Natural hybridization between diploid *Ficaria calthifolia* and tetraploid *Ficaria verna* subsp. *verna* in central Europe: evidence from morphology, ecology and life-history traits

Přirozená hybridizace mezi diploidní *Ficaria calthifolia* a tetraploidní *Ficaria verna* subsp. *verna* zjištěná na základě studia morfologie, ekologie a životní historie

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The genus *Ficaria* is a taxonomically intricate group in which polyploidization and hybridization contribute to taxa diversification. In central Europe, populations of diploid *F. calthifolia* and tetraploid *F. verna* subsp. *verna* occasionally come into contact, which results in an interspecific triploid hybrid, as recently demonstrated using molecular markers, genome size estimation and experimental crossing. In this study, we aimed to estimate the frequency and distribution of the triploid hybrid in central Europe, to identify those phenotypic traits that can be used to discriminate between hybrid and parental taxa and compare the phenology, pollen viability, sexual and asexual reproduction and niche differentiation of the triploid hybrid and parental taxa. Flow-cytometry analyses of 1171 individuals sampled from 67 localities revealed that triploid hybrids were regularly found at 89% of the sites sampled where there were sympatric populations of both parental taxa, with a mean percentage of 19.4% occurring at sites where there was a mixture of cytotypes. No pure hybrid population was found. The hybrids were intermediate between the parental taxa in most morphological characters and did not show any novel morphological characters. The characters that best differentiate the taxonomic groups were the production of axillary bulbils, aborted and well-developed achenes, leaf shape and plant habit. Almost all hybrids were sexually sterile: the pollen viability was considerably reduced (mean 8.4%) and on average 98% of the achenes were aborted. Local dispersal of the hybrids is possible through the production of bulbils in the leaf axils, although the number of bulbils per node and the mean weight of one bulbil were two and three times lower in the hybrid than in *F. verna* subsp. *verna.* The hybrid niche was intermediate between those of the parental taxa but shifted slightly towards that of *F. verna* subsp. *verna*. In addition, the taxonomic and nomenclatural treatment of the hybrid, which is described as *Ficaria* ×*sellii* Duchoslav, Popelka et Trávn., as well as a key for identifying the central European taxa of *Ficaria*, are presented.

K e y w o r d s: bulbils, contact zone, ecological differentiation, *Ficaria calthifolia*, *Ficaria* ×*sellii*, *Ficaria verna* subsp. *verna*, flow-cytometry, hybridization, multivariate morphometrics, phenology, pollen viability, polyploidy, taxonomy

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Introduction

Interspecific hybridization is a frequent phenomenon with an important role in the evolution of plants (Hegarty & Hiscok 2004, Mallet 2005, Rieseberg & Willis 2007, Soltis & Soltis 2009). Whitney et al. (2010) report that hybrids occur in 40% of 282 plant families surveyed, but generally, the occurrence of spontaneous hybridization is recorded in a small fraction of families and an even smaller fraction of genera. These genera are generally characterized by a perennial habit, an outcrossing breeding system and reproductive modes that are able to stabilize hybridity (Ellstrand et al. 1996, Preston & Pearmann 2015).

The exchange of genes between evolutionary lineages requires imperfect reproductive isolation and contact that allows them to meet and mate, resulting in at least offspring of mixed ancestry (Harrison 1993). The areas where hybridization occurs are called hybrid zones (Barton & Hewitt 1985). Most hybrid zones are considered to arise from the secondary contact of two formerly allopatric or parapatric taxa (Hewitt 1988). Existence of a hybrid zone might be an ephemeral event when hybrids are quickly removed by selection and cause no further effects. However, even in the case of infertile first-generation hybrids, interbreeding might waste parental reproductive effort and hybrid individuals might compete with parental species, and negatively affect their population growth (Senanan et al. 2004, Fitzpatrick et al. 2015). For long-lasting hybrid zones, several models have been developed to explain their structuring and maintenance. Models vary according to the roles of the intrinsic reductions in hybrid fitness and hybrid fitness variation that are tied to environmental gradients (Barton & Hewitt 1985, 1989, Harrison & Rand 1989, Arnold 1997). Hybrid zones are thus recognized as 'windows on evolutionary processes' (Harrison 1990).

Hybridization can have several important evolutionary consequences. Hybridization may contribute directly to the completion of reproductive isolation between hybridizing species as a result of reinforcement (Servedio & Noor 2003) or to the origin of new ecotypes or even new species (Mallet 2007), including infrequently generated homoploid hybrid species (Abbott et al. 2010, Abbott 2017) and more commonly generated allopolyploid species (Coyne & Orr 2004, Wood et al. 2009, Soltis et al. 2010, Barker et al. 2016). However, hybridization might also result in transient or permanent introgression across species barriers (Lowe et al. 2004). Incomplete crossing barriers against parental taxa may lead to uni- or bidirectional gene flow between hybridizing species that can result in various outcomes (e.g. collapse or expansion of a hybrid zone, adaptive evolution) depending on the fertility of the hybrids, the rate of hybridization and the relatedness between and population sizes of the hybridizing species (Arnold et al. 1999, Chapman & Burke 2006, Abbott et al. 2013). However, the role of introgression across species barriers might by modified when diploids hybridize with polyploids (i.e. heteroploid hybridization; Rieseberg & Willis 2007). Because of strong postzygotic barriers due to the triploid block (Ramsey & Schemske 1998), polyploids (tetraploids) are, to a high degree, reproductively isolated from diploids. However, partial fertility of triploids might facilitate gene flow between diploids and tetraploids that, in turn, might affect the evolution of the parental species (Comai 2005). Moreover, alternative pathways of gene flow between diploids and tetraploids might be enhanced by the relatively high production of unreduced gametes in some diploids (Kreiner et al. 2017), which might lead to one step introgression into tetraploids.

Assessing the role of interspecific hybridization in a specific taxonomic group depends on the identification of hybrids in the field, which is often difficult. The formerly accepted simple assumption that hybrids are morphologically intermediate between parental taxa (Wilson 1992) has been overcome with the advent of cytogenetic and molecular tools. Actually, hybrid phenotypes are a mosaic of parental, intermediate and novel characters, depending on the differences between the contributing genomes, rate of introgression and hybrid generation (Rieseberg & Ellstrand 1993, Rieseberg 1995, Rieseberg et al. 1999, Mallet 2005, Abbott et al. 2013). In addition, ecological divergence from parental taxa that occurs in the early generations of hybrids might be to slight to observe in the field, but may provide the raw material for adaptation (Rieseberg et al. 1999). The genotypes produced by recombination in hybrids should facilitate further exploration of ecological niches different from those of the parental taxa. Generally, however, little is known about the niches of heteroploid hybrid cytotypes and whether they differ from their parents in terms of their ecology (Ståhlberg 2009, Pinheiro et al. 2010, Hülber et al. 2015).

Hybridization and polyploidization are considered to be significant evolutionary mechanisms resulting in the diversification and speciation within the tribe *Ranunculeae* of the family *Ranunculaceae* (Hörandl et al. 2005, Emadzade et al. 2010, Hörandl & Emadzade 2012). Frequent hybridization is documented mainly for the apomictic and/or polyploid groups, for example in *Ranunculus auricomus* agg. (Hörandl et al. 2005, Hörandl & Emadzade 2012) and *R.* sect. *Batrachium* DC. (Prančl et al. 2018). The genus *Ficaria* Guett. is another group in the tribe *Ranunculeae*, in which polyploidization and hybridization may have been important in taxa diversification (Sell 1994, Zonneveld 2015, Drenckhahn et al. 2017). The genus *Ficaria* comprises small early spring-flowering geophytes with numerous root tubers, cordate-reniform to reniform leaf blades and shiny yellow flowers, which commonly grow in moist to wet habitats (Sell 1994). This genus is native to Europe and adjacent regions in Asia and Africa (Taylor & Markham 1978, Sell 1994) and was introduced into the United States and Canada (Post et al. 2009, Axtell et al. 2010), New Zealand (Webb et al. 1995) and Australia (Foreman & Walsh 1993).

Two *Ficaria* taxa are native to central Europe: diploid *F. calthifolia* Rchb. and tetraploid *F. verna* subsp. *verna* (Futák 1982, Křísa 1997, Zając & Zając 2001, Kästner & Fischer 2006, Marhold et al. 2007, Danihelka et al. 2012). *Ficaria calthifolia* is a relatively small tufted plant consisting of a rosette of shortened erect stems and leaves, which reproduces mainly by seeds and additionally also by the disintegration of root tubers (Sell 1994, Drenckhahn 2016). The northern limit to the distribution of this species is in central Europe and it is relatively more frequent only in warm lowland and adjoining hilly regions where it inhabits various habitats ranging from mesic and semidry grasslands through urban lawns and adjacent scrub to mesic and semidry deciduous forests (Towpasz 1971, Křísa 1997, Kästner & Fischer 2006, Illig & Ristow 2015). In contrast, *F. verna* subsp. *verna* (herefrom *F. *verna*) is tufted stand-forming plant with elongated stems and loose spreading habit, which reproduces mainly vegetatively by axillary bulbils and disintegration of root tubers and rarely by seed (Marsden-Jones 1935, Metcalfe 1939, Wcisło & Pogan 1981, Sell 1994, Reisch & Scheitler 2009). *Ficaria *verna* is widespread and common in a number of moist to wet habitats ranging from grasslands through roadsides, ditches to shaded habitats such as city parks, gardens, mesic and wet deciduous woodlands and scrubland (Taylor & Markham 1978, Křísa 1997, Kubát et al. 2002, Kästner & Fischer 2006). Despite the differences in the niches of these taxa, their populations occasionally come into contact, which

may result in interspecific hybridization. Several times in the past it has been suggested that the morphologically intermediate (triploid) plants occurring at sites where both the above mentioned taxa are locally sympatric are of hybrid origin (e.g. Towpasz 1971, Pogan & Wcisło 1974, Kästner & Fischer 2006).

We recently investigated sympatric populations of *F. calthifolia* and *F. *verna* using cytogenetic and molecular methods and found that sympatric populations were in fact composed of three genetic clusters corresponding to diploid *F. calthifolia*, tetraploid *F. *verna* and triploid plants (2n = 24). All the triploids were of hybrid origin based on the holoploid genome size and AFLP data, which also ruled out their formation via autopolyploidization in *F. calthifolia*. Experimental crosses between parental taxa and plastid sequencing of *in situ* sampled putative parental taxa and triploids confirmed that both parental taxa serve as pollen acceptors. However, experimental hybridization was found to be asymmetric, i.e. triploid offspring are more successfully produced in crosses involving *F. *verna* as a pollen acceptor (Popelka et al. 2019). On the other hand, only limited data are available on the frequency of hybrids in the field, their potential for sexual and asexual reproduction and morphological and ecological divergence from the parental taxa (Pogan & Wcisło 1974, 1983, 1986).

Using flow cytometry, multivariate morphometrics, phenological observations of plants growing in a common garden, pollen viability testing, sprouting experiment with bulbils and *in situ* study of niche breath at a local scale we addressed five main questions: (i) What is the frequency and distribution of hybrids between *F. calthifolia* and *F. *verna* in central Europe? (ii) Which phenotypic traits can be used to discriminate between a hybrid and its parental taxa? Is the hybrid phenotype intermediate between phenotypes of its parental taxa or does it frequently have novel traits? (iii) Does the phenology, pollen size and viability and sexual and asexual reproduction in the hybrid differ from that in its parental taxa? Which reproductive traits enable the hybrid to maintain its populations and disperse? (iv) Does the niche of the hybrid differ from that of its parental taxa? (v) What are the taxonomic and nomenclatural positions of the hybrid?

Material and methods

Sampling of plant material

Plants were collected at 67 localities in the Czech Republic, Austria, Slovakia, Hungary and neighbouring regions in Poland and Germany (Electronic Appendix 1). Because the main emphasis was placed on discovering hybrid plants, we focused on the contact zones between *F. calthifolia* and *F. *verna*; therefore, we visited localities where populations of the scarce *F. calthifolia* were likely to co-occur with the common *F. *verna*. At each locality, we primarily searched for and sampled plants that differed in phenotype from typical plants of *F. calthifolia* and *F. *verna*. If available, we completed sampling by collecting typical specimens of *F. calthifolia* and *F. *verna*. In addition, we sampled several supposed ‘pure’ populations of *F. calthifolia* and *F. *verna* inside and those of *F. *verna* outside contact zones. The plants were sampled throughout the sites, spaced at least 2 m apart in order to minimize collecting clones. The number of sampled individuals differed depending on the population size. Sampled plants were dug-out, transported and planted separately into plastic pots (8 × 8 × 8 cm) filled with a mixture of commercial substrate

and natural soil from the garden (3:1). Subsequently, pots were buried in the soil of a bed in the garden of the Department of Botany, Palacký University in Olomouc. Plants were watered mostly by precipitation (occasional watering was done during long dry periods) and shaded by light shade fabric (relative irradiation 70%) during the growing period to simulate natural conditions.

