumbreras et al., 2011), we can state that the variety of abiotic characteristic of Habitat 3260 is related to the variety of occurring *Batrachium* species. Therefore, the differences in habitat characteristic of the analysed species indicate the need to adapt the conservation, preservation and restoration measures of water crowfoot habitats to particular species, and undertaking these measures for the entire group of water crowfoots as such (Pedersen et al., 2006) cannot always be sufficient.

The study results showed preferences of *R. circinatus* × *R. fluitans* for rhithral rivers, whereas its reaction to nutrients was not clear. Hence, we recommend considering the hybrid as an indicator of Habitat 3260 according to the EU Habitat Directive. As yet, the completed research is not sufficient to recognize the hybrid as a habitat status indicator for the purpose of the Water Framework Directive. However, more extensive hydrochemical research would allow us to more accurately define its preferences in terms of ecological quality. By demonstrating the relatively abundant presence of the hybrid, the value of conducting further ecological research has been confirmed.

Conclusions

This is the first attempt in the literature to prepare ecological profiles for *Batrachium* taxa molecularly identified on the basis of polymorphisms detected in the sequences obtained by direct sequencing of selected DNA regions (ITS, *rpl32-trnL* and *psbE-petL*). This kind of research increases the efficiency of water crowfoot conservation.

The preferences of *R. circinatus* × *R. fluitans* have not been reported before in the scientific literature. Our observations have shown that the preferences of this taxon are favourable to rhithral conditions, and the reaction to this factor is weaker than that of *R. fluitans*. It was also found that *R. circinatus* × *R. fluitans* develops well in large watercourses.

It was shown that genetically, morphologically and ecologically, *R. fluitans* and *R. circinatus* × *R. fluitans* are closer to each other than to *R. circinatus*. The revelation of many new localities of this hybrid in Poland using molecular markers may establish that *R. circinatus* × *R. fluitans* is more widely distributed through Europe than has been known to date.

Of key importance to conservation programmes for *R. fluitans* is the maintenance of rhithral habitats, while the presence of hydromorphological modifications that do not affect this type of flow dynamics and water pollution is definitely marginal. The development of conservation programmes dedicated specifically to *R. circinatus* or *R. circinatus* × *R. fluitans* is still unlikely, although the obtained results might provide various information on their efficient maintenance.

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Author contributions The concept of the paper was initiated by DG, JZG and KSz. DG and KSz have performed the field studies, *Batrachium* sampling and ecological analysis, as well as interpretation of the results and writing of the manuscript. JZG and MJ collected *Batrachium* samples, performed molecular analysis and took part in manuscript preparation. All co-authors have approved publication of this manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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