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Reproductive biology in the genus *Ficaria*: reproductive modes, pollen viability and size, and experimental homoploid hybridization between selected taxa

Master's Thesis

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Declaration

I declare that I have written this Master's Thesis independently under the supervision of RNDr. Martin Duchoslav, Ph.D., with the use of cited literature.

25th July, Olomouc ……………………………….

signature

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Abstrakt

Rod *Ficaria* zahrnuje běžně rozšířené, jarně kvetoucí geofyty vyznačující se vysokou fenotypovou plasticitou a existencí několika ploidních úrovní (od 2x do 6x). Vysoká morfologická a genetická variabilita je patrně do značné míry způsobena následkem působení procesů hybridizace a polyploidizace, taxonomická problematika uvnitř rodu *Ficaria* tak není stále uspokojivě vyřešena. Pro pochopení fylogenetické struktury rodu *Ficaria* byla tedy zhodnocena reprodukční biologie, prezygotické a postzygotické reprodukčně izolační mechanismy pomocí studia schopnosti autonomní apomixie, autonomního autogamie, životaschopnosti pylových zrn a velikosti pylových zrn u většiny rozlišovaných taxonů a experimentální homoploidní hybridizace mezi vybranými taxony v kombinaci s odhadem velikosti genomu rodičovských taxonů a jejich hybridů, včetně stanovení dalších reprodukčních systémů u rodičovských taxonů. Autonomní apomixie, autogamie nebyla u studovaných taxonů, bez ohledu na ploidní stupeň zjištěna. Všechny studované taxony byly alogamní. Počet dobře vyvinutých, alogamicky vzniklých nažek odpovídal pylové viabilitě, pylová viabilita byla redukována u vyšších ploidních stupňů. Pylová délka rostla se zvyšující se velikostí genomu, ale byla výrazně heterogenní, nemohla být tedy využita k odhadu jednotlivých ploidních stupňů u studovaných taxonů. Abnormálně velká pylová zrna byla detekována u několika polyploidních taxonů. Prezygotické bariéry, jako autonomní apomixie a autogamie, viabilita tedy dohromady nepřispívají k reprodukční izolaci studovaných taxonů. Proto, následná mezitaxonová kompatibilita umožňuje snadnou obousměrnou homoploidní hybridizaci mezi vybranými taxony rodu *Ficaria* v kontrolovaných podmínkách.

Klíčová slova: Alogamie, Autonomní apomixie, Autonomní autogamie, Hybridizační experiment, Prezygotické reprodukčně izolační bariéry, Postzygotické reprodukčně izolační bariéry

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Abstract

Ficaria is a polyploid complex with high phenotypic diversity and the existence of several ploidy levels (from 2x to 6x). Both hybridization and polyploidization probably can be a major source of morphological and genetic variation that have given the taxonomic uncertainties in the genus *Ficaria*. Despite this taxonomic complexity, the phylogeny and taxonomy of the genus *Ficaria*, up to now remains poorly understood. Quantitative and qualitative studies of autonomous apomixis, autonomous selfing, pollen viability, and pollen length of most taxa/ploidy levels, and experimental homoploid crosses between the selected taxa with assessment of other reproductive modes (autonomous apomixis, autonomous selfing, outcrossing), and inference of the paternity via the estimation of the genome size of parental taxa and their hybrids were employed in evaluating of the reproductive biology, prezygotic and postzygotic reproductive isolation barriers within the genus *Ficaria.* Autonomous apomixis and autonomous selfing were absent in the studied *Ficaria* taxa regardless of ploidy level. All investigated taxa were allogamous. Number of well-developed achenes formed by outcrossing corresponded to pollen viability, pollen viability was reduced in high ploidy levels. Pollen length was increasing with genome size, but the pollen length was heterogenous, so that was not suitaible for the estimation of ploidy level of the studied taxa. Abnormally large pollen was detected in several polyploid taxa. Assemblages of the lack of autonomous apomixis and autonomous selfing and high pollen viability do not act as a prezygotic barrier to prevent mating between *Ficaria* taxa. Therefore, subsequent intertaxa compatibility allowed easy reciprocal asymmetric homoploid hybridization between selected taxa in the genus *Ficaria* in controlled conditions.

Key words: Autonomous apomixis, Autonomous selfing, Crossing experiment, Outcrossing, Postzygotic reproductive barriers, Prezygotic reproductive barriers

Number of pages: 93 **Number of appendices:** 12

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Contects

1. Introduction

Hybridization is considered as a major driver of evolution and speciation in vascular plants (Ramsey & Schemske 1998; Mallet 2007; Soltis & Soltis 2009). A recent study by Whitney et al. (2010) estimates that hybridization occurs in 40 % of families and 16 % of genera of vascular plants. Nevertheless, approximations of the occurrence of hybridization vary among authors depending on the methodology applied (Folk et al. 2018). Moreover, the estimates likely remain underestimated due to the generally challenging detection of hybrids (Mallet 2007; Whitney et al. 2010; Abbott et al. 2013). However, the actual occurrence of hybridization is unevenly distributed across different taxonomic groups (Ellstrand et al. 1996; Whitney et al. 2010; Abbott 2017). In general, the frequency of hybridization is considerably higher in taxa from evolutionary lineages characterized by perennial habits, longevity, clonal reproduction, outcrossing, selfing (favouring the persistence of once-formed hybrids), and less variable within-genus genome sizes (increasing the potential of hybridization, Ellstrand et al. 1996; Rieseberg 1997; Mallet 2007; Brys et al. 2016; Mitchell et al. 2019).

Hybridization has both positive and negative evolutionary consequences (e.g., Rieseberg 1997; Barton 2001; Abbott et al. 2013). Hybridization between taxa with a high degree of genome difference may contribute to the strengthen of reproductive barriers of hybridizing parental taxa (Paun et al. 2009), but in the case of closely related taxa, the hybridization may generate novel genotypic and phenotypic diversity that may result in speciation (Rieseberg 1997; Soltis & Soltis 2009; Abbott et al. 2013; Nieto Feliner et al. 2017). Backcrossing of the hybrid with one parental taxon (Rieseberg & Willis 2007) could lead to transfer of beneficial alleles between different taxa. Moreover, a hybrid by transgression, i.e., the formation of extreme phenotypes (Rieseberg & Ellstrand 1993; Rieseberg 1997; Seehausen 2004; Soltis & Soltis 2009; Abbott et al. 2013) could exhibit novel functional traits. These traits provide an elevated adaptive potential of hybrids (Barton 2001; Abbott et al. 2013; Soltis 2013) in the first filiar generation (F1) (Rieseberg & Ellstrand 1993) and especially in subsequent filiar generations (Abbott et al. 2013). Increased adaptive potential is usually reflected by the ability of hybrids to colonize of ecological niches not occupied by the parental taxa (Seehausen 2004), as hybrid phenotypes are a mosaic parent-like, and novel trait rather than intermediate ones in the subsequent generations

(Rieseberg & Ellstrand 1993, Rieseberg 1995; Rieseberg et al. 1999; Mallet 2005, Abbott et al. 2013). However, if partially fertile hybrid without morphological, phenological, or ecological differentiation to parents occurrs in the parental habitat, recurrent hybridization and introgression also can contribute to breakdown of the genetic integrity of parental taxa (Rhymer & Simberloff 1996; Otto & Whitton 2000; Mallet 2007; Brennan et al. 2014; Abbott 2017). The rapid breakdown of the genetic integrity prevalent between closely related taxa (reflected by reduced sterility of hybrids). Breakdown of the genetic integrity of parental taxa is reflected by the local complex hybrid swarms of primary contact between sympatric taxa (Rhymer & Simberloff 1996) and by the hybrid zones of secondary contact between allopatric taxa (Barton & Hewitt 1985; Rieseberg et al. 1999; Abbott 2017). The formation of hybrid zones results in the morphological and genetic continuum between parental taxa (Otto & Whitton 2000; Mallet 2007; Macková et al. 2017). The competition for abiotic and biotic resources may eventually lead to extinction, i.e., demographic exclusion of parental taxa by hybrids (Rhymer & Simberloff 1996; Bleeker et al. 2007; Todesco et al. 2016; Abbott 2017).

Therefore, to maintain the integrity of the different taxa, reproductive isolation mechanisms have evolved. These reproductive isolation mechanisms can be distinguished into two main categories, based on the developmental stage in which they appear: (a) prezygotic (before fertilization of the egg cell) and (b) postzygotic (after fertilization of the egg cell). Prezygotic reproductive isolation mechanisms include ecological temporal/spatial isolation, i.e., pollinator specificity, different flowering phenology, ecogeographical differentiation (e.g., Rieseberg & Carney 1998; Lowry et al. 2008; Abbott et al. 2013; Vallejo-Marín & Hiscock 2016), and reproductive isolation, i.e, the prevalence of prior selfing or mentor effect (Brys et al. 2016), and autonomous apomixis (e.g., Petit et al. 1999). Postzygotic reproductive isolation mechanisms include reproductive barriers that can be further classified into: (a) extrinsic, i.e., environment-dependent barriers such as ecological low viability of hybrids, minority cytotype exclusion and (b) intrinsic, i.e. environment-independent barriers such as low viability of hybrids, their sterility, reduction or loss of pollen viability, endosperm failure (Rieseberg & Carney 1998; Rieseberg et al. 1999; Lowry et al. 2008; Abbott et al. 2013; Lafon-Placette & Köhler 2016; Vallejo-Marín & Hiscock 2016). However, the above mentioned prezygotic and external postzygotic reproductive isolation mechanisms might be overcome by natural or human-mediated disturbances (Rieseberg et al. 1999; Ellstrand & Schierenbeck 2000; Orians 2000; Todesco et al. 2016; Vallejo-Marín & Hiscock 2016). Hence, the effectiveness of prezygotic and postzygotic barriers may be different among hybridizing taxa (Vallejo-Marín & Hiscock 2016). Effective reproductive barriers to reduce gene flow between taxa are required for the existence of a separate, genetically delimited taxon (Rieseberg & Willis 2007). Consequently, homoploid hybrids that can be recognized as evolutionarily separated taxa have developed ecogeographical differentiation (Abbott et al. 2010), different asexual reproduction (clonal growth, apomixis), and sexual ones (autonomous selfing). These changes reduce the breakdown of the genetic integrity of parental taxa by introgressive hybridization (Rhymer & Simberloff 1996; Rieseberg et al. 1999). Changes in reproductive modes are often associated with polyploidization resulting in evolutionary more stable hybrids (Otto & Whitton 2000; Mallet 2007; Rieseberg & Willis 2007; Siopa et al. 2020). Multiplication of complete chromosome sets can usually reduce between ploidy mating ("triploid block", Ramsey & Schemske 1998), inbreeding depression (Siopa et al. 2020), manifestation of recessive (often harmful) alleles, maintain high level of fixed heterozygosity in allopolyploids, and cause changes in gene expression, subsequently leading to changes of ecological niche (niche expansion, niche shift) via increasing of genetic variability (Ramsey & Schemske 2002; Otto & Whitton 2000; Adams & Wendel 2005; Jackson & Chen 2010; Soltis et al. 2016).