To estimate the frequency of hybrid and parental taxa within populations more precisely, localities where the hybrid occurred were visited once more, early in the season before *Ficaria* started flowering. The plants sampled were at least 1 m apart along several transects through each locality. An identical sampling scheme was applied at several localities where just one of the parental taxa occurred in order to check for any evidence of 'hidden' mixed-ploidy populations, which might indicate the resolution of the previous coarse sampling was insufficient, to detect traces of either previous hybridization or autopolyploidization. In total, 1171 plants were sampled; the dataset also includes plants sampled and analysed for an accompanying study (Popelka et al. 2019). Basic information about populations included in the various analyses is specified in Electronic Appendix 1.

Flow cytometry

The DNA ploidy level of each of the plants collected was determined by flow cytometry (FCM) using a Partec CyFlow ML Flow Cytometer (Partec GmbH., Münster, Germany) equipped with a Partec UV LED kit (365 nm, 10 mW) or a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, California) equipped with a blue laser (488 nm, 20 mW, BD Accuri™; BD Biosciences, San Jose). Data calibration was done using measurements of the same individuals as used for counting the chromosomes (Popelka et al. 2019) and also cultivated in the garden, using different instruments, stains and internal standards. Either *Secale cereale* L. 'Dankovske' (2C = 16.19 pg; Doležel et al. 1998) or *Pisum sativum* L. 'Ctirad' (2C = 9.09 pg; Doležel et al. 2007) was used as an internal standard.

In the first instrument (Partec CyFlow ML flow cytometer), 4',6-diamidino-2-phenylindole (DAPI) was used for staining. The samples were prepared using a simplified protocol and LB01 isolation buffer (Doležel et al. 2007). Fresh leaves of the internal standard and of the sample (~0.5 cm^2) were chopped together using a razor blade in a Petri dish containing 700 µl of ice-cold LB01 isolation buffer. The suspension was filtered through a 42-µm nylon mesh into a tube containing 300 µl of the same buffer. The nuclei suspension was stained with DAPI (4 $\mu g \cdot ml^{-1}$). The relative fluorescence intensity of the DAPI staining was recorded for 3000 nuclei. The ploidy level of each sample was determined by the position of its G_0/G_1 peak relative to the G_0/G_1 peak of the internal standard. One to three individuals from the same population were analysed together, and when more ploidy levels were suspected in the bulked sample, each individual was reanalysed separately.

In the second instrument (BD Accuri C6), propidium iodide (PI) was used as a stain. Samples were prepared using a similar protocol as for DAPI with the following differences: 550 µl of LB01 isolation buffer were used, 30 µl of RNase A type IIA (50 $\mu g \cdot ml^{-1}$) was added before staining and suspension was stained with PI (50 $\mu g \cdot ml^{-1}$). Each individual was analysed separately. *Ficaria calthifolia* was not measured with *Secale cereale* L. 'Dankovske' as peak positions overlapped.

Generally, for measurements using both PI and DAPI, histograms with coefficients of variation (CV) for the G0/G1 peaks of the analysed sample and a standard of less than 5% were accepted. The relative DNA contents (= fluorescence ratios between the positions of the sample and internal reference standard G_0/G_1 peaks for DAPI and PI) of the individual taxa of *Ficaria* are provided in Table 1.

Table 1. – Relative DNA content (= fluorescence ratios between the positions of the sample and internal reference standard G_0/G_1 peaks) of the *Ficaria* taxa assessed using flow cytometry; the stain was either DAPI or PI. All values are calculated relative to the respective internal standard (*Secale cereale* 'Dankovske' or *Pisum sativum* 'Ctirad', respectively). n = number of samples analysed; SD = standard deviation.

Taxon	DAPI			
		Standard = *Pisum sativum* 'Ctirad'		Standard = *Secale cereale* 'Dankovske'
	n	Mean ratio to the standard ± SD (min–max)	n	Mean ratio to the standard ± SD (min–max)
F. calthifolia (2n ~ 2x ~ 16)	159	1.68±0.03 (1.56–1.83)	103	1.31±0.03 (1.27–1.39)
Hybrid (2n ~ 3x ~ 24)	82	2.59±0.07 (2.40–2.81)	20	1.95±0.08 (1.79–2.18)
*F. *verna* (2n ~ 4x ~ 32)	199	3.45±0.10 (3.08–3.72)	191	2.59±0.07 (2.41–2.77)
	PI			
		Standard = *Pisum sativum* 'Ctirad'		Standard = *Secale cereale* 'Dankovske'
	n	Mean ratio to the standard ± SD (min–max)	n	Mean ratio to the standard ± SD (min–max)
F. calthifolia (2n ~ 2x ~ 16)	239	1.70±0.06 (1.59–1.89)	–	–
Hybrid (2n ~ 3x ~ 24)	39	2.60±0.07 (2.41–2.78)	16	1.39±0.01 (1.38–1.43)
*F. *verna* (2n ~ 4x ~ 32)	90	3.54±0.07 (3.39–3.65)	23	1.91±0.02 (1.86–1.94)

Morphometric analyses

In total, 124 individuals (*F. calthifolia*: 46, hybrid: 39, *F. *verna*: 39) from 23 sites (Electronic Appendix 1), which had been cultivated for at least two years in a garden, were analysed. For each individual, 17 morphological characters were measured or scored, and 8 ratio characters were calculated (Table 2). Characters traditionally used in the determination keys for discrimination between the species of *Ficaria* studied, as well as several new characters for the leaf blade (Electronic Appendix 2), were recorded from fresh material. One to three flowers, collective fruits, and flowering stems per individual were measured. Flower characters and stem length were measured at full flowering (i.e. between anther dehiscence of the outer flower stamens and the onset of anther dehiscence of the inner stamens of each flower measured). Other characters were recorded when plants had mature achenes. Characters of the three largest ground (basal) leaves growing up from root tubers were measured. Stem length, number of leaves per stem and per node were recorded only for the epigeic part of each individual. The achenes were divided into well-developed (mature) and aborted, separately counted and their numbers expressed per collective fruit. All multiply measured characters were averaged per individual before statistical comparison.

Table 2. – Descriptive statistics of all characters studied (mean, standard deviation, minimum, maximum) for the taxonomic groups (*F. calthifolia*, n = 46; *F. *verna*, n = 39; hybrid, n = 39). All multiply measured characters were averaged per individual before data visualization and statistical comparison. One-way ANOVA was used for univariate tests. Significant tests after Bonferroni correction are indicated by * in the last column. Bonferroni multiple comparison test was used after significant result of ANOVA; different letters indicate significant differences at $P \leq 0.05$. [1]Selected characters measured on leaf blade of ground leaves are schematically drawn in Electronic Appendix 2 and their alternative description according to Ellis et al. (2009) is added within square parentheses. [2] Leaf blade shape = relative distance of the widest part of the blade along the midvein from the tip of a blade (LLc/LLb). [3] Character was recorded for epigeic part of the stem only. In fact, many plants also had hypogeic node(s) with leaves but it was not possible to count leaves on this node without disturbance of the individual causing damage to the stem and loss of flowers and/or ripening achenes. [4]*F. calthifolia* was excluded from statistical comparison due to zero values and zero variance for the character. [5]Log (x+1) transformed before analysis. [6]Variables used in PCoA. [7]Variables used in PCA

Character	Abbr.	*Ficaria calthifolia*			Hybrid			*F. *verna*			ANOVA	
		Min.	Mean±SD	Max.	Min.	Mean±SD	Max.	Min.	Mean±SD	Max.	F	P
Leaf blade length A [midvein length; cm] [1]	LLa[6,7]	1.6	2.5±0.3[a]	3.3	1.8	2.4±0.3[b]	3.0	1.8	2.4±0.4[b]	3.2	4.37	0.015
Leaf blade length B [blade length; cm] [1]	LLb[6,7]	2.0	3.1±0.4[a]	4.0	2.4	3.1±0.3[ab]	3.6	2.6	3.3±0.5[b]	4.5	4.03	0.020
Leaf blade width [cm] [1]	LW[6,7]	1.7	2.7±0.3[a]	3.3	2.2	2.8±0.2[a]	3.2	2.5	3.2±0.4[b]	4.2	19.50	< 0.001*
Leaf blade length/width (LLb/LW)	LBLW[6,7]	1.027	1.144±0.059[a]	1.29	0.985	1.117±0.058[a]	1.268	0.892	1.055±0.063[b]	1.165	24.08	< 0.001*
Leaf blade shape (LLc/LLb) [1,2]	LS[6,7]	0.625	0.694±0.042[a]	0.812	0.558	0.684±0.062[a]	0.842	0.532	0.641±0.051[b]	0.756	11.92	< 0.001*
Depth of blade notch [Length of basal extension; LLb – LLa; cm] [1]	DBN[7]	0.3	0.5±0.1[a]	0.9	0.2	0.8±0.1[b]	1.0	0.6	1.0±0.2[c]	1.3	87.21	< 0.001*
Relative depth of blade notch (DBN/LLb)	RDN[6,7]	0.115	0.172±0.029[a]	0.246	0.187	0.241±0.039[b]	0.298	0.237	0.289±0.024[c]	0.331	160.57	< 0.001*
Petiole length [mm]	PL[6,7]	38.1	76.7±15.4[a]	115.1	37.82	89.9±15.8[b]	117.8	51.2	85.7±13.1[b]	108.1	8.87	< 0.001*
Stem length during flowering [cm]	SL[6,7]	32.0	65.3±19.7[a]	120.2	61.3	102.8±20.7[b]	160.3	68.3	119.1±27.8[c]	200.5	62.54	< 0.001*
Depth of blade notch/petiole length*100	DBNPL	0.325	0.713±0.208[a]	1.316	0.238	0.855±0.194[b]	1.522	0.664	1.134±0.243[c]	1.709	40.83	< 0.001*
Number of nodes per stem	NN[6,7]	1.0	1.3±0.4[a]	2.5	1.0	2.5±0.7[b]	4.0	2.0	3.6±0.8[c]	5.0	137.96	< 0.001*
Number of leaves per epigeic part of stem[3]	NLS	0.0	0.4±0.7[a]	2.5	0.0	2.2±1.0[b]	4.0	1.0	3.3±0.9[c]	6.0	118.98	< 0.001*
Number of leaves per epigeic node[3]	NLN[6,7]	0.0	0.2±0.3[a]	1.0	0.0	0.8±0.3[b]	1.3	0.5	0.9±0.2[b]	1.4	89.89	< 0.001*
Flower diameter [mm]	FD	21.0	31.0±4.0	41.0	25.0	30.0±4.0	40.0	24.0	32.0±4.0	40.0	1.11	0.331
Number of petals per flower	NPF[6,7]	7.0	9.0±1.6[a]	16.0	8.0	9.0±1.0[ab]	11.0	8.0	10.0±2.0[b]	13.0	3.52	0.032
Petal length [mm]	PL	11.0	14.9±2.1	19.2	12.1	14.6±1.9	19.3	11.6	15.2±1.9	19.1	0.79	0.457
Petal width [mm]	PW[6,7]	4.5	5.6±0.7[a]	7.7	3.4	5.2±0.8[b]	6.9	4.0	5.0±0.6[b]	6.3	7.60	0.0007*
Petal length/width (PL/PW)	PLW[6,7]	1.83	2.69±0.31[a]	3.27	2.31	2.86±0.34[b]	3.92	2.44	3.04±0.26[c]	3.57	13.36	< 0.001*
Aborted achenes per collective fruit	NAA	1.7	9.0±5.9[a]	35.7	10.0	17.8±5.4[b]	36.0	5.0	13.1±4.0[c]	19.8	36.03	< 0.001*
Mature achenes per collective fruit	NDA[6]	3.3	12.4±5.0[a]	27.0	0.0	0.3±0.6[b]	3.0	0.5	4.1±3.1[c]	11.3	271.08	< 0.001*
Total number of achenes per collective fruit	NA[6]	9.0	21.6±6.5[a]	39.0	10.0	18.1±5.3[b]	36.0	6.0	17.2±5.0[b]	28.0	7.28	0.001*
Proportion of aborted achenes (NAA/NA)	PAA[6]	0.12	0.39±0.18[a]	0.91	0.77	0.98±0.04[b]	1.0	0.37	0.77±0.13[c]	0.97	204.98	< 0.001*
Number of axillary bulbils per stem[4]	NBS[6]	0.0	0.0	0.0	0.0	2.0±0.2[a]	5.3	2.0	3.7±1.1[b]	6.5	40.67	< 0.001*
Number of axillary bulbils per node (NBS/NN)[4]	NBN[6]	0.0	0.0	0.0	0.0	0.85±0.52[a]	2.13	0.5	1.11±0.38[b]	2.11	6.58	0.012
Weight of one axillary bulbil [mg] [4, 5]	WB[6]	0.0	0.0	0.0	0.0	9.5±8.7[a]	50.3	2.3	29.5±20.1[b]	81.9	35.13	< 0.001*

Parental taxa and their hybrids were defined based on DNA ploidy levels estimated using flow cytometry (Table 1). The data were first analysed using univariate statistics (one-way ANOVA with Bonferroni multiple comparison test; Bonferroni correction of P-values in ANOVA was also applied). Before statistical tests, some characters were log-transformed to improve their normality. Descriptive statistics based on original (untransformed) values are presented in tables and visualized in boxplots.

Multivariate analyses (principal coordinate analysis, PCoA; principal component analysis, PCA; Legendre & Legendre 2012) based on morphological (incl. ratio) characters were used to display the overall pattern in the variation along the first two ordination axes, extracting most of the original multidimensional character space. The analyses were run with individual plants as objects (OTU). After a correlation analysis of characters, we selected a smaller set of relatively uncorrelated characters ($|r| < 0.9$; see Table 2 for survey). Because some characters were invariant for *F. calthifolia* (Table 2: NBS, NBN, WB), PCoA with Gower's dissimilarity coefficient for mixed data (Legendre & Legendre 2012) including these characters was used to quantify resemblances between OTUs. The second analysis (PCA) was based on the correlation matrix of the characters, with the achene and bulbil characters excluded.

To determine the extent of morphological separation among taxonomic groups, canonical discriminant analysis (CDA; Legendre & Legendre 2012) was computed using the same data-set as for the PCA. Three characters with zero variance for one taxonomic group (*F. calthifolia*: NBS, NBN, WB) were not included in this analysis. The best predictors (characters) were chosen using stepwise selection. Only characters whose adjusted P (Bonferroni correction) fell below 0.05 during stepwise selection were included in the final model. The significance of the canonical axes of the final model was tested using a Monte Carlo permutation test with 999 permutations. Parametric classificatory discriminant analysis (PCDA) was used to estimate the percentage of plants correctly assigned to the predetermined taxonomic groups, based on the characters used in the final CDA model. PCoA, PCA and CDA were run using software Canoco 5.0 (ter Braak & Šmilauer 2012) and PCDA using NCSS 9 (NCSS 9 Statistical Software. NCSS, LLC. Kaysville, Utah, USA).

Phenology

The phenology of the parental taxa and the hybrids was recorded for 76 plants originating from six sites with sympatric populations of parental species and their hybrids (Electronic Appendix 1). From each population plants were randomly sampled, transported and cultivated in a common garden for at least three years before the observations. The observations began on 1 April 2013 and terminated on 20 May 2013. During this period, phenological surveys were carried out at two- to four-day intervals on several plants (4–5) of each taxon per original locality. The four phenophases distinguished were: 1, flower opening; 2, anther opening – full flowering; 3, flower fading and onset of achene development; and 4, ripe achenes. Phenophases were recorded for the first and the last flower of each plant. To compare the flowering phenology of the taxa, for each individual plant, we calculated the census day from the beginning of the year when the plant enters every phenophase using data recorded for the first flower. To compare the overlap in flowering among parental taxa and hybrids, an index of overlap was calculated according

to Husband & Schemske (2000). The number of flowering plants per taxonomic category at each census was expressed first as a percentage of the total flowering plants observed for that taxonomic category across all census periods and then for each census date taking the value for the category that had the lower percentage of flowering plants and summing these values across all census dates. The flowering period of the population was the period between the onset of phase 1 of the earliest flower and the onset of the phase 3 of the last flower. The index was calculated separately for each pair (i.e. *F. calthifolia* – *F. *verna*, *F. calthifolia* – hybrid, *F. *verna* – hybrid) from each locality and then averaged. The index is equal to zero when flowering is completely asynchronous and is equal to one when there is complete overlap. Differences in the onset of the respective phenophases among the taxonomic categories and the index of overlap among pairs of taxa were compared using linear mixed models (LMM) with an identity link function and normal distribution function. The taxonomic category or pairs of taxa were considered as fixed-effect factors and locality as a random factor (block). Significance of a fixed factor was estimated using a F-test and Satterthwaite's approximation of the degrees of freedom in lme4 (Bates et al. 2017) and lmerTest libraries (Kuznetsova et al. 2017) in R.

Pollen viability and size

Eighty cultivated plants originating from 23 sites (*F. calthifolia*: 33, hybrid: 26, *F. *verna*: 21 individuals; Electronic Appendix 1) were subjected to a pollen viability test. Mature anthers were removed from a flower early in the morning and fresh pollen grains were released from these anthers into a drop of a solution of fluorescein diacetate made up in a concentration of ~10^{-6} M in sucrose on a microscope slide (Heslop-Harrison & Heslop-Harrison 1970). The suspension was mixed and incubated at room temperature for 5 minutes. Subsequently, the suspension was covered by a glass coverslip and examined under a fluorescence microscope at 100× magnification (Olympus Bx60, Olympus Optical Co. (Europa) GmbH). Viable grains rapidly accumulate free fluorescein, which can be detected due to its fluorescence (Heslop-Harrison & Heslop-Harrison 1970). At least, 200 grains per slide were counted. Pollen viability was expressed as the percentage of total grains counted that were viable. We also measured the size of the pollen grains for a limited subset of cultivated populations; these grains were also used for pollen viability analysis. Because normally developed pollen grains are almost spherical in *Ficaria*, we measured their diameter. However, aborted pollen grains are wrinkled in *Ficaria*. Therefore, we measured their length along the longer axis. For microscopic measurement of the pollen size, we used Olympus cellSens software (Olympus Corporation). The data were analysed using LMM with populations nested within the taxonomic groups.

Sprouting of bulbils and growth of young plants

This experiment was designed to compare the sprouting of axillary bulbils and growth of young plants of *F. *verna* and the hybrid in the first season. In May 2015, mature bulbils were randomly collected from well-developed plants cultivated for at least two years in the garden. In total, 86 bulbils from 17 plants of *F. *verna* from eight localities and 50 bulbils from 10 hybrid plants from 4 localities were used in this experiment (Electronic Appendix 1). Bulbils were immediately weighed and planted separately (5 cm apart, 0.5 cm below soil level) in a randomized pattern in parallel rows in natural soil in the garden. Soil

was watered mostly by precipitation (occasional watering was done during dry periods) and shaded by light shade fabric (relative irradiation 70%) during the growing period. The experimental plot was repeatedly checked at three to four-day intervals, and sprouting of each bulbil was recorded during spring of 2016. Sprouted plants were monitored during the season to check their flowering and production of above ground bulbils. After entering dormancy in early summer 2016, the plants were dug up and the number of below ground tubers and axillary bulbils was counted for each individual that sprouted. The bulbils that did not grow were dug up and checked. Because the hybrid plants do not produce axillary bulbils and do not flower, these two variables were not statistically assessed. The percentage sprouting and total number of vegetative propagules produced were analysed using (generalized) linear mixed models ((G)LMM) with a logit link function and binomial distribution function (percentage sprouting) or an identity link function and normal distribution (total number of vegetative propagules produced = below-ground tubers + axillary bulbils). The taxonomic category was considered to be a fixed-effect factor, initial weight of bulbil a covariate and population a random factor nested within taxonomic category in the analyses. Initial weight of bulbil was used in the mixed models to separate the effect of size and ploidy. Differences in the initial weight of the bulbils between the taxonomic categories were tested using LMM. The taxonomic category was considered to be a fixed-effect factor and population as a random factor nested within the taxonomic category. The weight of the bulbil was log-transformed before all analyses. Calculations were done using the lme4 and lmerTest libraries in R. The significance of fixed factors was estimated either by the Wald test (in the case GLMM) or the F-test (in the case of LMM) using the Satterthwaite approximations of the degrees of freedom.

Ecological differentiation among parental taxa and hybrid

Two localities with large populations of all three taxa and visible microhabitat heterogeneity were selected (nos 5 and 47; Electronic Appendix 1) and visited during May 2015 in order to search for microhabitat differentiation between parental taxa and their hybrids. At each locality, we selected several points (no. 5: 22, no. 47: 31) using habitat-stratified random sampling to cover microhabitat variation at both localities. At each point, a 0.5 × 0.5 m plot was established and one leaf from each *Ficaria* individual growing in the plot was collected and placed in plastic bags. An individual *Ficaria* was defined as a rosette of leaves distinctly separated from that of other plants. Then, the vegetation composition (vascular plants) within the plot was recorded using the 7-grade Braun-Blanquet abundance-dominance scale (van der Maarel & Franklin 2013). Subsequently, a soil probe was used to take a sample from the top 10 cm of soil in the middle of each plot. Each soil sample was stored in a plastic bag and immediately weighed. The percentage of light reaching the herbaceous plant layer was estimated using hemispherical photography (Chianucci & Cutini 2012). At each point, a hemispherical image was recorded using an Olympus E-500 camera, equipped with Fish Eye lens. The Gap Light Analyser software (Fraser et al. 1999) was used for image processing, and one parameter, the percentage of site openness (RI), which is the percentage of open sky seen from beneath a canopy, was calculated for each point.

After transport to the laboratory, the DNA-ploidy level of each leaf was determined using flow cytometry (see above), and the percentages of each parental taxa and hybrid

within each plot were calculated. Soil samples were oven dried at 60 °C for 48 hours to constant weight. The soil relative water content (SRWC) was calculated by dividing the dry weight by the initial weight of the sample. For each plot, a mean Ellenberg indicator value (Ellenberg et al. 1992) for several environmental factors (i.e. nutrients, light, temperature, moisture and soil reaction) was calculated from species data without species weighting. *Ficaria* taxa were excluded from the calculation of mean indicator values.

The data were analysed using multivariate analyses. Before the analyses, cover data were transformed by ordinal transformation (van der Maarel 1979). First, a partial detrended correspondence analysis (pDCA; Legendre & Legendre 2012) of the floristic composition was done. DCA was used because preliminary analysis showed that the response data had a gradient > 6 SD units long (ter Braak & Šmilauer 2012). Floristic composition of plots with *Ficaria* taxa excluded were response data; localities were represented as blocks; and the RI, SRWC, Ellenberg indicator values, and percentage of each parental taxa and hybrid within each plot were supplementary variables.

Two methods were used to test microhabitat differentiation among parental taxa and their hybrid. First, response curves for each taxonomic group were modelled using generalized linear models (GLM). Due to overdispersion, GLMs using the quasi-binomial distribution and logit-link function were calculated. The position of samples along the first ordination axis of pDCA was used as a predictor. Model complexity (null, linear or quadratic) was evaluated using the Akaike Information Criterion statistic (AIC; Šmilauer & Lepš 2014). Second, a partial canonical correspondence analysis (pCCA; Legendre & Legendre 2012) was applied to the floristic composition. Percentage of parental taxa within plots were considered as explanatory variables, and localities are covariates (blocks). The effect of the taxonomic group was tested using a Monte Carlo permutation test with 999 permutations limited within the blocks. All analyses were performed using CANOCO 5 package. Nomenclature of the taxa recorded in plots follows Kubát et al. (2002).

Results

Distribution of different ploidy levels in central Europe

Three DNA-ploidy levels were identified by the FCM analyses of 1171 plants collected throughout central Europe: diploid plants morphologically corresponding to *F. calthifolia*, tetraploid plants morphologically corresponding to *F. *verna* and triploid (= hybrid) plants (Table 1). The relative genome size of each taxon determined by us corresponds well with previous measurements calibrated using chromosome-counted plants (Popelka et al. 2019). Outside the contact zone between *F. calthifolia* and *F. *verna*, all 13 populations sampled were pure tetraploid *F. *verna*.