Hybridization accompanied by polyploidization probably also contributed to the taxonomic complexity of a seemingly negligible polyploid complex of the genus *Ficaria* of the family Ranunculaceae Juss. (Zonneveld 2015; Drenckhahn 2016). The occurrence of polyploidization and hybridization is inferred from the existence of individuals with an intermediate phenotype (Marsden-Jones & Turrill 1952; Towpasz 1971; Gill et al. 1972; Sell 1994; Kästner & Fischer 2006; Drenckhahn 2016; Popelka et al. 2019b). Despite this, experimental and molecular studies that would confirm the impact of occurrence of the polyploidization and hybridization on the taxonomic complexity of the genus *Ficaria* are scarce (Popelka et al. 2019a; Sochor unpubl.). The genus *Ficaria* comprises widespread spring-flowering geophytes that commonly occupy predominantly wet and moist habitats (Post et al. 2009). The genus *Ficaria* is distributed throughout most parts of Europe and adjacent areas of Asia and Africa (Taylor & Markham 1978; Tutin & Cook 1993; Sell 1994; Veldkamp 2015)

Table 1: Summary of distinguished taxa within the genus *Ficaria* (sensu Veldkamp 2015), their distribution, ecology, ploidy levels according to the literature and present records. * Alternativelly, all these subspecies might be considered as species (see Zonneveld 2015)

Taxon*	Distribution	Ecology	Ploidy	References
Ficaria verna subsp. *verna*	Europe (except Mediterranean), secondarily Canada, USA and New Zeeland	moist deciduous forests, ravine forests, moist meadows and scrubs	3x, 4x, 5x, 6x	Anders-Gasser 1985, Sell 1994, Křísa in Hejný & Slavík 1988, Post et al. 2009, Veldkamp 2015, Zonneveld 2015
Ficaria verna subsp. *calthifolia*	Central, south-eastern Europe, southern Ukraine, Russia, Transcaucasia, secondarily the USA and New Zealand	meadows, dry hillside, bright forests	2x	Sell 1994, Post et al. 2009, Veldkamp 2015
Ficaria verna subsp. *fertiis*	western, southwestern Europe, secondarily the USA	moist deciduous forests, edges of banks and streams	2x	López Gonzáles 1986, Sell 1994, Post et al. 2009, Veldkamp 2015, Zonneveld 2015
Ficaria verna subsp. *ficariiformis*	Mediterranean, secondarily Great Britain, USA, New Zealand	waterlogged deciduous forests on mineral-rich soils, river edges, sandy substrates	4x, 5x, 6x	Sell 1994, Post et al. 2009, Stace 2010, Veldkamp 2015
Ficaria verna subsp. *chrysocephala*	Western Mediterranean, secondarily the USA	?	4x	Sell 1994, Post et al. 2009, Veldkamp 2015, Zonneveld 2015
Ficaria verna subsp. *ficarioides*	Greece (especially Karpathos, Kasos), southern Turkey (Anti-Taurus Mountains, Cilicia), Caucasus	mountains	2x	Veldkamp 2015, Zonneveld 2015
Ficaria verna subsp. *kochii*	Caucasus and southern Turkey (Anatolia), Iraq, Iran	mountains	4x	Veldkamp 2015, Zonneveld 2015

but its members have been introduced to the North America (Post et al. 2009; Axtell et al. 2010), and New Zealand (Webb et al. 1995; Howell 2008). Within the genus, only one species, *Ranunculus Ficaria* L., in the broad sense has been originally considered (Sell 1994). Based on its considerable morphological variability and the existence of several ploidy levels, many taxa with unclear taxonomic values have been described later (e.g., Allen 1958; Löve & Löve 1961; Clapham et al. 1962; Tutin & Cook 1993; Hess et al. 1997). Moreover, many taxa have probably been described repeatedly at various taxonomic levels from different parts of Europe, leading to a substantial nomenclatural confusion (Veldkamp 2015).

Recently, seven subspecies of the species *Ficaria verna* Huds (sensu Veldkamp 2015) are recognized (Table 1), but an alternative approach suggests that these subspecies might be considered at the species level (Zonneveld 2015). In total, five ploidy levels have been recorded so far (Popelka unpubl.). Althought, only few studies have adressesd the ploidy level structure in populations and distribution of each ploidy level of *Ficaria* taxa, one ploidy level is usually recognized for each single taxon (Table 1). More common are diploids (2n=2x=16, based on x=8; Gill et al. 1972; Pogan & Wcisło 1974; Sell 1994; Zonneveld 2015; Konečná 2018; Popelka unpubl.) with the possible presence from one to seven (exceptionally eight) B chromosomes (Larter 1932; Marsden-Jones & Turrill 1952; Gill et al. 1972; Marchant & Brighton 1974; Pogan & Wcisło 1981b; Sell 1994), and tetraploids (2n=4x=32, based on x=8; Pogan & Wcisło 1974; Sell 1994; Zonneveld 2015; Konečná 2018; Popelka unpubl.). In contrary, triploids (2n=3x=24, based on x=8), pentaploids (2n=5x=40, based on x=8), and hexaploids (2n=6x=48, based on x=8) are the minority cytotypes (Neves 1942; Soó & Borhidi 1964; Pogan & Wcisło 1974; Tröhler 1976; Anders-Gasser 1985; Sell 1994; Zonneveld 2015; Drenckhahn et al. 2017; Konečná 2018; Popelka unpubl.).

Mixed populations comprising more cytotypes/taxa and populations comprising single, minority cytotype found to be extremely rare. Coexistence of the following ploidy levels/taxa were reported so far: triploids of *F. ×sellii* with diploids of *F. verna* subsp. *calthifolia* and tetraploids of *F. verna* subsp. *verna* (Pogan & Wcisło 1974, 1986; Popelka et al. 2019a, b); diploids of *F. verna* subsp. *fertilis* with tetraploids of *F. verna* subsp. *verna* (Marsden-Jones & Turrill 1952; Gill et al. 1972; Popelka unpubl.) tetraploids of *F. verna* subsp. *verna* with tetraploids of *F. verna* subsp. *ficariiformis* (Popelka unpubl.); triploids, tetraploids, and pentaploids of *F. verna* subsp. *verna* (Tröhler 1976; Anders-Gasser 1985). Populations containing single, minority cytotype were recorded just for triploids of *F. ×sellii* (Popelka unpubl.). These mixed populations provide evidence for the polyploid establishment, inter-taxa/ploidy coexistence and the potential of subsequent ecological segregation such as *F. ×sellii* (Popelka et al. 2019a).

All *Ficaria* taxa that have been studied so far reproduce vegetatively by the fragmentation of below-ground tubers (Marsden-Jones 1933), in the case of tetraploids *F. verna* subsp. *verna* and *F. verna* subsp. *ficariiformis* additionally also by axillary bulbils (Sell 1994), and reproduce sexually through production of seeds, although in polyploids success of sexual reproduction is (substantially) reduced (Marsden-Jones 1933; Gill et al. 1972; Wcisło & Pogan 1981; but see Popelka et al. 2019a). In addition to these reproductive modes, the results reported by Metcalfe (1939) suggest the minor occurrence of autonomous selfing in diploids *F. verna* subsp. *fertilis* and in tetraploids *F. verna* subsp. *verna*, autonomous apomixis in diploids *F. verna* subsp. *fertilis* and pseudogamy or autonomous selfing in *F. verna* subsp. *fertilis* and *F. verna* subsp. *verna* (Metcalfe 1939). Unfortunately, the germination of such seeds was not investigated (Metcalfe 1939). In contrast to Metcalfe (1939), the occurrence of autonomous selfing (Pogan & Wcisło 1981a) and autonomous apomixis (Popelka et al. 2019a) was not later recorded for diploids of *F. verna* subsp. *calthifolia* and tetraploids of *F. verna* subsp. *verna* (Pogan & Wcisło 1981a; Popelka et al. 2019a). Experimental crosses (Popelka et al. 2019a) and the study of genetic (Pogan & Wcisło 1974, 1983, 1986; Popelka et al. 2019a) and morphological variability (Towpasz 1971; Kästner & Fischer 2006; Drenckhahn 2016; Popelka et al. 2019b) have revealed recent heteroploid, reciprocal, asymmetric hybridization between *F. verna* subsp. *verna* ($2n = 4x = 32$) and *F. verna* subsp. *calthifolia* ($2n = 2x = 16$), resulting in triploid, morphologicaly intermediate hybrids ($2n = 3x = 24$), beeing mostly sterile and persisting by vegetative propagation (Popelka et al. 2019a, 2019b). Early studies have found that the occurrence of heteroploid hybridization between *F. verna* subsp. *verna* ($2n = 4x = 32$) and *F. verna* subsp. *fertilis* ($2n = 2x = 16$) could not be also excluded ($2n = 3x = 24$; Marsden-Jones & Turrill 1952; Gill et al. 1972). On the contrary to heteroploid hybridization, homoploid hybridization has not been so far performed, although a polyphyletic origin of some recent polyploid taxa via homoploid hybridization and subsequent polyploidization has been hypothesised (e.g. origin of *F. verna* subsp. *verna*, see below).