Within the contact zone, several types of population compositions were identified at the 54 localities sampled (Fig. 1, Electronic Appendix 1). The most commonly identified were localities with pure tetraploid populations of *F. *verna* (23 localities, 42.6%). There were 13 localities (24.1%) with pure diploid *F. calthifolia* populations. One third of the sites (18 localities) were composed of mixed populations of different *Ficaria* cytotypes. Triploid hybrid individuals were found at 16 localities (29.6%) sampled within the contact zone. The most frequent mixed-population sites were those containing diploids,

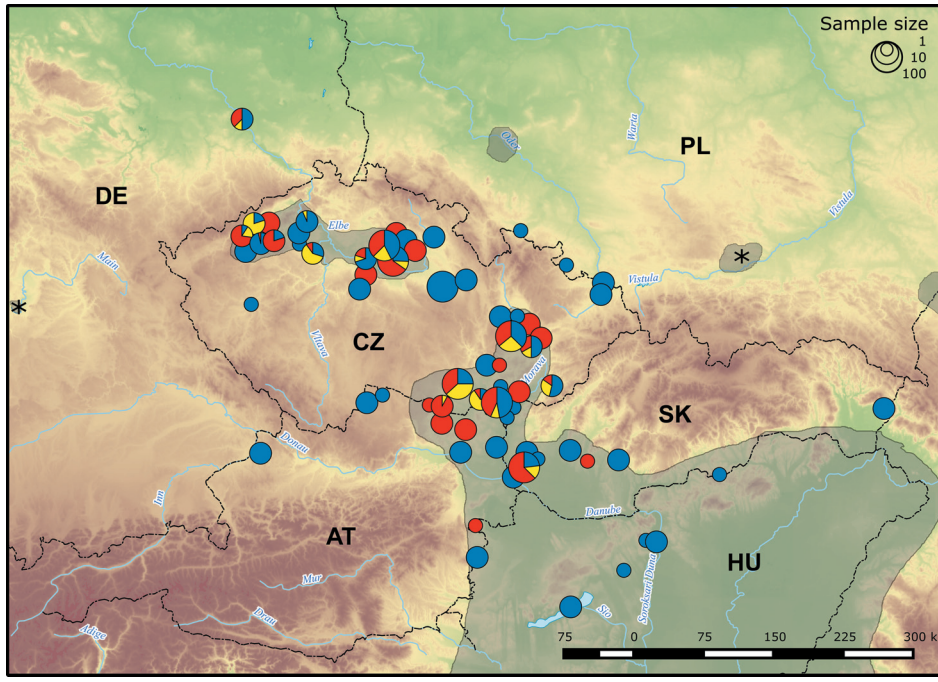


Fig. 1. – Population composition of *Ficaria* at sites inside (grey shading) and outside the contact zone between *F. calthifolia* and *F. *verna* in central Europe. Note small and isolated contact zones outside the continuous distribution of *F. calthifolia* in Poland and Germany. Contact zones between *F. calthifolia* and *F. *verna* are based on the distribution of *F. calthifolia* extracted from various sources (Soó & Borhidi 1966, Dostál 1977, Futák 1982, Křísa 1997, Zając & Zając 2001, Kästner & Fischer 2006, Illig & Ristow 2015, Veldkamp 2015, Drenckhahn 2016, Drenckhahn et al. 2017) and our unpublished data. Except at high altitudes, *Ficaria *verna* is common almost throughout central Europe (Veldkamp 2015). Explanations of colouring: red: *F. calthifolia*, blue: *F. *verna*, yellow: triploid hybrid, *: occurrence of both parental taxa and triploid plants (= supposed hybrids) at site(s) are reported in other sources (Towpasz 1971, Pogan & Wcisło 1974, 1983, 1986, Drenckhahn 2016).

tetraploids (i.e. both *Ficaria* species) and triploid hybrids (13 localities, 24.1%), but we also found three localities with mixed populations containing just triploids and one of the *Ficaria* species (*F. calthifolia* and triploids: one locality, 1.8%; *F. *verna* and triploids: two localities, 3.7%). No pure hybrid triploid population was recorded. Both *Ficaria* taxa without triploids co-occurred at two localities (3.7%). *Ficaria *verna* was the most frequent taxon at mixed-population sites (mean±SD; 40.2±22.7%), followed by *F. calthifolia* (35.7±26.0%) and triploid hybrids (24.1±21.4%) (Fig. 1, Electronic Appendix 1).

Morphological differentiation

The taxa *F. calthifolia* and *F. *verna* differed in all but five characters after Bonferroni correction was applied (Table 2 and Fig. 2). Hybrids were either intermediate between parental taxa in most of the characters analysed (52% of the characters; e.g. numbers of nodes and number of leaves per epigeic part of stem, relative length of blade notch) or differed from *F. calthifolia* but not from *F. *verna* (20% of the characters; e.g. leaf blade

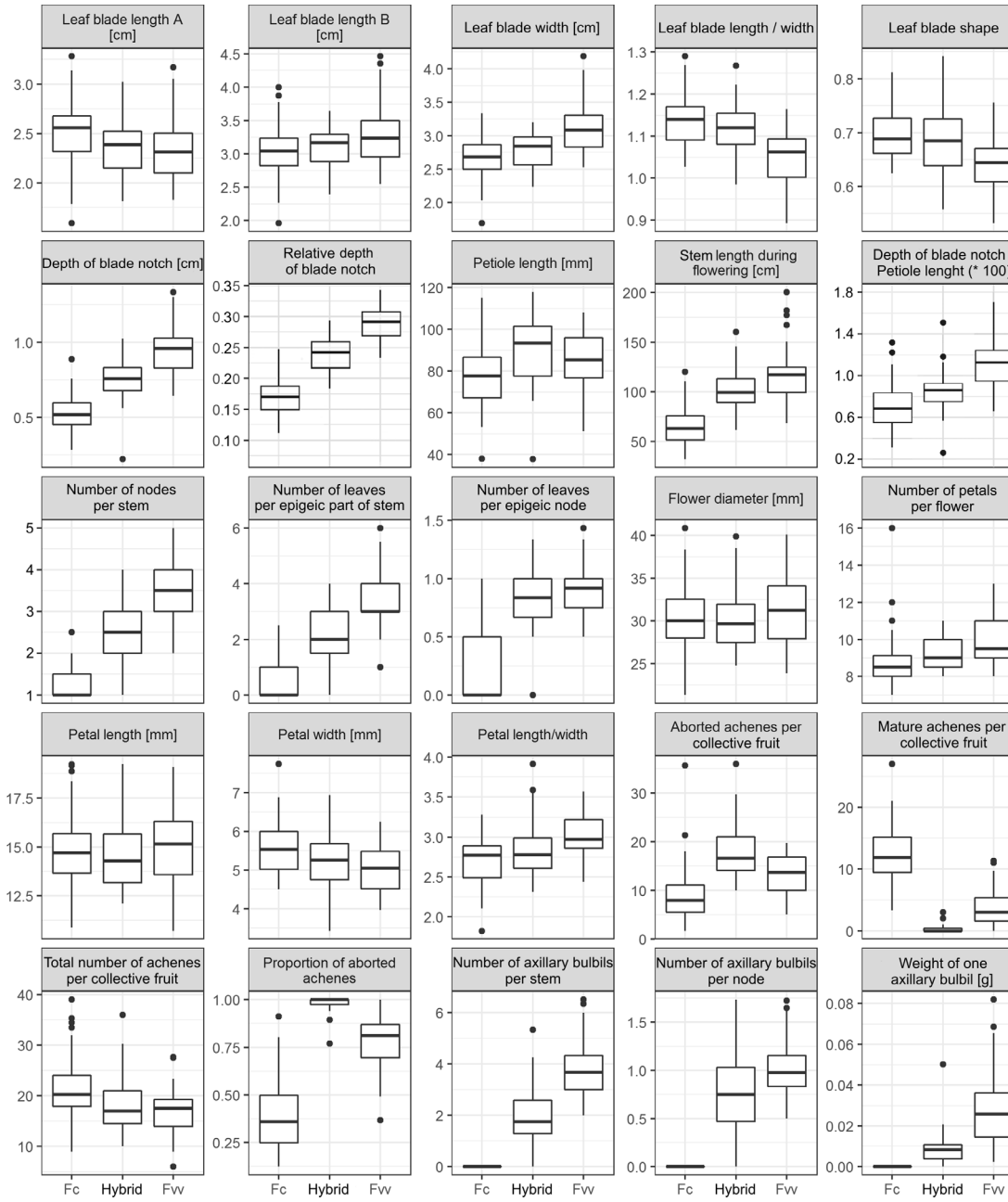


Fig. 2. – Boxplots of the morphological characters and their ratios for *Ficaria calthifolia* (Fc), *F. *verna* (Fvv) and their hybrid grown in a common garden. All multiple measurements of characters were averaged per individual before statistical comparison. Only ground (basal) leaves were used for measurement of leaf size characters.

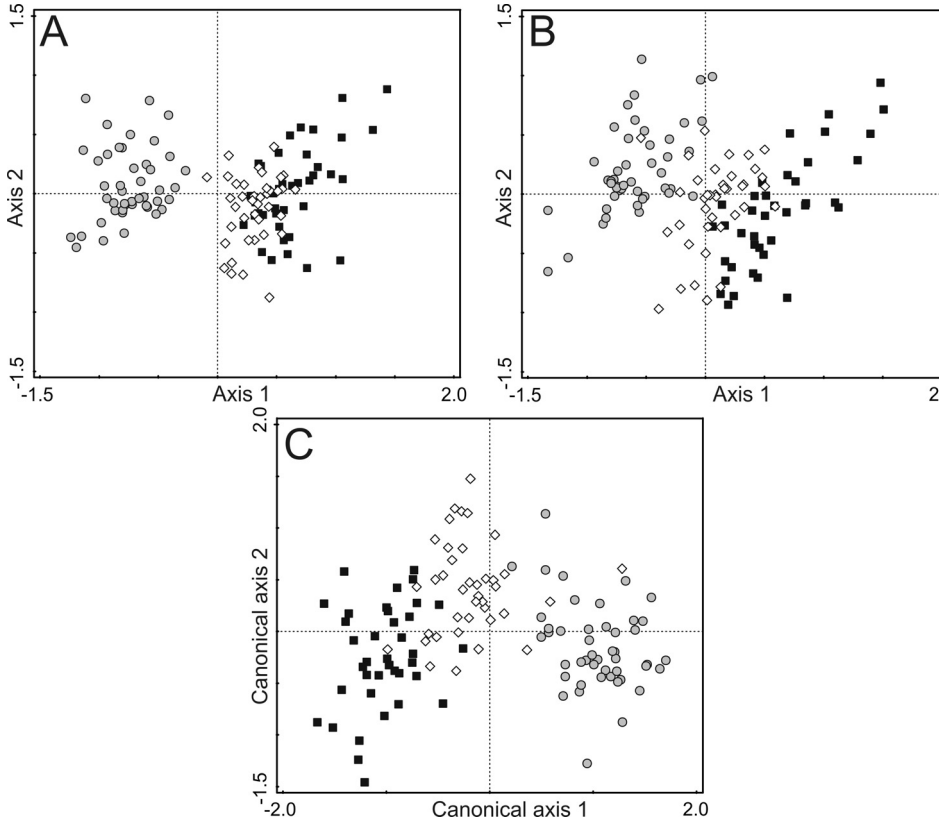


Fig. 3. – Results of multivariate analyses of morphological characters of 124 *Ficaria* plants. (A) Principal coordinate analysis based on 19 vegetative and reproductive characters. The first and second ordination axes explain 42.5% and 11.0% of total variation, respectively. (B) Principal component analysis based on 15 morphological characters with characters on achenes and bulbils excluded. The first and second ordination axes explain 36.3% and 18.8% of total variation, respectively. (C) Canonical discriminant analyses with forward selection that selected six characters (see Table 3). Symbols: *F. *verna* – black squares, *F. calthifolia* – grey circles, hybrid – white diamonds.

length, petiole length). Hybrids differed from *F. *verna* but not from *F. calthifolia* in 12% of the characters (e.g. leaf blade width, leaf blade shape). The characters that differentiate the taxonomic groups best are related to the production of axillary bulbils, aborted and well-developed achenes, leaf shape and plant habit. *Ficaria *verna* and *F. calthifolia* produced 13 and 40 times more well-developed achenes, respectively, than the hybrids, which on average aborted 98% of their achenes. On the other hand, both *F. *verna* and the hybrids produced axillary bulbils, but the number of bulbils per node and the mean weight of one bulbil were two and three-times lower in the hybrid than in *F. *verna,* respectively. No novel (transgressive) trait was recorded for the hybrid (Table 2, Fig. 2).

The PCoA based on 19 vegetative and reproductive characters and the PCA based on 15 characters (with characters on achenes and bulbils excluded) revealed two separate groups of OTUs corresponding to parental taxa (Fig. 3A, B). The hybrids were clearly differentiated from *F. calthifolia* but overlapped with *F. *verna* when the characters on the achenes and bulbils were included in the analysis (Fig. 3A). The hybrids occupied an intermediate position between parental taxa when the characters on the achenes and bulbils were excluded from the analysis (Fig. 3B).

Discriminant analysis using forward selection resulted in six selected characters and showed complete separation of parental taxa but weak separation of the hybrids from both parental taxa along the first canonical axis. Hybrids filled the space between the parental taxa (Monte Carlo permutation test, 1st axis: F = 88.4, P = 0.001; all axes: F = 26.1, P = 0.001; Table 3, Fig. 3C). Three characters (relative depth of blade notch, number of nodes per stem and number of leaves per epigeic node) had the highest explanatory contribution in this analysis (Table 3). Parametric CDA with selected characters (Table 3) had fairly high classification success (89%). Three plants of *F. calthifolia* and four of *F. *verna* were erroneously classified as hybrids and three and four hybrid plants were erroneously classified as either *F. calthifolia* or *F. *verna*, respectively.

Table 3. – List of morphological characters measured in *Ficaria* (excl. characters on achenes and bulbils) selected by forward analysis and their correlations with the first (Can1) and second (Can2) canonical axes in the canonical discriminant analysis (Fig. 3C). Percentage (%) means explanatory contribution of each selected character to the whole set of characters considered during the forward selection. Characters with the highest absolute correlations with any axis are in bold.

Character	Can1	Can2	%
Petiole length (PL)	–0.297	**0.429**	5.8
Relative depth of blade notch (RDN)	**–0.907**	0.001	55.9
Leaf blade length/width (LBLW)	0.546	0.317	4.3
Depth of blade notch (DBN)	**–0.826**	–0.115	3.6
Number of inodes per stem (NN)	**–0.898**	–0.064	9.2
Number of leaves per epigeic node (NLN)	**–0.802**	0.397	11.4

Phenology in a common garden

Taxa differed from each other in the timing of every phenological phase. The earliest was *F. calthifolia*, followed by the hybrids, and the latest was *F. *verna* (Table 4). The measure of flowering overlap between *F. calthifolia* and *F. *verna* reached intermediate values (57.8%) and was significantly lower than that found between the hybrids and either of the parental taxa (Table 4).

Pollen viability and size

Taxa differed from each other in mean pollen viability (LMM; F = 1034.9, P < 0.001; individual comparison tests with Bonferroni correction at P ≤ 0.05). Pollen viability of *F. calthifolia* always exceeded 65%, with that of most of the samples higher than 80% (mean±SD, 81.8±5.0%). The pollen viability of *F. *verna* was lower (58.1±7.1%) and only rarely exceeded 65%, whereas the pollen viability of the hybrids was very low (8.4±2.9%) and usually did not exceed 10% (Fig. 4A).

Table 4. – Comparison of the onset (x±SE) of every phenophase studied (F1–F4) in *Ficaria calthifolia*, *F. *verna* and hybrid cultivated in a common garden and index of flowering overlap between pairs of taxa. Mean values characterizing onset of each phenophase represent number of days from the beginning of the year. Values with different superscripts (within columns in the case of phenophases and within a row in the case of the index of flowering overlap) are significantly different (P ≤ 0.05).

Taxon/phenophase	F1	F2	F3	F4
	x±SE	x±SE	x±SE	x±SE
F. calthifolia	99.4±0.8[a]	102.7±0.8[a]	106.0±0.8[a]	110.3±0.8[a]
Hybrid	103.6±0.6[b]	107±0.8[b]	110.5±0.7[b]	114.4±0.7[b]
*F. *verna*	107.2±0.7[c]	110.8±0.7[c]	114.2±0.7[c]	118.3±0.8[c]
$F_{2, 73}$	28.8	28.6	28.6	24.1
P	< 0.001	< 0.001	< 0.001	< 0.001
	F. calthifolia – *F. *verna*	*F. calthifolia* – hybrid	*F. *verna* – hybrid	$F_{2, 8}$ P
Index of flowering overlap (%)	57.8±3.6[a]	72.8±3.6[b]	74.0±3.6[b]	10.3 0.006

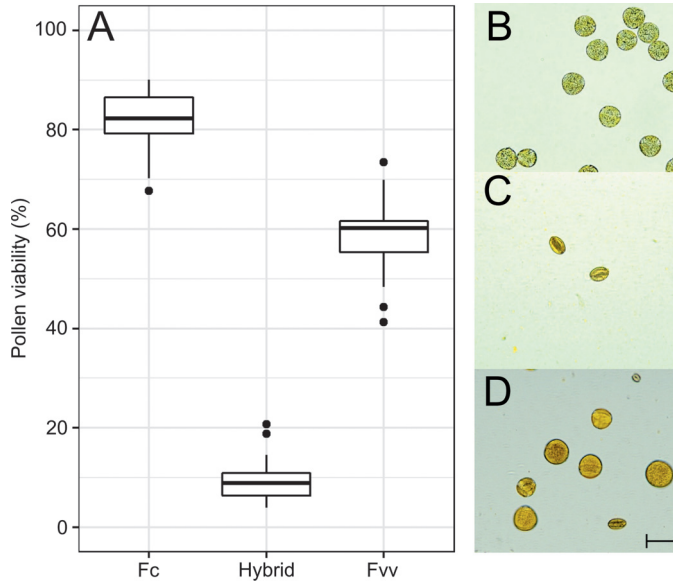


Fig. 4. – Pollen viability, pollen shape and size in *Ficaria* taxa. (A) Box-plots of pollen viability (%) in diploid *Ficaria calthifolia* (Fc), tetraploid *F. *verna* (Fvv) and triploid hybrid estimated using a fluorescein diacetate fluorescence analysis. (B–D) Pollen grains of *Ficaria calthifolia* (B), hybrid (C) and *F. *verna* (D). Length of bar is 50 µm.

Taxa differed significantly in the size of viable, normally developed pollen grains (LMM; F = 42.3, P < 0.001) and non-viable aborted pollen grains (LMM; F = 27.2, P < 0.001). The diameters of normally developed pollen grains increased with increase in ploidy level of the taxa but only the diameter of the pollen of *F. *verna* was significantly larger than that of the other taxa, which did not differ from each other in this trait (mean±SD; *F. calthifolia*: 33.9±2.8 µm, hybrid: 35.4±3.1 µm; *F. *verna*: 37.9±3.1 µm;

individual comparison tests with Bonferroni correction at $P \leq 0.05$; Fig. 4B–D). We did not observe any 'gigas' pollen grains indicating unreduced gametes.

Aborted pollen grains were wrinkled, flattened and generally smaller than normally developed pollen grains (Fig. 4C). The lengths of the pollen grains were shortest in the hybrids (23.5±2.1 µm), intermediate in *F. calthifolia* (24.8±1.9 µm) and longest in *F. *verna* (26.4±2.6 µm). However, only *F. *verna* had significantly longer aborted pollen grains than the other taxa (individual comparison tests with Bonferroni correction at $P \leq 0.05$).

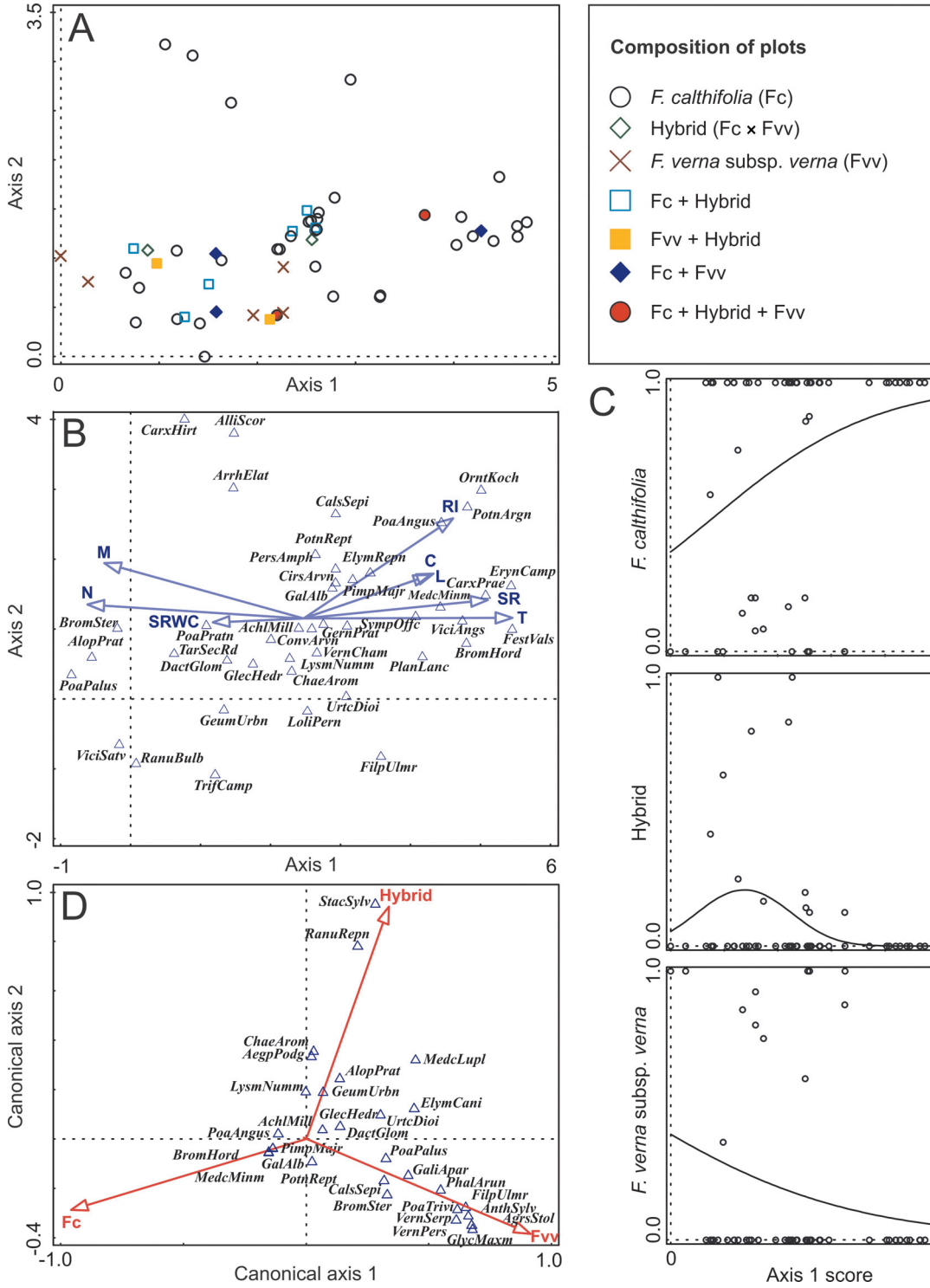
Sprouting of bulbils and growth of young plants

The initial weight of the bulbils was significantly higher for *F. *verna* (mean±SD; 72±40 mg) than for the hybrids (40±20 mg; LMM, $F = 7.053$, $P = 0.024$). Bulbils of the hybrids and tetraploid *F. *verna* did not differ in the percentage sprouting and the initial weight of the bulbil did not affect the percentage that sprouted (GLMM, bulbil weight: $z = 0.844$, $P = 0.399$, taxon: $z = 1.081$, $P = 0.279$, bulbil weight × taxon: $z = -0.292$, $P = 0.770$). The percentage sprouting was high for both taxa (hybrid: 98%, *F. *verna*: 88%) and slightly lower for *F. verna* due the poor sprouting of bulbils from one population. Just two *F. *verna* plants (2.6%) and no hybrid plants flowered during the first season. Approximately fifteen percent of the *F. *verna* plants and no hybrid plants produced at least one axillary bulbil during the first season. The number of underground tubers and axillary bulbils produced depended on the initial weight of the bulbil (LMM, bulbil weight: $F = 108.97$, $P < 0.001$, taxon: $F = 0.617$, $P = 0.999$, bulbil weight × taxon: $F = 0.569$, $P = 0.452$).

Frequency of hybrids in the field and ecological differentiation of parental taxa and hybrids

At both localities studied in detail (Svätý Jur [no. 47] in Slovakia and Zbraň [no. 5] in Czechia) there was a big variation in the relative percentages (range 0–100%) of the parental taxa and hybrids in the different plots. The most frequent was *F. calthifolia* (mean percentage, 25–75 percentile; Svätý Jur: 72%, 20–100%; Zbraň: 69%, 13–100%), followed by *F. *verna* (Svätý Jur: 18%, 0–0%; Zbraň: 20%–15%) and the least frequent was the hybrid (Svätý Jur: 10%, 0–0%; Zbraň: 11%–13%). Most plots contained just one taxon (Svätý Jur: 77%, Zbraň: 75%), with one plot per locality with only hybrid plants. Hybrids were found in all possible combinations with parental taxa at both localities, but the most frequent combination was that of *F. calthifolia* and hybrids, recorded in three plots per locality, whereas other combinations were recorded just once per locality.