Existence of strong phenotypic plasticity (Post et al. 2009; Uhlířová 2019), shared chloroplast haplotypes between individuals of different taxa (Sochor unpubl.), and the occurrence of several ploidy levels (from 2x to 6x; e.g., Soó & Borhidi 1964; Anders-Gasser 1985; Sell 1994 Zonneveld 2015; Drenckhahn et al. 2017) with high variability within-cytotype genome sizes in diploids *F. verna* subsp. *calthifolia* and tetraploids *F. verna* subsp. *verna* (Konečná 2018), suggest a possible role of homoploid hybridization followed by subsequent introgression or polyploidization in the genus *Ficaria*. Evaluation of the phylogenetic complexity of the polyploid complex of the genus *Ficaria* based on a cytogenetic approach (assessment of absolute genome size and DNA ploidy level) was first drawn by Zonneveld (2015). A widely distributed, tetraploid, bulbils-producing *F. verna* subsp. *verna* is supposed to be of allotetraploid origin, resulting from homoploid hybridization between the diploid taxa of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis*, followed by polyploidization (Zonneveld 2015). However, further study of the genome size variability in tetraploid *F. verna* subsp. *verna* on a broad geographical scale revealed the existence of genome-size delimited lineages/populations, i.e., western and eastern ones (Drenckhahn et al. 2017). Therefore, Drenckhahn et al. (2017) concluded that the tetraploid of *F. verna* subsp. *verna* contains two different taxa with divergent origin. However, phylogenetic origin and spatial distribution of *F. verna* subsp. *verna* is unknown. Moreover, Popelka (unpubl.) suggests a minor occurrence of diploid plants morphologically similar to the tetraploid cytotype of *F. verna* subsp. *verna*, considered as diploid of *F. verna* subsp. *verna* in the present study.

In addition, there have not been published any studies about the origin of other polyploid taxa in the genus, including *F. verna* subsp. *chrysocephala* and *F. verna* subsp. *ficariiformis*. Therefore, the origin and evolutionary role of homo- and heteroploid hybridization and associated introgressions within the polyploid, taxonomically complicated complex of the genus *Ficaria* are still not clear. Despite of various karyological (e.g., Pogan & Wcisło 1974; 1981a, 1981b, 1986; Trinajstić 1979; Zonneveld 2015; Drenckhahn et al. 2017; Konečná 2018; Popelka et al. 2019a; Sochor unpubl.), morphological (e.g., Veselá 1969; Marchant & Brighton 1974; Tröhler 1976; Taylor &Markham 1978; Trinajstić 1979; Sell 1994; Post et al. 2009; Veldkamp 2015; Drenckhahn 2016; Vazquez 2016; Popelka et al. 2019b; Uhlířová 2019), and ecological (e.g., Marsden-Jones 1933;

Metcalfe 1938; Marchant & Brighton 1974; Nicholson 1983; Popelka et al. 2019b) studies, no comprehensive molecular phylogenetic studies, crossing experiments, reproductive modes investigation, pollen viability & size, in most taxa have been performed yet.

To provide insight to the potential hybridization and possibly come up with taxonomic implication, the intra-cytotype compatibility, direction of crosses and reproductive output as assessment of postzygotic reproductive barriers are investigated in the selected taxa. Reproductive modes, pollen viability & size, and correlation between pollen viability and reproductive percentage of well-developed achenes, and correlation between pollen size and genome size as assessment of the prezygotic reproductive barriers are elucidated in most *Ficaria* taxa/ploidy levels. Such approach as a useful tool to reveal prezygotic and postzygotic reproductive isolation barriers was also applied in other polyploid complexes (e. g., *Hieracium* s. str., Mráz & Paule 2006; *Cyanus* Mill., Olšavská & Löser 2013). Variation of reproductive modes, pollen viability & size is compared between taxa/ploidy levels.

2. Objectives of thesis

The evolutionary relationships among the taxa of the genus *Ficaria* are yet unresolved, owing to the occurrence of polyploidization and hybridization. Therefore, the present study investigates the reproductive modes and pollen viability & size of most *Ficaria* taxa. Furthermore, this study evaluates the postzygotic barriers between three diploid taxa and within groups of populations from different parts of the distribution range of one tetraploid taxon. At least, this study examines the possibility of recurrent polyploidization via one step model or triploid bridge in the genus *Ficaria*. The following questions were adressed:

1. What is the diversity of reproductive modes in the studied taxa? Does the pattern of reproductive modes relate to the ploidy level of the taxon? Does the percentage of well-developed achenes (seeds) per collective fruit of the studied taxa relate to their pollen viabilities?
2. What is the variability of pollen viability in the studied taxa? Does the pollen viability relate to hybrid or polyploid origins?
3. What is the variability of pollen length in the studied taxa? Do the patterns of pollen lengths relate to the genome size of the ploidy level of the taxa? Are there any differences in pollen lengths suggesting the production of viable "gigas" (unreduced) pollens in the studied taxa?
4. Do homoploid crosses and intrataxa outcroses between/within the diploid cytotypes of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and diploid plants morphologically similar to the tetraploid cytotype of *F. verna* subsp. *verna* and between/within eastern and western populations of the tetraploid cytotype of *F. verna* subsp. *verna*, result in production of viable seeds? If so, what is the genome size and morphology of hybrids?

3. Materials and methods

3.1 Sampling of plant material

Plants were provided by members of the research *Ficaria* team from natural populations covering the entire area of distribution of the studied taxa in Europe (sensu Veldkamp 2015) between 2011 – 2019 (Appendix 1). Within each population, the sampled plants were spaced at least two metres apart to minimize the collection of clones. The collected plants were transported to the outdoor conditions of the common garden of the Department of Botany, Faculty of Science, Palacký University in Olomouc, and cultivated individually in plastic pots (8 x 8 x 8 cm) filled with a mixture of commercial substrate and natural soil substrate in the proportion of 3:1. Pots were immersed into the soil to limit the drying of plants. In long dry periods, the plants were occasionally watered. Plants were shaded by light shade fabric (relative irradiation 70 %) during the whole growing period to simulate natural conditions.

Each plant was assigned to a specific taxon (see Table 1) by available keys (especially according to Sell 1994; Veldkamp 2015). The ploidy levels of individual plants were derived based on measurements of the genome sizes of the same individuals as previously used for counting the chromosomes (Popelka et al. 2019a; Kobrlová unpubl.). The DNA ploidy of plants that were not previously included to counting of chromosomes was assessed based on the genome size estimated by ML CyFlow (Partec GmbH, Münster) equipped with a green laser (532 nm, 100 mW, Cobolt Samba; Cobolt AB, Stockholm, Sweden, Popelka et al. 2019a; Kobrlová unpubl.).

The maps visualising the distribution of populations of *Ficaria* taxa used for estimation of reproductive modes, pollen viability & size in the present study were created in R software version 3.5.2 (RStudio Team 2020) using the packages "tidyverse", "rnaturalearth", "rnaturalearthdata", "sf", "rgeos", "ggspatial".

3.2 Flow cytometry

The genome sizes were estimated for the offspring and parental taxa used in the crossing experiments. Samples were prepared according to a simplified protocol of Doležel et al. (2007). Fresh leaves of the sample (~0.5 cm^2) and an appropriate volume of the internal standard (*Secale cereale* L. 'Daňkovské' 2C = 16.19 pg, for tetraploid individuals, *Pisum sativum* L. 'Ctirad' 2C = 9.09 pg for diploid individuals, Doležel et al. 1998) were chopped together using a sharp razor blade in a Petri dish containing 1 ml of ice-cold LB01 isolation buffer (Doležel et al. 2007). The suspension was filtered through a 42-µm nylon mesh into a tube. Then, 50 µl of RNA-sy (50 µg-ml^{-1}) was added to prevent RNA staining, and the nuclei suspension was stained with 50 µl of fluorochrome PI (propidium iodide, 50 µg-ml^{-1}) and vortexed briefly. The relative fluorescence intensity of the PI staining was recorded for 5000 nuclei of each sample. The estimated genome size of the sample was determined on a linear scale of the graphical output based on the ratios of the distances of the peaks of the standard and the sample in the G1 phase. The resulting genome size of a given plant is derived from a single measurement.

3.3 Reproductive modes

The following reproductive modes were tested in the studied *Ficaria* taxa: autonomous apomixis, autonomous selfing, and outcrossing. In total, 180 plants from 64 populations were examined (Fig. 1).

The reproductive modes of the studied taxa were determined by using pollen exclusion bags (Kearns & Inouye 1993). Before flowering, the flowers used for testing

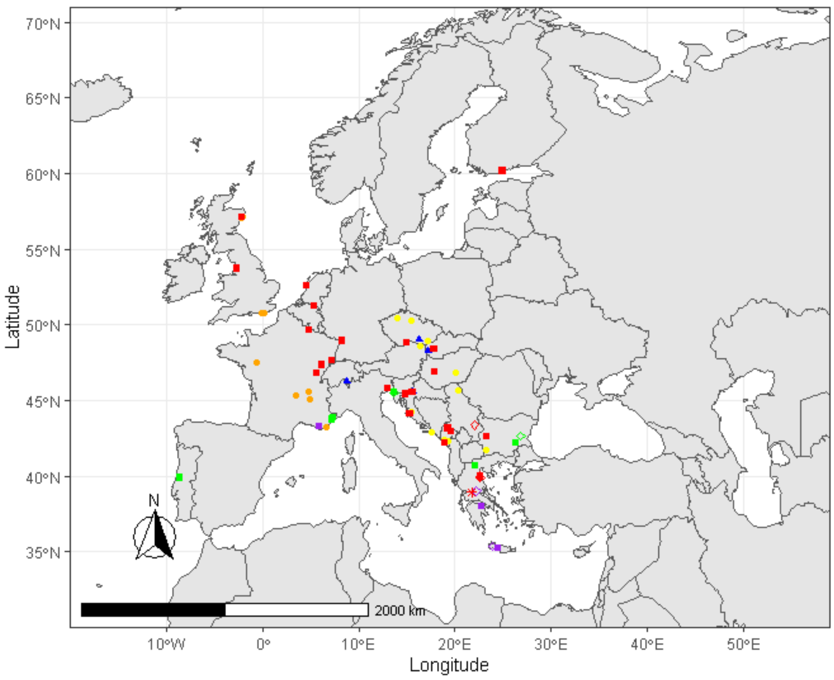

Figure 1: Distribution of populations of *Ficaria* taxa used to reproductive modes investigation in the present study. **yellow circle** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **orange circle** the diploid cytotype of *F. verna* subsp. *fertilis*, **green square** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **green diamond** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **purple square** the tetraploid cytotype of *F. verna* subsp. *chrysocephala*, **purple diamond** the pentaploid cytotype of *F. verna* subsp. *chrysocephala*, **blue triangle** the triploid cytotype of *F.* ×*sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **red square** the tetraploid cytotype of *F. verna* subsp. *verna*, **red diamond** the pentaploid cytotype of *F. verna* subsp. *verna*, **red star** the hexaploid cytotype of *F. verna* subsp. *verna*.

of autonomous apomixis were emasculated and wrapped with non-woven synthetic textile for pollinator exclusion. Flowers used for testing of autonomous selfing were not emasculated and wrapped in non-woven synthetic textile bags for pollinator exclusion.