A partial DCA revealed a distinct pattern along the first axis from wet-to-moist, nutrient-rich and often-shaded microhabitats on the left to open, mesic to semidry and less nutrient-rich microhabitats on the right of the diagram (Fig. 5B). This gradient correlates well with the pattern of occurrences of taxonomic groups (Fig. 5A). *Ficaria *verna* showed the highest probability of occurring on the left and significantly decreased along the first axis ($F = 238.2$, $P < 0.001$), whereas *F. calthifolia* showed the opposite trend ($F = 6.5$, $P = 0.013$). The hybrids showed a unimodal response with the optimum between the parental taxa ($F = 3.9$, $P = 0.026$), but shifted slightly left towards *F. *verna* (Fig. 5C). A partial CCA showed significant differences in the floristic composition of plots in which the composition of taxonomic groups differed (Monte Carlo permutation test, 1st canonical axis: $F = 2.9$, $P = 0.002$; all canonical axes: $F = 2.1$, $P = 0.002$). Indicators of


A
Axis 2
Axis 1
3.5
0.0
0
5
Composition of plots
F. calthifolia (Fc)
Hybrid (Fc × Fvv)
F. verna subsp. verna (Fvv)
Fc + Hybrid
Fvv + Hybrid
Fc + Fvv
Fc + Hybrid + Fvv
B
CarxHirt
AlliScor
ArrhElat
OrntKoch
CalsSepi
RI
PotnArgn
PoaAngus
PotnRept
M
PersAmpl
ElymRepn
C
L
ErynCamp
CirsArvn
CarxPrae
N
GalAlb
PimpMajr
MedcMinm
SR
BromSter
SRWC
AchlMill
SympOffc
T
ViciAngs
AlopPrat
PoaPratn
ConvArvn
GernPrat
FestVals
TarSecRd
VernCham
BromHord
DactGlom
LysmNumm
PlanLanc
GlecHedr
ChaeArom
PoaPalus
UrtcDioi
GeumUrbn
LoliPern
ViciSatv
RanuBulb
FilpUlmr
TrifCamp
4
-2
-1
6
Axis 2
Axis 1
D
StacSylv
Hybrid
RanuRepn
ChaeArom
AegpPodg
MedcLupl
AlopPrat
LysmNumm
GeumUrbn
ElymCani
GlecHedr
AchlMill
UrtcDioi
PoaAngus
DactGlom
PimpMajr
PoaPalus
BromHord
GalAlb
GaliApar
MedcMinm
PotnRept
PhalArun
CalsSepi
FilpUlmr
BromSter
PoaTrivi
AnthSylv
VernSerp
AgrsStol
VernPers
GlycMaxm
Fc
Fvv
1.0
-0.4
-1.0
1.0
Canonical axis 2
Canonical axis 1
C
F. calthifolia
Hybrid
F. verna subsp. verna
1.0
0.0
0
5
Axis 1 score

nutrient-rich and wet-to-moist soils (e.g. *Agrostis stolonifera*, *Glyceria maxima*, *Phalaris arundinacea*, and *Poa trivialis*) were associated with *F. *verna*, whereas indicators of mesic to semidry conditions (e.g. *Achillea millefolium* agg., *Medicago minima*, and *Poa angustifolia*) were associated with *F. calthifolia* (Fig. 5D). Hybrids were in an intermediate position between parental taxa in the ordination diagram.

Discussion

Hybrids are frequent at contact sites of parental taxa but apparently do not disperse beyond their boundaries

We observed striking differences in the compositions of the populations at *Ficaria* sites depending on whether they occurred inside or outside the contact zone between putative parents (Fig. 1). Outside the contact zone, only tetraploid *F. *verna* occurred; that is, all sampled populations were pure tetraploid, and no admixtures of other cytotypes were recorded. In contrast, almost one-third of the sites sampled within the contact zone between parental taxa were cytotype-mixed, and more importantly at approximately three-quarters of these cytotype-mixed sites there were also triploid plants, whose occurrence might be theoretically explained by their autotriploid origin from *F. calthifolia*

◀ Fig. 5. – Microhabitat differentiation of parental taxa (*Ficaria calthifolia*, *F. *verna*) and the hybrid. (A) Partial DCA (1st and 2nd axis) of vegetation data sampled at two localities (Svätý Jur in Slovakia and Zbraň in Czechia) where both the parental taxa and hybrid occurred. Occurrence of taxonomic groups (*F. calthifolia*, hybrid, *F. *verna*) at each plot is distinguished (i.e. pure and different types of mixtures within a plot). The first two axes explained 14.0% and 7.6% of variation after fitting covariates, respectively. (B) Species-supplementary variables plot of pDCA of vegetation data sampled. Only species with the highest weight in the analysis are shown. Arrows of supplementary variables show the marginal effect of the particular environmental variable upon the sample scores in the ordination diagram (Ellenberg indicator values: C = continentality, M = moisture, N = nutrients, L = light, SR = soil reaction, T = temperature; SRWC = soil relative water content, RI = relative irradiation). (C) Results of GLM testing the effect of axis 1 score in pDCA on the probability of occurrence of taxonomic groups. The figure provides the best models selected by the lowest AIC value. In all models, a quasi-binomial model type with logit-link function was used. All models were significant at $P \leq 0.05$. (D) Species-environmental variables plot of pCCA of vegetation data sampled, testing the effect of percentage of the taxonomic group within a plot on the floristic composition of sampled plots. Only species with high fit in the analysis (> 6%) are shown. Species abbreviations: *AegpPodg* (*Aegopodium podagraria*), *AgrsStol* (*Agrostis stolonifera*), *AchlMill* (*Achillea millefolium*), *AlliScor* (*Allium scorodoprasum*), *AlopPrat* (*Alopecurus pratensis*), *AnthSylv* (*Anthriscus sylvestris*), *ArrhElat* (*Arrhenatherum elatius*), *BromHord* (*Bromus hordeaceus*), *BromSter* (*Bromus sterilis*), *CalsSepi* (*Calystegia sepium*), *CarxHirt* (*Carex hirta*), *CarxPrae* (*Carex praecox*), *ChaeArom* (*Chaerophyllum aromaticum*), *CirsArvn* (*Cirsium arvense*), *ConvArvn* (*Convolvulus arvensis*), *DactGlom* (*Dactylis glomerata*), *ElymCani* (*Elymus caninus*), *ElymRepn* (*Elymus repens*), *ErynCamp* (*Eryngium campestre*), *FestVals* (*Festuca valesiaca*), *FilpUlmr* (*Filipendula ulmaria*), *GalAlb* (*Galium album*), *GaliApar* (*Galium aparine*), *GernPrat* (*Geranium pratense*), *GeumUrbn* (*Geum urbanum*), *GlecHedr* (*Glechoma hederacea*), *GlycMaxm* (*Glyceria maxima*), *LoliPern* (*Lolium perenne*), *LysmNumm* (*Lysimachia nummularia*), *MedcLupl* (*Medicago lupulina*), *MedcMinm* (*Medicago minima*), *OrntKoch* (*Ornithogalum kochii*), *PersAmph* (*Persicaria amphibia*), *PhalArun* (*Phalaris arundinacea*), *PimpMajr* (*Pimpinella major*), *PlanLanc* (*Plantago lanceolata*), *PoaAngus* (*Poa angustifolia*), *PoaPalus* (*Poa palustris*), *PoaPratn* (*Poa pratensis*), *PoaTrivi* (*Poa trivialis*), *PotnArgn* (*Potentilla argentea*), *PotnRept* (*Potentilla reptans*), *RanuBulb* (*Ranunculus bulbosus*), *RanuRepn* (*Ranunculus repens*), *StacSylv* (*Stachys sylvatica*), *SympOffc* (*Symphytum officinale*), *TarSecRd* (*Taraxacum* sect. *Ruderalia*), *TrifCamp* (*Trifolium campestre*), *UrtcDioi* (*Urtica dioica*), *VernCham* (*Veronica chamaedrys*), *VernPers* (*Veronica persica*), *VernSerp* (*Veronica serpyllifolia*), *ViciAngs* (*Vicia angustifolia*), *ViciSatv* (*Vicia sativa*)

and/or are hybrids between *F. calthifolia* and *F. *verna*. However, a recent study of molecular markers, genome size and the results of a crossing experiment using *Ficaria* (Popelka et al. 2019) together with the present results showing the regular occurrences of triploids at contact sites of parental taxa and the absence of triploids in otherwise diploid *F. calthifolia* populations that are not in close contact with *F. *verna* populations (Fig. 1) clearly corroborate a previous hypothesis (Pogan & Wcisło 1974, 1983, 1986) that triploid plants are hybrids that originate independently at many contact sites between both parents.

An occasional occurrence of *Ficaria* triploids is also reported in several southern and central European countries (Negodi 1930, 1937, Barros Neves 1942, Tröhler 1976, Anders-Gasser 1985, Diosdado & Pastor 1996, Drenckhahn 2016) but their origin needs to be resolved. There have been detailed studies on the frequency and distribution of chromosomal races of the *F. verna* agg., including triploids in Great Britain (Gill et al. 1972, Marchant & Brighton 1974, Nicholson 1983). Both Gill et al. (1972) and Nicholson (1983) report ~13% of sites with mixed populations of different cytotypes, but at three-quarters of these sites there is only a mixture of diploids (= *F. verna* subsp. *fertilis* [Laegaard] Stace) and tetraploids (= *F. *verna*), which seems to be in marked contrast with the situation recorded in central Europe. However, these contrasting results are probably due to differences in the sizes of the population samples collected in these two studies: only 39% of the samples were of three or more plants in the study of Gill et al. (1972), whereas we sampled, on average, 17 individuals per site. Aside from these differences, both Gill et al. (1972) and in the present study mixed-ploidy populations containing only one parent and the hybrid were rarely recorded. We interpret this simply as an accidental extinction of one of the parents; most of these sites were in town lawns and parks that are occasionally disturbed by building works and park maintenance. On the other hand, we did not find a pure triploid population. Gill et al. (1972) report only two pure triploid populations (~1% of all populations analysed), but these populations may also have been mixed because only one and two plants were sampled. Moreover, within mixed-cytotype sites with the occurrence of both parents and their hybrid, usually one of the parental taxa dominated, and the hybrid was usually in a minority. All these data are indicative of poor dispersal capacity and/or weak competiveness of the hybrid, which limits its spread beyond the site of origin (see below).

Morphological differentiation

The parental *Ficaria* taxa are morphologically well differentiated by several characters traditionally considered as useful for discriminating between them. These characters include plant habit, appearance of axillary bulbils and number, and percentage of well-developed achenes (Sell 1994, Axtell et al. 2010, Veldkamp 2015, Drenckhahn 2016). On the other hand, several dimensions of the flowers (flower diameter, petal length and petal width) were mentioned by some authors (Veselá 1969, Veldkamp 2015) as useful discriminant characters, but these characters can not be used to distinguish between the parental taxa as only weak trends were recorded (Fig. 2). In agreement with the statement of Veselá (1969), *F. calthifolia* tends to have ovate petals, whereas *F. *verna* has narrow ovate petals. Relatively poor efficiency of floral characters for discriminating between taxa is partially due to the fact that *Ficaria* petals continue to grow and become narrower

during flower development and thus examination of flowers at different phases in their development can give heterogeneous and incomparable data. We controlled for this by examining flowers at the same developmental phase but this did not resolve the problem. Using several ratio characters describing leaf shape, on the other hand, proved more useful as discriminant characters (Table 2). In particular, the relative depth of the notch in the blade had high discriminating power for distinguishing between parental taxa (see also Kästner & Fischer 2006).

Because we studied *Ficaria* taxa grown in the common garden the morphological variation could be narrower and mean values might have shifted differently for each parental taxon in comparison with in situ conditions (Fialová et al. 2014). However, Post et al. (2009), studying herbarium specimens of *Ficaria* from in situ conditions in an invaded range (U.S.A.), found differences in several characters between the parental taxa similar to the differences observed by us between the parental taxa in the garden. Some dimensions of the sepals (e.g. sepal width), nectarium shape and innervation of the petals, which are mentioned by Veselá (1969) as a way of discriminating between parental taxa, were not considered by us in this study. Austrian authors (Kästner & Fischer 2006, Fischer et al. 2008) mention the position of hydathodes on a leaf blade as another useful character for discriminating between parental taxa, but our preliminary investigations found this character to be highly variable in most of the populations studied. Therefore, we decided not to include this character in the morphometric analyses.

Hybrid plants were intermediate between parental taxa in 52% of the characters studied. This is in accordance with the mean percentage of intermediate characters reported in 46 studies of F1 homoploid hybrids (Rieseberg & Ellstrand 1993). Intermediate characteristics of hybrids are usually interpreted as a consequence of the polygenic control of quantitative characters with simple additive effects (Rieseberg & Ellstrand 1993). However, that hybrids are more similar to *F. *verna* in their characteristics than to *F. calthifolia* might be explained by at least three processes: (i) some characters (e.g. production of axillary bulbils) might result from one or a few loci with dominant effects (López-Caamal & Tovar-Sánchez 2014), (ii) a dosage effect might affect the gene expression in heteroploid hybrids (Ståhlberg 2009, De hert et al. 2011, Yao et al. 2013), or (iii) subgenomic expression dominance might occur immediately following hybridization (Edger et al. 2017). On the other hand, only a slight morphological novelty (i.e. a transgressive character) was recorded in hybrids, with the hybrids having the longest leaf petioles. This might be explained by the rather similar genomes of the parental taxa – Drenckhahn et al. (2017) speculate that central and eastern European *F. *verna* is an allotetraploid derivative of a *F. calthifolia* precursor – and/or by the absence of a later generation of hybrid plants in the populations (Popelka et al. 2019) that usually express an increase in transgressive characters compared to F1 hybrids (Rieseberg & Ellstrand 1993). Nearly analogous patterns of morphological similarity are reported by Aagaard et al. (2005) between allotriploid hybrids and their parental taxa, i.e. the diploid *Dactylorhiza incarnata* subsp. *cruenta* and its putative allotetraploid derivative *D. lapponica*.

Flowering asynchrony of parental taxa and intermediate phenology of hybrid

Phenological observation on plants of parental taxa grown in a common garden showed that *F. calthifolia* and *F. *verna* have slightly different flowering phenologies. Every phenophase started approximately one week earlier in *F. calthifolia* than in *F. *verna*. This resulted in an overlap in flowering of 57.8%. This pattern is in line with that reported in several parts of the range of the taxa studied (e.g. Schur 1866, Křísa 1997, Kästner & Fischer 2006) and demonstrates that flowering phenology is a significant prezygotic barrier that increases assortative mating in parental taxa (Husband & Schemske 2000, Ferriol et al. 2015).

Despite flowering asynchrony between *F. calthifolia* and *F. *verna*, our data show that there was sufficient time for pollinators to cross-pollinate both species. When taking into account natural conditions, however, flowering asynchrony between parental taxa may be more pronounced due to their preferences for different microhabitats at the contact sites (see also below), which may affect the rate of growth, and subsequently, the time of flowering. The reciprocal crossing between parental taxa (Popelka et al. 2019) results in offspring, but this cannot be taken as direct evidence of bidirectional gene flow under natural conditions because the flowering of the parental taxa was synchronized in a greenhouse. However, genetic markers prove the hybrid origin of triploids in sympatric parental populations (Popelka et al. 2019), confirming that the flowering periods of the parental taxa overlap. Therefore, there is a potential for multidirectional gene transfer between the parental taxa.

Greater variability in the overlap of the flowering periods of the hybrid plants and their progenitors usually suggest that these hybrid populations consist of a mix of different genotype classes representing also backcrosses with progenitors (LeBoldus et al. 2013). In contrast, nonoverlapping flowering times would result in hybrid plants crossing primarily with each other, thereby delaying the immediate introgression into progenitors (Snow at al. 1998). However, hybrid *Ficaria* plants had uniformly intermediate flowering phenologies as the onset of every phenophase was intermediate between those recorded for the parental taxa. Intermediate phenology is typical of earlier hybrids (as also observed in our case, Popelka et al. 2019) and is reported in, e.g. hybrids between diploid *Centaurea aspera* L. and allotetraploid *Centaurea seridis* L. (Ferriol et al. 2015).

Pollen viability in parental species and the hybrids: consequences for seed production and gene flow

We found that, in contrast to the very low pollen viability of the hybrids, more than a half of the pollen grains of the parental taxa are viable and they have a regular spherical shape. However, a poorer production of viable pollen was recorded for *F. *verna* than *F. calthifolia*. Almost identical patterns of pollen viability are reported by Pogan & Wcisło (1973, 1974, 1981a, b, 1983) in Polish populations of parental taxa and their hybrids and by Marchant & Brighton (1974) in British populations of diploid *F. verna* subsp. *fertilis*, tetraploid *F. *verna,* and their triploid hybrid. The low pollen fertility of *F. *verna* might be explained by its tetraploidy adversely affecting microsporogenesis, as is reported for plants from several Polish populations by Pogan & Wcisło (1981a). The high pollen sterility of the hybrids is caused by the production of highly misbalanced (aneuploid) gametes due to its odd ploidy (Pogan & Wcisło 1983), which indicates that the hybrids are likely to only produce

few or no seeds. Ramsey & Schemske (1998) summarize the data on pollen fertility in 18 different 'allotriploid' hybrids and report that, on average, it is as high as 23.7%, which is higher than the pollen viability recorded by us for the *Ficaria* hybrid.

We also observed that pollen diameter increases with increasing ploidy level in the *Ficaria* taxa studied. This is consistent with the results of several previous investigations at the microevolutionary level, in which congeneric taxa tend to support the trend of increasing pollen width with increasing genome size, especially when the divergence between taxa is associated with variation in the ploidy level (see Knight et al. 2010 and the references therein). Inspection of pollen shape and frequencies of different pollen shape classes (viable ~spherical, inviable ~wrinkled) under a microscope provide rapid and simple evidence of ploidy level and pollen viability of the *Ficaria* taxa studied.

Indeed, the pattern in the production of well-developed achenes in parental and hybrid plants cultivated in a common garden closely matched that of pollen viability. Our estimates of number and percentage of well-developed achenes per collective fruit agree almost perfectly with other reports on *F. calthifolia* (Drenckhahn 2016) but differ from several reports on *F. *verna* (Marsden-Jones 1935, Taylor & Markham 1978, Wcisło & Pogan 1981, Jung et al. 2008, but see Drenckhahn 2016). This is particularly curious when an average of 23% of well-developed achenes per collective fruit were recorded by us in *F. *verna* compared to the 7% and 2% that are reported by Wcisło & Pogan (1981) and Marsden-Jones (1935), respectively. Most of the achenes (on average 98%) produced by hybrids were aborted, and only some hybrid plants produced up to three well-developed achenes, which agrees with Pogan & Wcisło (1974, 1983) and Drenckhahn (2016). Although several factors (e.g. genetic differences between populations, different environmental and weather conditions and disturbance; Jung et al. 2008) might explain the discrepancies in seed-set estimations between studies, we guess that both ours and Drenckhahn (2016) estimates of achene production in *F. *verna* are characteristic of the situation in genetically diverse populations with intensive intra- and perhaps also interspecific outcrossing (Popelka et al. 2019).

However, whether the production of well-developed achenes is proof of fecundity in *Ficaria* is uncertain. Marsden-Jones & Turrill (1952) note that the majority of 'good' achenes produced by *F. *verna* fail to germinate, and others (Marsden-Jones 1935, Taylor & Markham 1978) also report an overall poor percentage germination (below 40%) of achenes for *F. *verna*. Wcisło & Pogan (1981) analysed the embryology of well-developed achenes of *F. *verna* and report that most of them (86%) had degenerated embryos and were in fact non-viable. Also attempts to germinate a few achenes produced by the hybrid (Pogan & Wcisło 1983, Popelka pers. obs.) failed because all achenes decayed. In contrast, a study by Popelka et al. (2019) reports a high percentage germination of well-developed achenes produced by experimental crosses mimicking xenogamy not only in *F. calthifolia* (mean total germination 91%) but also in *F. *verna* (61%). However, *in situ* observation of many *F. calthifolia* seedlings (Pogan & Wcisło 1983, Drenckhahn 2016, Popelka pers. obs.), low production of well-developed achenes by *F. *verna* and hybrids and zero (hybrid) to intermediate (*F. *verna*) vs. the extremely high (*F. calthifolia*) genotypic diversity found in populations growing in sympatry at three sites (Popelka et al. 2019) suggest almost total sterility of hybrids, poor sexual fecundity of *F. *verna* and high fecundity of *F. calthifolia*.

The frequent occurrence of partially fit triploid hybrids at sites with both parental taxa growing in sympatry indicates the possibility of introgression across species barriers. Some studies dealing with various polyploid complexes report gene flow from diploids to tetraploids through partially fit triploids (i.e. a triploid bridge; Husband & Sabara 2003, Aagaard et al. 2005, Slotte et al. 2008, Zohren et al. 2016). Alternatively, backcrossing between a triploid hybrid and diploid ancestor might occur (Ramsey & Schemske 1998, Sutherland & Galloway 2017). Based on the previous discussion, the mentioned mechanisms of gene flow between *F. calthifolia* and *F. *verna* are probably extremely rare. Indeed, no traces of recent gene flow between the parental taxa are reported by Popelka et al. (2019). However, a recent finding of a complex cytotypic structure at one contact site of *F. calthifolia* and *F. *verna* in Germany containing, in addition to di-, tri- and tetraploids, also pentaploids (Drenckhahn 2016), might indicate that postzygotic reproductive isolation between parental taxa may not be as complete as our data indicate.

Maintenance and local dispersal of hybrid is enhanced by production of bulbils in leaf axils

Almost complete sexual sterility of the triploid hybrids questions their ability to regenerate and disperse at the sites of origin. However, we found that hybrids express one character of *F. *verna* that allow them to disperse not only locally but potentially also beyond the borders of the population: axillary bulbils (see also Pogan & Wcisło 1983, Drenckhahn 2016). Production of bulbils is considered by several authors as the only efficient mode of spread and colonization of tetraploid *F. *verna* (Marsden-Jones 1935, Verheyen & Hermy 2004). In both taxa, the percentage of bulbils sprouting was high (88–98%) in the common garden experiment, which is in accordance with the data on percentage of bulbils of *F. *verna* sprouting in Great Britain (Marsden-Jones 1935, Taylor & Markham 1978) and Belgium (Verheyen & Hermy 2004), and in other studies on sprouting of aerial bulbils of various species (e.g. Fialová et al. 2014, Fialová & Duchoslav 2014 and references therein). However, we found that bulbils of the hybrids were approximately three times lighter and two times less frequently produced per stem than by *F. *verna*. Consequently, smaller hybrid juveniles might be at a competitive disadvantage (Leishman 2001), even greater when growing with large juveniles originating from *F. *verna.* Gill et al. (1972) also report that the triploid hybrid between *F. verna* subsp. *fertilis* and *F. *verna* produces smaller bulbils than *F. *verna*. However, the consequences of these differences for establishment and survival of the hybrid under natural conditions remain unclear. Gustafsson et al. (2002) show that, in bulbil-producing *Cardamine bulbifera* (L.) Crantz, early phases of recruitment *in situ* are influenced neither by competition from surrounding vegetation nor increasing bulbil densities, which indicates that the effects of inter- and intraspecific competition on the establishment of *Cardamine* plants are not important. The weak effect of cover of surrounding vegetation as a proxy of interspecific competition on survivorship of juveniles originating from bulbils of *F. *verna* is also reported by Verheyen & Hermy (2004). Both studies, however, show that short-term population densities and population spread are limited by bulbil availability that is suggestive of clear handicap of hybrid *Ficaria*, which is likely to be the reason why we did not observe pure populations of hybrids.

Hybrid seems to be ecologically intermediate between parental taxa: consequences for hybrid zone persistence

When all the available data gathered by us is considered, the hybrid zone between *F. calthifolia* and *F. *verna* has a mosaic structure, in which populations of parental taxa alternate with mixed populations (see also Coustau et al. 1991, Daguin et al. 2001). The mosaic structure is usually explained by differential adaptation in a patchy environment (Harrison & Rand 1989) that also serves as a major isolating mechanism between potentially hybridizing species (Arnold 2006). The literature and our data indicate that niches of hybridizing *Ficaria* taxa are not identical but slightly overlap. Moreover, their parental habitats frequently occur in close proximity. Once hybridizing *Ficaria* taxa meet, hybrid plants may result and become established interspersed mostly between parental habitats. We find that the niche of the hybrid is intermediate between but also slightly overlaps the niches of the parental taxa. Intermediate niche of the hybrid might be interpreted in two opposing ways. First, the ecological niche of the hybrid might be related to the hybrid's higher fitness than the parental taxa in the intermediate habitat. If true, the hybrid would be able to establish a long-lived evolutionary lineage and potentially spread beyond the borders of the contact sites. Alternatively, the fitness of the hybrid is lower than that of the parental taxa regardless of habitat and is selected against but might be recurrently formed (= the mosaic model; Barton & Hewitt 1985). If true, the microhabitat separation of the hybrid is simply an artefact of the localized dispersal of triploid seeds that are produced by hybridizing, tightly growing individuals of parental taxa at ecologically transient microsites (see also Ståhlberg 2009). To rigorously test which of these models is correct will require a reciprocal transplanting experiment. Hybrid sterility however limits formation of subsequent hybrid generations via sexual reproduction (Popelka et al. 2019), potentially leading to selection of later generation superior hybrid plants with novel genotypes allowing invasion of novel habitats (Arnold 1997). One potential way of restoring sexual reproduction in hybrids might be the generation of hexaploids by crossing of various triploid hybrid individuals, a process observed in several other triploid hybrids (Ramsey & Schemske 1998).