The ability of intrataxa (interpopulation) outcrossing was analysed for taxa used in the crossing experiment, realised in two years. Specifically, flowers used for testing of intrataxa (interpopulation) outcrossing were emasculated, and flowers were pollinated using the fresh pollen of plants from different populations of the same taxon in three consecutive days. Paternal plants from three different populations were crossed in all possible combinations with three maternal plants within respective population. Individuals used for the study of intrataxa (interpopulation) outcrossing were also involved in the crossing experiment (see chapter 3.5). The bags were kept until maturity of achenes to prevent achene loss. Achenes were harvested month after flowering.

Ripening achenes were harvested and stored in paper bags at room temperature for four months. After this period, the achenes were classified as mature (well-developed achenes) or aborted (wrinkled and small achenes). The reproductive success (%) was calculated as the number of well-developed achenes/total number of produced achenes*100. Aborted achenes were excluded from further analysis.

3.4 Pollen viability & length

The pollen viability of 360 plants from 145 populations were examined (Fig. 3), the pollen length of well-developed pollen was analysed on the subset of individuals used for the study of pollen viability; in total 335 plants from 139 populations were examined (Fig. 3).

Mature anthers on the onset of anther dehiscence were removed from a flower per individual early in the morning. Fresh pollen grains were released from those anthers onto the slide into a drop of a solution of fluorescein diacetate ($\sim 10^{6}$ M in sucrose, Heslop-Harrison & Heslop-Harrison 1970). The suspension was homogenized and incubated at room temperature for five minutes. Subsequently, the suspension was covered by a glass coverslip and observed under a fluorescence microscope at 100× magnification (Olympus Bx60, Olympus Optical Co. (Europa) GmbH) and images taken by Quick PHOTO CAMERA 3 software (Fig. 2, Appendix 4)

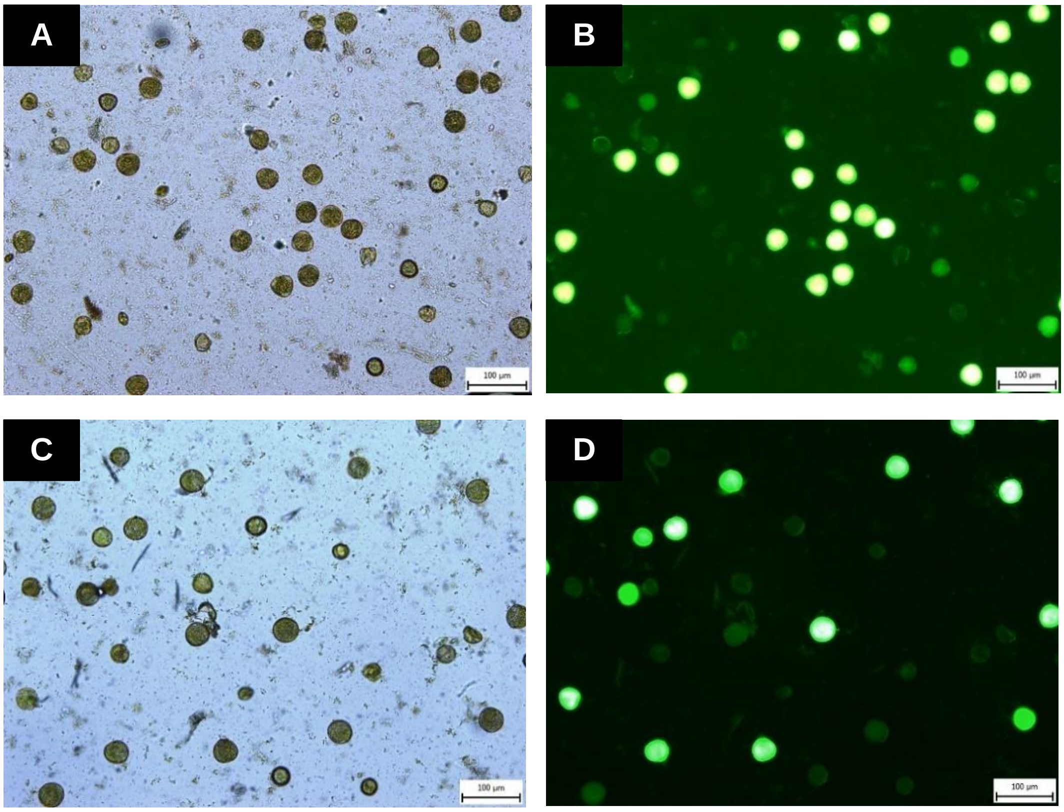

Figure 2: Example of graphical output from a microscope Olympus BX60: observing of pollen viability &length, pollen viability is estimated by fluorescein diacetate, **A, B** *F. verna* subsp. *calthifolia*, **C, D** *F. verna* subsp. *verna*, **A, B** all pollen grains**, B, D** viable, fluorescently detected pollen grains.

from ten microscopic areas were used for estimation of pollen viability & length. The pollens that accumulated free fluorescein were considered viable, unstained pollen grains were considered inviable (Heslop-Harrison & Heslop-Harrison 1970). At least 300 pollens per each plant for estimation of the pollen viability (%) were counted. The well-developed viable pollen grains are almost spherical in *Ficaria*; therefore, their length was measured as the diameter of the circle. At least 100 pollen lengths per each plant were measured. The lengths of aborted pollen grains were not measured. The measurements were performed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016.). Data were analysed in R software using the package "lme4" for Linear mixed models (Bates et al. 2018), "multcomp" for multiple comparisons after Type-III analysis of variance (Hothorn et al. 2016), "gg2plot" for histograms. Pollen lengths were visualised using histograms, bin widths were estimated according to Sturge's Rule. Differences in pollen viability & length among different taxa/ploidy levels were tested using Linear mixed models with the effect of the population nested within the fixed effect of the taxon/ploidy level and followed by

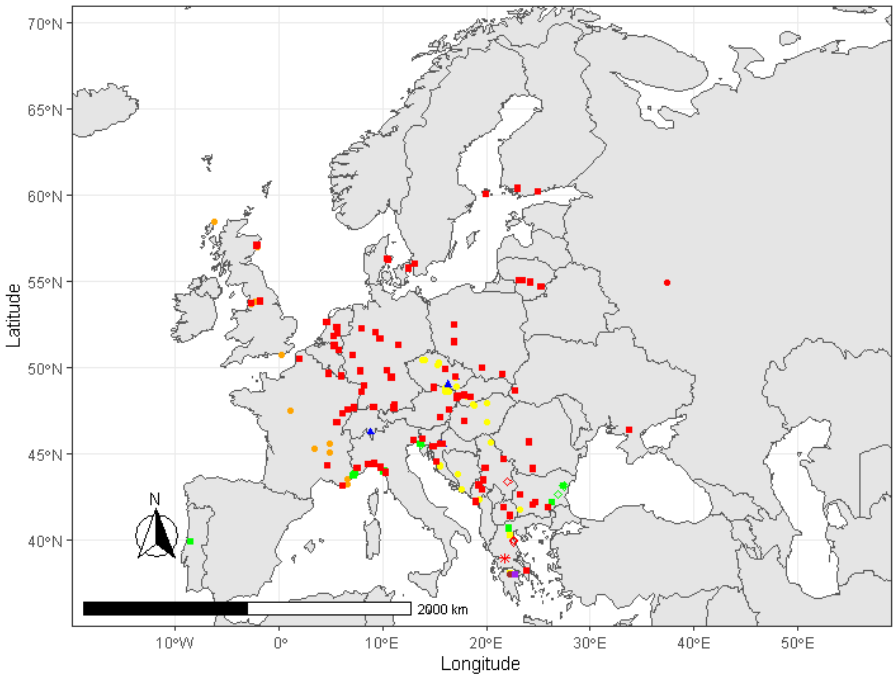

Figure 3: Distribution of populations of *Ficaria* taxa used to examination of pollen viability &length in the present study. **yellow circle** the diploid cytotype of *F. verna* subsp. *calthifolia*, **yellow square** the tetraploid cytotype of *F. verna* subsp. *calthifolia***, orange circle** the diploid cytotype of *F. verna* subsp. *fertilis*, **green square** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **green diamond** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **brown circle**, the diploid cytotype of *F. verna* subsp. *ficaroides* , **purple square** the tetraploid cytotype of *F. verna* subsp. *chrysocephala*, **blue triangle** the triploid cytotype of *F.* ×*sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **red circle** the diploid cytotype of *F. verna* subsp. *verna*, **red square** the tetraploid cytotype of *F. verna* subsp. *verna*, **red diamond** the pentaploid cytotype of *F. verna* subsp. *verna*, **red star** the hexaploid cytotype of *F. verna* subsp. *verna*.

post hoc comparisons using Tukey-Kramer Multiple Comparison test. A parametric bootstrap was used for calculations of p-value. Correlations between pollen lengths and genome sizes, between percentage of well-developed achenes (seeds) per collective fruit by spontaneous xenogamy and pollen viability, between numbers of well-developed achenes by spontaneous xenogamy and pollen viability, between longitude and pollen viability, between latitude and pollen viability were analysed using Pearson linear correlations. Genome sizes and reproductive outputs (percentage of well-developed achenes (seeds) per collective fruit by spontaneous xenogamy were adopted for the subset of plants as were previously measured by Konečná (2018) and by Uhlířová (unpubl.), respectively.