Taxonomy and nomenclature of the interspecific Ficaria hybrid

Ferdinand Schur was probably the first author to write about an 'intermediate form' between the taxa *F. *verna* (as *F. ranunculoides*) and *F. calthifolia* in his description (name protologue) of *F. intermedia* Schur (Schur 1866) from the vicinity of the Romanian town of Sibiu. For a full insight into his concept of *Ficaria* taxa in this region one needs to study the chapter on *Ficaria* in his publication on Transylvanian plants (Schur 1866: 13, 14) and carefully examine his herbarium specimens from the region, currently deposited in herbarium LW as part of his 'Transylvanian herbarium collection'. From several localities in the wider neighbourhood of Sibiu, he reports four species in the genus *Ficaria*: *F. ranunculoides* Roth. (= *F. *verna*), *F.* '*calthaefolia*' Rchb., *F. intermedia* Schur and *F. transsilvanica* Schur. The first taxon is reported by him in forests, thickets and orchards in the vicinity of Sibiu and flowering in April and May. The voucher specimen in herbarium LW (no. 201828) consists of two plants with axillary bulbils collected in an orchard in the vicinity of Sibiu, which undoubtedly is *F. *verna*. The second species Schur mentions from wet salt meadows and flowering in April, but there

is no voucher specimen in his herbarium collection. Nevertheless, the ecological characteristics he reports do not match those of the real *F. calthifolia*, but correspond with those of one of the many ecotypes of *F. *verna*. In addition, the flowering time (April) does not conflict with this suggestion. Moreover, Schur (1866: 14) describes in detail ecological characteristics ('on dry sunny hills together with *Carex humilis* and *Viola ambigua*, at an altitude of approximately 600 m') and the early flowering time (February, March) of his newly described species, *F. transsilvanica*. These two pieces of evidence indicate that this taxon is, in fact, *F. calthifolia*. This accords well with the fact that specimens on both the relevant herbarium sheets (syntypes, nos. 201832 and 201833) in Schur's collection in herbarium LW unambiguously belong to *F. calthifolia*. In addition, current authors (Sell 1994, Veldkamp 2015) consider the name *F. transsilvanica* as a synonym of *F. calthifolia*. The last taxon reported by Schur (1866) from the vicinity of Sibiu and at the same time described as a new species is *F. intermedia*. It was found on a track from the village of Răşinari (southeast of Sibiu) to the mountain Vârful Măgura Cisnădiei (1304 m a.s.l.), at an altitude of ~900 m a.s.l. and flowering in May. These characteristics alone indicate that the ecological requirements of this taxon are very different from that of *F. calthifolia* (= *F. transsilvanica* sensu Schur) in the area Schur studied. Only one sheet with one specimen of *F. intermedia* exists in Schur's collection. Careful study of this dried plant undoubtedly revealed it is *F. *verna* with a somewhat shortened one-flowered stem, but with distinctly alternate leaves. Thus, we cannot agree with the statement of Sell (1994) and Veldkamp (2015) that the name *F. intermedia* is a synonym of *F. calthifolia*. In this context in the chapter on the genus *Ficaria* in the publication of Schur (1866), this author writes that his *F. intermedia* is an 'intermediate form' between the ecotype of *F. *verna* from forests, thickets and orchards (*F.* '*ranunculoides*' in his nomenclature) and the ecotype of the same taxon from wet salt meadows (*F.* '*calthaefolia*' in his erroneous taxonomic concept). Because all these facts indicate an undeniable link, we can state that Schur's *F. intermedia* is not a designation for the hybrid between the taxa of *F. calthifolia* and *F. *verna*.

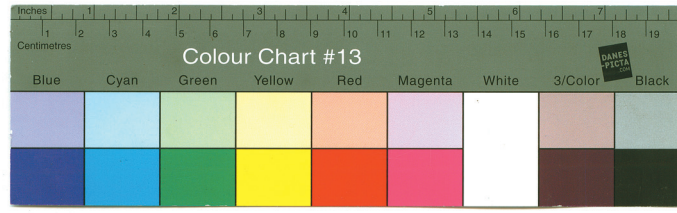
For the name of *F. transsilvanica* Schur Enum. Pl. Transsilv. 14 (1866), we select as the **lectotype (hoc loco)** sheet no. 201832 in herbarium LW collected by F. Schur in March 1850 with habitat and locality characteristics: "In collibus apricis inter frutices cum *Carex humilis* – an Lehmwänden und Mergelboden. Hammersdorf" [Hammersdorf = Guşteriţa village at the north-eastern margin of the town of Sibiu]. The second sheet (no. 201833) in herbarium LW, containing specimens from the same locality, collected on 21 April 1850, is the **paralectotype**.

Detailed observations of mixed populations of the taxa *F. calthifolia* and *F. *verna* in south-eastern Poland, accompanied by karyological investigations, led to the discovery of triploid morphologically intermediate plants, which the authors of these studies (Pogan & Wcisło 1974, 1983) considered as hybrids. Our studies using other methods (Popelka et al. 2019, this study) confirm their opinion. However, because the Polish authors considered the parental taxa to be two different subspecies of *F. verna* s. l., they did not need to propose names for these hybrids. Recently, Drenckhahn (2016) also mentions finding of intermediate (triploid) plants between *F. calthifolia* and *F. verna* s. s. in a park in Würzburg in northern Bavaria, in which both the parental taxa occur. However, this author did not attempt to propose a binomic name for these plants.

Because we could not find a published binomic name for this interspecific hybrid (repeatedly formed by the hybridization of *F. calthifolia* and *F. *verna*), we formally describe it here. For this hybrid taxon, we suggest the name *F. ×sellii*, which is derived from the surname of the outstanding British expert on plant taxonomy, Peter Derek Sell (born in 1929, died in 2013), who was also interested, among many other plant groups, in the genus *Ficaria*.

Ficaria ×sellii Duchoslav, Popelka et Trávn., **nothosp. nova**
[*Ficaria calthifolia* Rchb. × *Ficaria verna* Huds. subsp. *verna*]

Holotype: Czech Republic, Moravia, distr. Prostějov, village of Plumlov, mesic grassland in the valley of the Kleštínek brook (Nature reserve Kněží hora), 1.2 km SSE of the Plumlov chateau; 290 m a.s.l.; 49°27'16"N, 17°01'19"E; 26 April 2018, leg. O. Popelka & M. Duchoslav; OL (no. 36802) (Fig. 6). – Isotypes: OL (nos 36803-36806), BRNM (nos 809856, 809857). Note: All specimens of the holotype and isotypes were estimated as triploid (2n ~ 24) using flow-cytometry.



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FLORA MORAVICA
Ficaria x *sellii* Duchoslav, Popelka et Trávn. ined.
Česká republika: 71c. Drahanské podhůří, distr. Prostějov,
Plumlov, mesic grassland in the valley of the Kleštínek brook (Nature reserve Kněží hora), 1.2 km SSE of the Plumlov chateau - 290 m s. m.
N 49° 27' 16", E 017° 01' 19" (WGS-84) Quadr.: 6568ac
det.: Popelka O., Duchoslav M. et Trávníček B.
Pozn.: HOLOTYPE! Specimen(s) was (were) estimated as triploid (2n ~ 24) by the flow-cytometry method.
Dat: 26.4.2018 leg.: Popelka O. et Duchoslav M.

Herbář katedry botaniky PřF UP Olomouc - OL
Ficaria x *sellii* Duchoslav, Popelka et Trávn. ined.
HOLOTYPE!

Fig. 6. – Holotype of *Ficaria ×sellii* deposited in OL (no. 36802).

Diagnosis

Ficaria ×*sellii* differs from the first parent, *F. calthifolia*, particularly in the presence of bulbils in the axils of lower stem leaves at the end and after flowering, an almost total absence of well-developed achenes in collective fruits and a predominance of deformed, wrinkled (not ± spherical) pollen grains in its anthers. Usually, it differs by having more elongate stems, which are often partly procumbent, with more (usually 2–3) nodes and by alternate leaves on these stems.

From the second parent, *F.* **verna*, the hybrid taxon differs especially in the usual absence of bulbils in axils of upper stem leaves, smaller bulbils in axils of lower stem leaves, usually by more crowded basal leaves and shorter stems, with fewer nodes (usually 2–3), sometimes also with opposite leaves, as well as in having even fewer (usually zero) well-developed achenes in collective fruits.

From the parental taxa, *F.* ×*sellii* is distinguishable in being triploid, and in having a very low percentage (less than 10%) of spherical, viable pollen grains in its anthers. Morphologically *F.* ×*sellii* occupies a nearly intermediate position between the parental taxa. At the onset of flowering, it more closely resembles *F. calthifolia* in having somewhat crowded leaves and short stems, whereas at the end of flowering, *F.* ×*sellii* is morphologically closer to the second parent in having long stems, which are often somewhat ascending or procumbent, with bulbils in axils of lower stem leaves. Blade notch of *F.* ×*sellii* has length of 20–25% of the total blade length whereby it differs from both *F. calthifolia* (notch length less than 20% of the blade length) and *F.* **verna* (notch length more than 27% of the blade length). Because *F.* **verna* is distinctly more variable in the growth form of its stems than *F. calthifolia*, at first sight the separation of hybrid plants from this first parental taxon is usually more difficult. Ecology, phenology and distribution of *F.* ×*sellii* are characterized in detail above.

Identification key to the central-European *Ficaria* taxa

1a Bulbils absent from leaf-axils, even after flowering; flowering stems short and ± erect, usually with one or two nodes; stem leaves densely crowded in seemingly basal rosette, sometimes large plants have an additional one or a pair of leaves on flowering stem; leaf blade of ground leaves with mild notch, which is shorter than 1/5 of the blade length; well-developed achenes per collective fruit plentiful, usually more than 10. – Most pollen grains well-developed, spherical; pollen viability (as is defined above) (67–) 78–87 (–90)% . . . . . *F. calthifolia*

1b Bulbils present in leaf-axils at the end and after flowering period; flowering stems usually elongate and ± procumbent or rarely ascending, usually with two to five nodes; stem leaves usually do not form a distinct dense rosette, but are opposite or alternate on flowering stem; leaf blade of ground leaves with deep notch, which is longer than 1/5 of the blade length; well-developed achenes per collective fruit scarce, usually less than 10 2

2a Flowering stems elongate and ± procumbent, usually with three to five nodes; leaf axillary bulbils well-developed and present in leaf axils along the entire length of stems (sometimes absent only in uppermost leaf); stem leaves ± equally distributed over the stem, usually alternate; well-developed achenes scarce, in number (1–) 2–6 (–12) per collective fruit. – More than half of pollen grains normally developed, spherical; pollen viability (44–) 55–63 (–73)% . . . . . *F. verna* subsp. *verna*

2b Flowering stems usually rather elongate and ascending or procumbent, usually with two to three nodes; leaf axillary bulbils often small and frequently absent on the upper part of stems; stem leaves sometimes crowded in a seemingly basal (somewhat diffuse) rosette; well-developed achenes usually absent, in number 0 (–3) per collective fruit. – Habit of plants intermediate between the taxa *F. calthifolia* and *F. verna* subsp. *verna*; majority of pollen grains wrinkled, flattened; pollen viability less than 10% . . . . . *F.* ×*sellii*

See www.preslia.cz for Electronic Appendices 1–3

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Souhrn

Rod orsej (*Ficaria*) je taxonomicky komplikovaná skupina jarních efemeroidů, na jejíž diverzifikaci se podílejí jak polyplodizace, tak hybridizace. Ve střední Evropě se vyskytují dva taxony rodu *Ficaria*: vzácnější diploidní *F. calthifolia* a běžně rozšířená tetraploidní *F. verna* subsp. *verna.* Ačkoliv se oba taxony liší ve svých ekologických nárocích, v teplejších územích se jejich populace mohou dostat do kontaktu. Polští autoři se v 80. letech 20. století zabývali studiem několika smíšených populací, ve kterých nalezli i triploidní jedince, o kterých se domnívali, že mohou být hybridy mezi diploidní *F. calthifolia* a tetraploidní *F. verna* subsp. *verna*. V předchozím výzkumu jsme potvrdili tuto hypotézu jak pomocí studia velikosti genomu a molekulárních markerů u triploidů a domnělých rodičů na několika lokalitách se smíšenými populacemi, tak i experimentálním křížením mezi rodiči (Popelka et al. 2019). Tento článek se zabývá studiem četnosti a výskytu triploidního hybrida na území střední Evropy a srovnává morfologickou variabilitu, fenologii, fertilitu pylu, sexuální a asexuální reprodukcí a ekologii hybrida s rodičovskými taxony. Triploidní hybrid byl nalezen na 89% lokalit, na kterých se v blízkosti vyskytovaly oba rodičovské taxony. Nebyly nalezeny žádné populace hybrida bez výskytu alespoň jednoho z rodičů. Hybrid byl morfologicky a fenologicky intermediární mezi rodiči, měl velmi nízkou fertilitu pylu (8,4%) a nevytvářel životaschopné nažky. Jeho reprodukce a lokální šíření jsou zajištěny tvorbou kořenových hlíz a především pacibulek v úžlabí listů; nicméně počet a hmotnost pacibulek produkovaných hybridem jsou nižší než je tomu u pacibulek produkovaných tetraploidní *F. verna* subsp. *verna.* Na dvou lokalitách s bohatými populacemi rodičovských taxonů a hybrida jsme provedli podrobnou studii jejich mikrostanovištních nároků. *Ficaria verna* subsp. *verna* preferovala na živiny bohatá a vlhká až mokrá, často stíněná mikrostanoviště, zatímco *F. calthifolia* preferovala mezická až subxerická, převážně osluněná mikrostanoviště. Hybrid osídloval ekologicky intermediární mikrostanoviště. Hybrid je popsán pod jménem *Ficaria* ×*sellii* Duchoslav, Popelka et Trávn. V závěru článku je uveden klíč k určení všech tří taxonů rodu, které jsou nyní známé z území střední Evropy.

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