3.5 Crossing experiments

In March 2019, 30 mature individuals were selected from three populations of *F. verna* subsp. *calthifolia* (from Bulgaria, Czech Republic, Montenegro), three populations of *F. verna* subsp. *fertilis* (from Great Britain [two populations], France). In March 2020, another 90 individuals were selected from six populations of *F. verna* subsp. *calthifolia* (from Austria, Bosnia and Herzegovina, Czech Republic [two populations], Hungary, Montenegro), three populations of *F. verna* subsp. *fertilis* (from Great Britain [two populations], France), population of the diploid cytotype *F. verna* subsp. *verna* (individual from Russia), six populations from the western part of the distribution range of *F. verna* subsp. *verna* (from France [five populations], borderline between France and Italy [population]) and six populations from the eastern part of the distribution range of *F. verna* subsp. *verna* (from Montenegro [four populations], Croatia [two populations]). Samples of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* consisted of individuals covering geographical variability and variability in genome sizes. Samples of *F. verna* subsp. *verna* consisted of individuals from the western part of the distribution range (represented by populations from France) and from the eastern part of the distribution range (represented by populations from the Balkans, Montenegro, and Croatia). The DNA ploidy levels of all experimental plants were assessed using flow cytometry, as described above.

Three plants from each population (one flower per treatment per individual) represented the acceptors of pollen (= maternal plants) and two plants from each population were used as a donor of pollen (= paternal plants). The maternal and paternal plants were grown separately in outdoor conditions of the common garden. Plants were regularly watered and partly shaded (relative irradiation 70 %) to simulate optimal growing conditions in field. Before flowering, plants were isolated from pollinators using pollinator exclusion cages covered with a layer of fine mesh fabric. In addition, all manipulated flowers were wrapped with non-woven textile bags (Kearns & Inouye 1993) to prevent contaminated pollination within the cage. The bags were kept until maturity of achenes to prevent achenes loss. In total, four types of treatments were performed: (a) autonomous apomixis, flowers were emasculated and left unpollinated (control flowers), (b) autonomous selfing, flowers were not emasculated (autogamy), (c) intrataxa (interpopulation) outcrossing, flowers were emasculated, flowers were pollinated using the fresh pollen of plants from different populations of the same taxon (xenogamy) and (d) intertaxa, homoploid crossing, flowers were emasculated, and pollinated with fresh pollen from flowers of the other taxon (homoploid crossing). At flowering, the receptive styles of the maternal plant were, in the case of intrataxa outcrossing and intertaxa homoploid crossing, gently brushed against the anthers of the three paternal plants, once every day for three consecutive days. Paternal plants from three different populations were crossed in all possible combinations with three maternal plants within the respective population. Achenes were harvested about month after flowering.

Ripening achenes were harvested and stored in paper bags at room temperature for four months. After this period, the achenes were classified as mature (well-developed achenes) or aborted (wrinkled and small achenes). All obtained well-developed achenes (seeds) were sown in autumn of a given year in pots (two achenes per one pot, 0.5 cm below the soil surface) filled with a mixture of commercial and natural soil substrates in the proportion of 1: 1 and placed in the outdoor conditions of the common garden. The following parameters were recorded during the next two seasons after sowing: germination rate of mature well-developed achenes (seeds) per ploidy level/taxon (%), pollen viability of seedlings (%), and percentage of well-developed achenes (seeds) per collective fruit (seeds) in seedlings derived by spontaneous xenogamy. A total 317 seedlings were transplanted.

Data were analysed in R. Differences in the percentage of well-developed achenes (seeds) per collective fruit, germination rate (%) across the pollination treatments ×taxa/ploidy level were tested using One-way ANOVA.

4. Results

4.1 Reproductive biology of *Ficaria* taxa as assesment of prezygotic reproductive barriers

4.1.1 Autonomous apomixis and autonomous selfing

Presence of autonomous apomixis in the emasculated flowers of the studied taxa was excluded, as no well-developed achenes were recorded in any collective fruit of experimentally treated plants. Rate of well-developed achenes formed by autonomous selfing was extremely low (tetraploid *F. verna* subsp. *verna*) or did not occur at all (other taxa/ploidy levels, Table 2).

Table 2: Summary of the percentage of well-developed and aborted achenes (seeds) per collective fruit formed by autonomous apomixis and autonomous selfing of *Ficaria* taxa under study.

	Apomixis				**Selfing**			
Taxon (Ploidy)	**Well-developed achenes [%]**	**Aborted achenes [%]**	**Number of flowers**	**Number of populations**	**Well developed achenes [%]**	**Aborted achenes [%]**	**Number of flowers**	**Number of populations**
FC (2x)	0.00	100	35	11	0.00	100	34	11
FFE (2x)	0.00	100	27	11	0.00	100	27	11
FFI (4x)	0.00	100	22	7	0.00	100	22	7
FFI (5x)	0.00	100	5	2	0.00	100	5	2
FCH (4x)	0.00	100	3	3	0.00	100	3	2
FCH (5x)	0.00	100	3	2	0.00	100	2	2
FS (3x)	0.00	100	12	4	0.00	100	12	4
FV (4x)	0.00	100	57	23	0.34	99.66	56	23
FV (5x)	0.00	100	10	3	0.00	100	10	3
FV (6x)	0.00	100	1	1	0.00	100	2	1

FC (2x) the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FCH (4x)** the tetraploid cytotype of *F. verna* subsp. *chrysocephala*, **FCH (5x)** the pentaploid cytotype of *F. verna* subsp. *chrysocephala*, **FS (3x)** the triploid cytotype of *F. ×sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp. *verna*, **FV(6x)** the hexaploid cytotype of *F. verna* subsp. *verna*.

4.1.2 Pollen viability

Pollen stainability was used as an approximation of pollen viability. The pollen viability was significantly different between the studied taxa/ploidy levels (LMM, χ^2=140.24, d.f.=7, $p < 0.001$, Fig. 4). The diploid cytotypes showed high pollen viability, whereas the tetraploid cytotypes showed a tendency for reduced pollen viability (but see the tetraploid *F. verna* subsp. *calthifolia*). Poor pollen viability occurred in odd ploidy levels (the triploid cytotype of *F.* ×*sellii*, the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, and of *F. verna* subsp. *verna*). Medium positive correlation between the mean value of the percentage of well-developed achenes (seeds) per collective fruit (according to Uhlířová unpubl.) per population and the mean value of pollen viability per maternal population was confirmed (r = 0.407, n = 67, p<0.001, Fig. 5). Percentage of well-developed achenes (seeds) per collective fruit of tetraploid *F. verna* subsp. *verna* (according to Uhlířová unpubl.) and the mean pollen viability per population were not correlated (r = 0.079, n = 44, p=0.61). Weak positive correlation between the mean number of well-developed achenes (seeds) per collective fruit (according to Uhlířová unpubl.) per population and the mean pollen viability per maternal population was confirmed (r = 0.345, n = 67, p<0.001, Fig. 6). Longitude and mean pollen viability per population and latitude and mean pollen viability per population of tetraploid *F. verna* subsp. *verna* were not correlated (r = 0.006, n = 71, p=0.961; r=0.192, n=71, p=0.102, respectively).

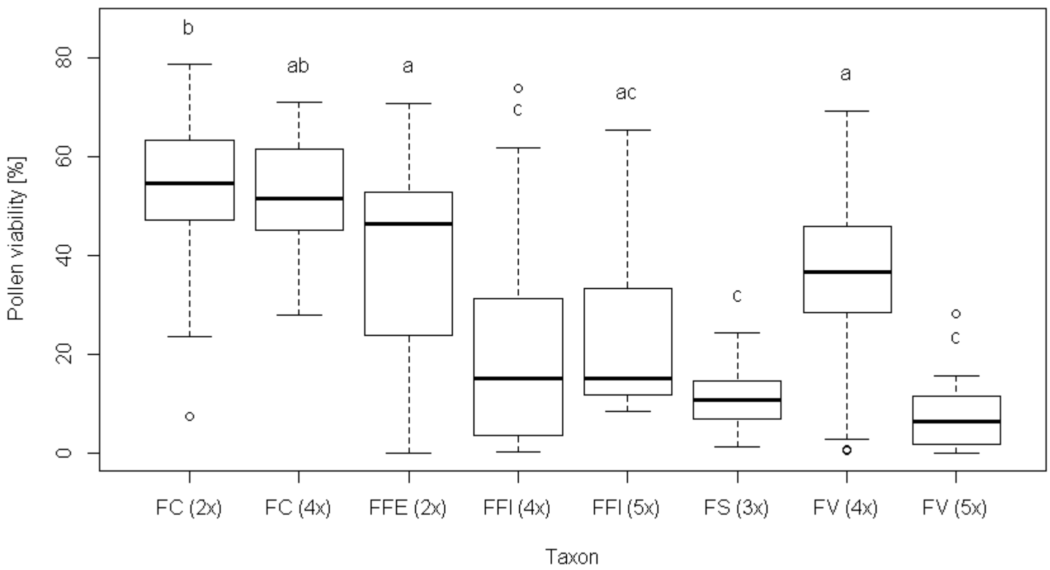

Figure 4: Comparison of variability of pollen viability of *Ficaria* taxa under study. The values of pollen viability are estimated as the average value per individual. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FC (4x)** the tetraploid cytotype of *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FS (3x)** the triploid cytotype of *F. ×sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp. *verna*. Letters indicate the results of comparisons between groups represented by combination taxon/ploidy level using Tukey-Kramer Multiple Comparison test. Taxa with the same letter do not differ significantly ($p \leq 0.001$).

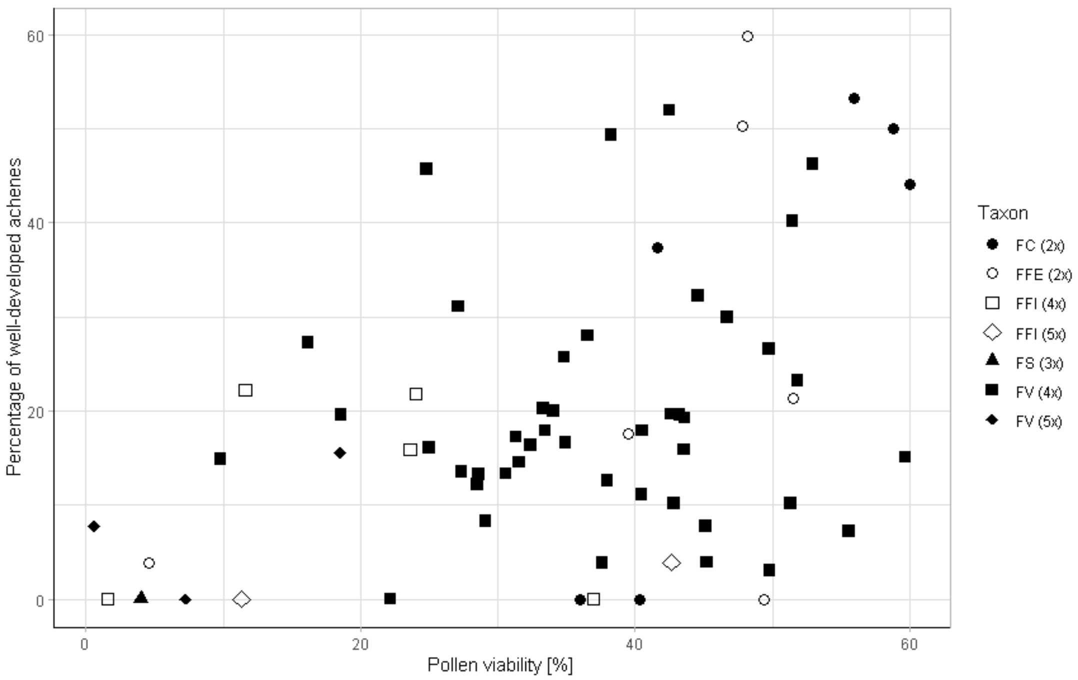

Figure 5: Relationship between pollen viability and percentage of well-developed achenes (seeds) per collective fruit * of *Ficaria* taxa under study. The values of pollen viability and percentage of well-developed achenes (seeds) per collective fruit are estimated as average values per each population. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FS (3x)** the triploid cytotype of *F. ×sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp. *verna*.* Data on the percentage of well-developed achenes (seeds) per collective fruit were adopted from Uhlířová (unpubl.).

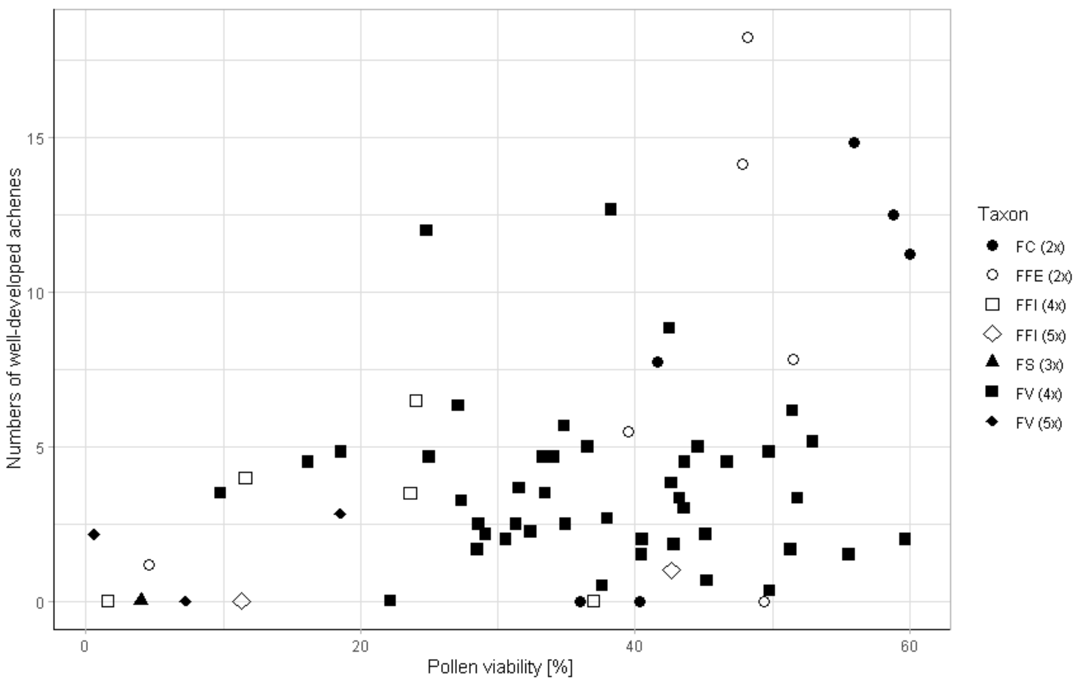

Figure 6: Relationship between pollen viability and numbers of well-developed achenes (seeds) per collective fruit* of *Ficaria* taxa under study. The values of pollen viability and numbers of well-developed achenes are estimated as average values per each population. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FS (3x)** the triploid cytotype of *F. ×sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp. *verna*.* Data on the number of well-developed achenes (seeds) per collective fruit were adopted from Uhlířová (unpubl.).

4.1.3 Pollen length

Aborted pollen grains were excluded from further analysis. Pollen length differed significantly among taxa (LMM, χ^2= 229.69, d.f.=7, p<0.001, Fig. 7), with diploid taxa having significantly shorter pollens than polyploid taxa. Accordingly, strong positive correlation between the mean value of pollen length per population and the mean value of absolute genome size (2C DNA; according to Konečná 2018) per population was confirmed (r = 0.779, n = 30, p<0.001, Fig. 8).

Negligible production of abnormal gametes indicating of "gigas" male gametes was recorded for the tetraploid cytotype of *F. verna* subsp. *verna* and especially for odd-ploidy levels, the triploid cytotype of *F.* × *sellii*, the pentaploid cytotype of *F. verna* subsp. *verna*, and for high even-ploidy level, the hexaploid cytotype of *F. verna* subsp. *verna* (Fig. 9).

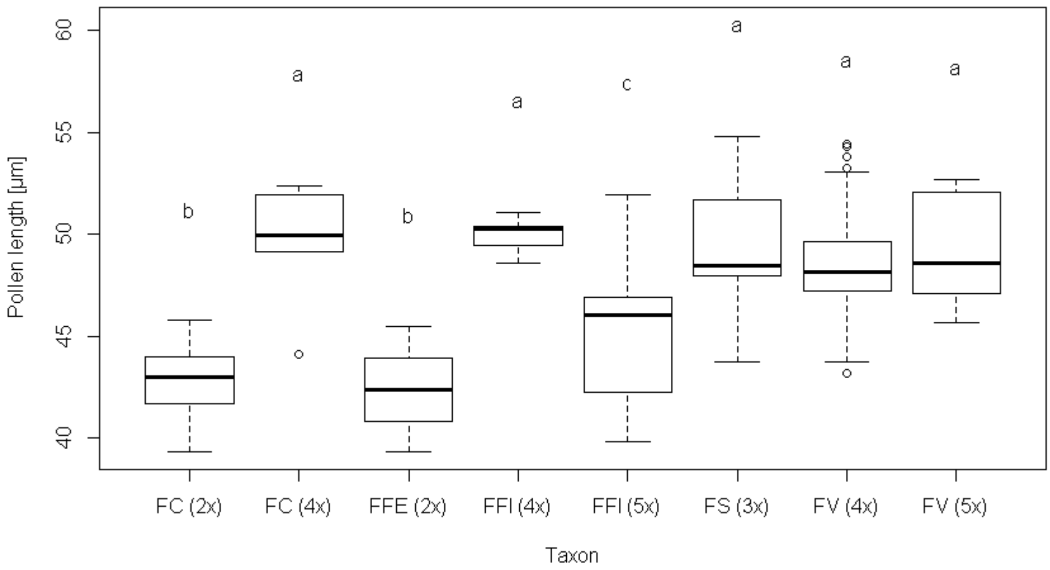

Figure 7: Comparison of variability of pollen length of *Ficaria* taxa under study. The values of pollen length are estimated as the average value per individual. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FC (4x)** the tetraploid cytotype of *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FS (3x)** the triploid cytotype of *F. verna* ×*sellii* (*F. verna* subsp. *calthifolia* ×*F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp.*verna***.** Letters indicate the results of comparisons between groups represented by combination taxon/ploidy using Tukey-Kramer Multiple Comparison test. Taxa with the same letter do not differ significantly ($p \leq$ 0.001).

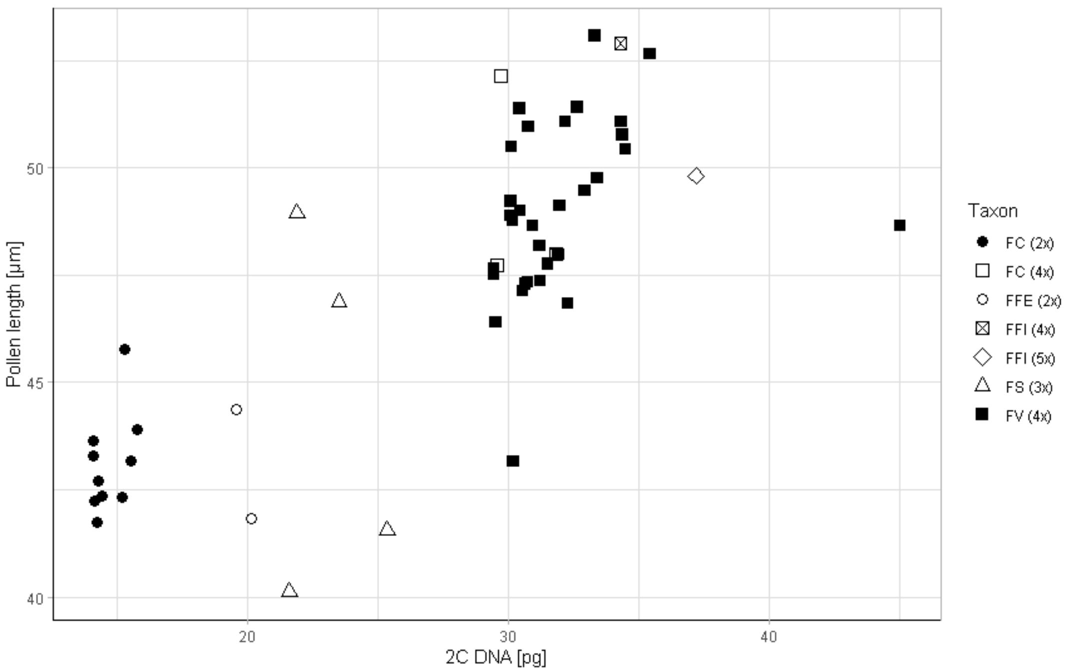

Figure 8: Relationship between pollen length and absolute genome size * of *Ficaria* taxa under study. The values of pollen length and genome size are estimated as average values per each population. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FC (4x)** the tetraploid cytotype of *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FS (3x)** the triploid cytotype of *F.* ×*sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*.* Data on the absolute genome size were adopted from Konečná (2018).

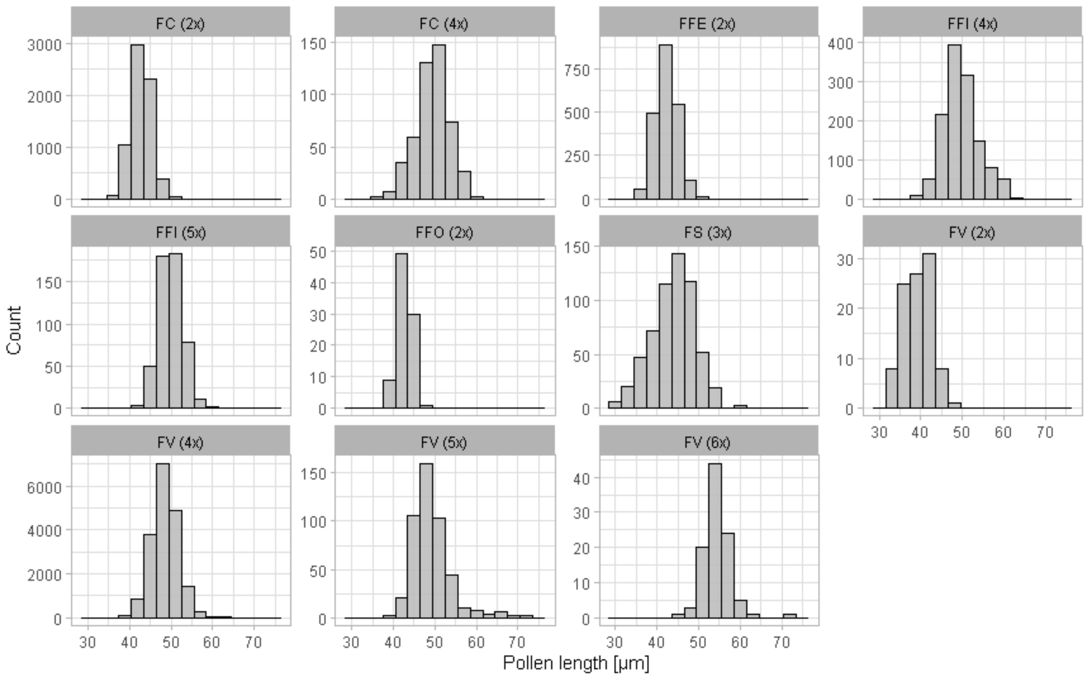

Figure 9: Comparisons of pollen length of stained, potentially viable pollen. These numbers are based on all measured pollen grains in individual flowers. **FC (2x)** the diploid cytotype of the *F. verna* subsp. *calthifolia*, **FC (4x)** the tetraploid cytotype of *F. verna* subsp. *calthifolia*, **FFE (2x)** the diploid cytotype of *F. verna* subsp. *fertilis*, **FFI (4x)** the tetraploid cytotype of *F. verna* subsp. *ficariiformis*, **FFI (5x)** the pentaploid cytotype of *F. verna* subsp. *ficariiformis*, **FFO (2x)** the diploid cytotype of *F. verna* subsp. *ficarioides*, **FS (3x)** the triploid cytotype of *F.* ×*sellii* (*F. verna* subsp. *calthifolia* × *F. verna* subsp. *verna*), **FV(2x)** the diploid cytotype of *F. verna* subsp. *verna*, **FV(4x)** the tetraploid cytotype of *F. verna* subsp. *verna*, **FV(5x)** the pentaploid cytotype of *F. verna* subsp.*verna*, **FV(6x)** the hexaploid cytotype of *F. verna* subsp. *verna*.

4.2 Postzygotic reproductive barriers

4.2.1 Crossing experiment

Percentage of well-developed achenes (seeds) per collective fruit of diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, and *F. verna* subsp. *verna* differed significantly across the pollination treatments ×taxa/ploidy level (one-way ANOVA, $F_{5,106}$=4.831, p<0.001, Fig. 10). Percentage of well-developed achenes (seeds) per collective fruit derived from intertaxa homoploid crosses between *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* in reciprocal crosses was lower than by intrataxa (interpopulation) outcrosses for both parental taxa. The percentage of well-developed achenes (seeds) per collective fruit derived from intrataxa (interpopulation) outcrosses was higher in *F. verna* subsp. *calthifolia* than in *F. verna* subsp. *fertilis*. Intertaxa homoploid crosses between *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal x paternal taxon) yielded a higher number of well-developed achenes (seeds) per collective fruit than reciprocal crosses. The percentage of well-developed achenes (seeds) per collective fruit derived from homoploid crosses of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* (maternal taxa) with diploid cytotype of *F. verna* subsp. *verna* (paternal taxon) was negligible (Table 3). The number of well-developed achenes (seeds) per collective fruit of diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and *F. verna* subsp. *verna* did not differ significantly across the pollination treatments ×taxa/ploidy level (one-way ANOVA, $F_{5,106}$= 1.371, p= 0.257, Fig. 10; Table 3).

Percentage of well-developed achenes (seeds) per collective fruit of tetraploids of *F. verna* subsp. *verna* did not differ significantly across the pollination treatments ×taxa/ploidy level (one-way ANOVA, $F_{3,48}$= 0.619, p=0.606, Fig. 11). The number of well-developed achenes (seeds) per collective fruit formed by intrataxa (interpopulation) outcrosses between/within eastern and western populations of the tetraploids of *F. verna* subsp. *verna* was negligible (Table 4). The number of well-developed achenes (seeds) per collective fruit of tetraploids of *F. verna* subsp. *verna* did not differ significantly across the pollination treatments ×taxa/ploidy level (one-way ANOVA, $F_{3,48}$= 0.805, p= 0.496, Fig. 11, Table 4).

Germination rate (%) of seeds (achenes) per collective fruit of diploids of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* did not differ significantly across the pollination treatments ×taxa/ploidy level (one-way ANOVA, $F_{3,64}$= 0.946, p=0.424,

Fig. 12). The germination rate of seeds (achenes) formed by intertaxa homoploid crosses between *F. verna* subsp. *calthifolia* (maternal taxon) with the diploid cytotype of *F. verna* subsp. *verna* (paternal taxon) was negligible and reached 3.41 %, but intertaxa homoploid crosses using the diploid cytotype of *F. verna* subsp. *verna* as a paternal taxon yielded the reduced number of well-developed achenes (seeds) per collective fruit, so that the germination rate of offspring was reduced, too (Table 3).

The seeds (achenes) produced by intrataxa (interpopulation) outcrosses between/within eastern and western populations of the tetraploids of *F. verna* subsp. *verna* did not germinate at all (Table 4).

Mean holoploid genome size (2C value) of offspring formed by intrataxa (interpopulation) outcrosses of *F. verna* subsp. *calthifolia* and of *F. verna* subsp. *fertilis* was comparable with the mean value of holoploid genome size (2C value) of parental taxa (Table 5, Figs 13, 14), with some deviating cases with either slightly higher (up to 20 % difference; *F. verna* subsp. *calthifolia*) or lower (up to 12% difference; *F. verna* subsp. *fertilis*) genome size than parents. Holoploid genome size of offspring derived from intertaxa homoploid crosses between *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* (maternal x paternal taxon) and from reciprocal crosses was intermediate between the 2C values of their parental taxa (Table 5, Figs 13, 14). Pollen viability (Table 6) and the percentage of well-developed achenes (seeds) per collective fruit formed by spontaneous xenogamy (Table 7) of flowering offspring from intrataxa (interpopulation) outcrosses of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* and intertaxa homoploid crosses between *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal x paternal taxon) were reduced. No unreduced male gametes formed by cultivated offspring from pollination treatments were recorded. Visual elucidation of the morphology of cultivated offspring formed by intertaxa homoploid crosses between diploids of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* revealed that the offspring in the first filial generation was morphologically intermediate between parental taxa.

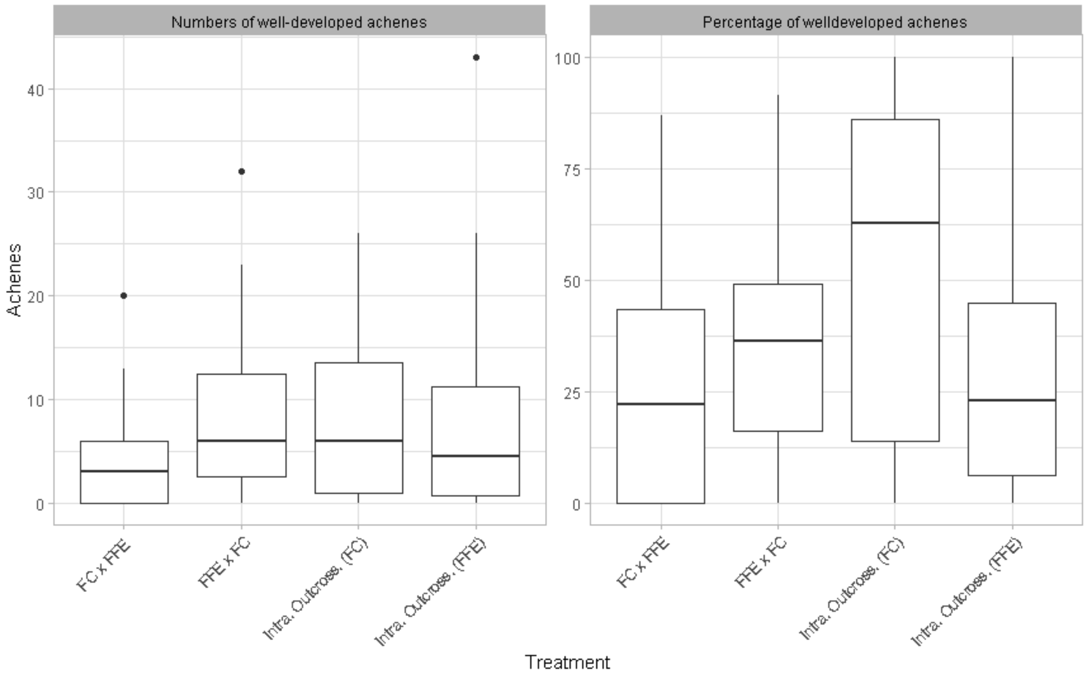

Figure 10: Percentages of well-developed achenes (seeds) per collective fruit formed by intertaxa homoploid crosses between diploids of *F. verna* subsp. *calthifolia* (maternal taxon) and *F. verna* subsp. *fertilis* (paternal taxon, **FC x FFE**), intertaxa homoploid crosses between diploid cytotypes of *F. verna* subsp. *fertilis* (maternal taxon) and *F. verna* subsp. *calthifolia* (paternal taxon), **FFE x FC**), intrataxa outcrossing of the diploid cytotype of *F. verna* subsp. *calthifolia* (**Intra. Outcross. (FC)**), intrataxa (interpopulation) outcrosses of diploids of *F. verna* subsp. *fertilis* (**Intra. Outcross. (FFE)**).

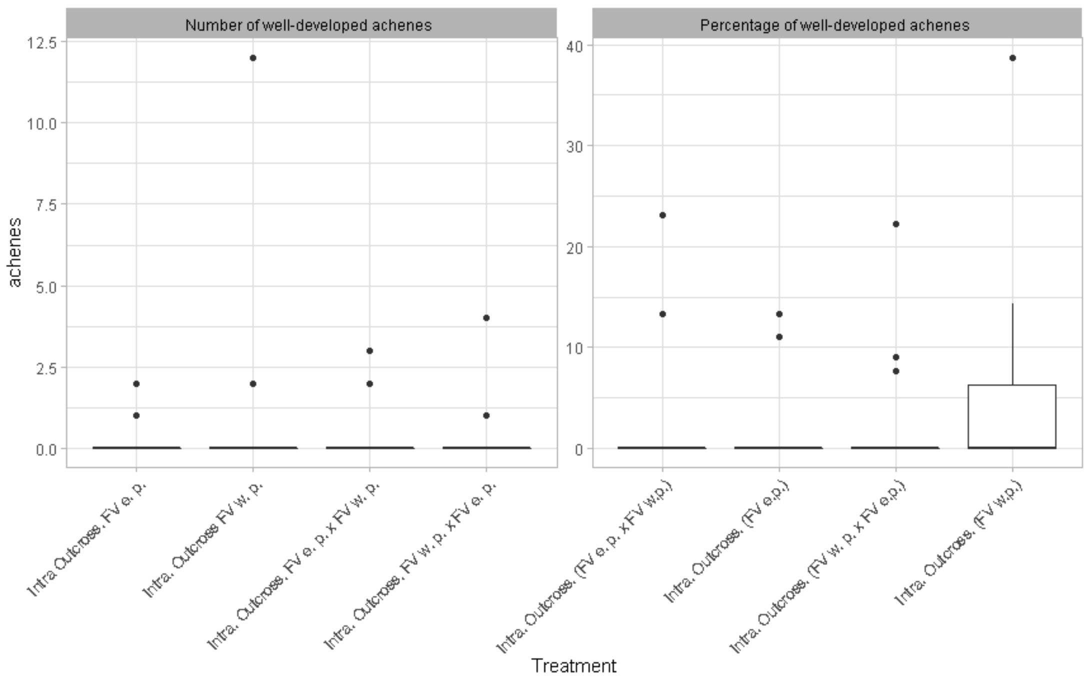

Figure 11: Percentages of well-developed achenes per collective fruit formed by intrataxa (interpopulation) outcrosses of eastern populations of the tetraploids of *F. verna* subsp. *verna* (**Intra. Outcross. (FV e. p.)**, intrataxa (interpopulation) outcrosses of western populations of the tetraploids of *F. verna* subsp. *verna* (**Intra. Outcross. (FV w. p).** intrataxa (interpopulation) outcrosses of eastern and western populations of the tetraploids of *F. verna* subsp. *verna* (**Intra. Outcross. (FV e. p. x FV w.p.)**, intrataxa (interpopulation) outcrosses of tetraploids of western and eastern populations of tetraploids of *F. verna* subsp. *verna* (**Intra. Outcross. (FV w. p. x FV e. p.)**.

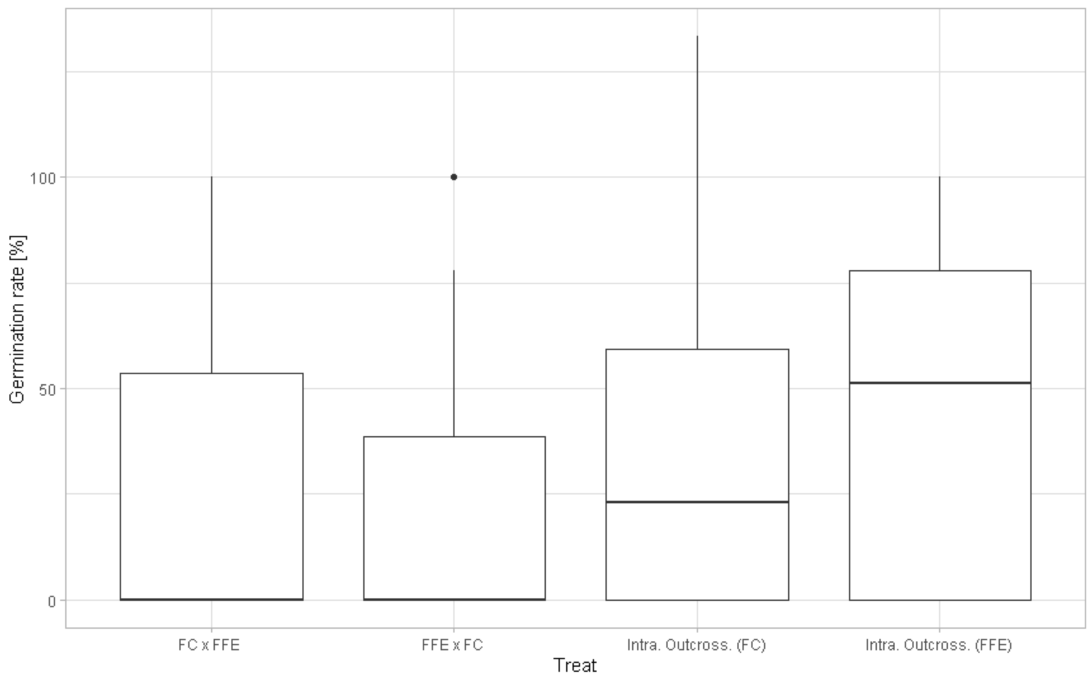

Figure 12: Germination rate of well-developed achenes (seeds) per collective fruit formed by intertaxa homoploid crosses between diploids of *F*. *verna* subsp. *calthifolia* (maternal taxon) and *F*. *verna* subsp. *fertilis* (paternal taxon, **FC x FFE**), intertaxa homoploid crosses between diploid cytotypes of *F*. *verna* subsp. *fertilis* (maternal taxon) and *F*. *verna* subsp. *calthifolia* (paternal taxon), **FFE x FC**), intrataxa outcrossing of the diploid cytotype of *F*. *verna* subsp. *calthifolia* (**Intra. Outcross. (FC)**), intrataxa (interpopulation) outcrosses of diploids of *F*. *verna* subsp. *fertilis* (**Intra. Outcross. (FFE)**).

Table 3: Summary of production of aborted and well-developed achenes between different treatments in the crossing experiment of diploids of *Ficaria* taxa under study

			n		Mean number of aborted achenes per collective fruit					Mean number of well-developed achenes per collective fruit					Germination rate [%]		
Taxon	**Ploidy**	**Treatment**	**Flow.**	**Pop.**	**n**	**Mean**	**± SD**	**Min.**	**Max.**	**n**	**Mean**	**± SD**	**Min.**	**Max.**	**In the 1. year**	**In the 2. year**	**Total**
F. verna subsp. *calthifolia*	2x	apomixis	21	7	311	14.8	4.83	6	23	0	0	0	0	0	0	0	0
F. verna subsp. *calthifolia*	2x	sefing	18	7	319	17.7	6.37	13	31	0	0	0	0	0	0	0	0
F. verna subsp. *calthifolia*	2x	intra. outcross.	22	7	189	8.22	6.53	0	18	214	9.3	8.87	0	26	25.2	6.3	31.5
F. verna subsp. *calthifolia*	2x	x FFE	21	7	228	10.9	4.96	3	19	95	4.52	5.05	0	20	20.26	5.06	25.3
F. verna subsp. *calthifolia*	2x	x FV	11	6	174	15.8	7.04	7	30	31	2.82	4.29	0	11	3.41	0	3.41
F. verna subsp. *fertilis*	2x	apomixis	20	6	423	21.15	8.58	8	34	0	0	0	0	0	0	0	0
F. verna subsp. *fertilis*	2x	sefing	21	6	441	21	7.92	8	35	0	0	0	0	0	0	0	0
F. verna subsp. *fertilis*	2x	intra. outcross.	20	6	207	10.35	6.18	0	22	162	8.1	10.8	0	43	43.32	0	43.3
F. verna subsp. *fertilis*	2x	x FC	27	6	363	13.4	7	3	41	232	8.59	8.32	0	32	21.1	3.02	24.1
F. verna subsp. *fertilis*	2x	x FV	11	4	237	19.75	9.94	8	34	12	1	1.86	0	6	0	0	0

n (count), **Flow**. (flowers), **Pop**. (populations), **SD** (standard deviation), **Min**. (minimum), **Max.** (maximum**), intra. outcross.** (intrataxa (interpopulation) outcrosses), **x FFE** (homoploid cross with *F. verna* subsp. *fertilis*), **x FV** (intertaxa homoploid cross with *F. verna* subsp. *verna*), **x FC** (intertaxa homoploid crosses with *F. verna* subsp. *calthifolia*)

Table 4: Summary of production of aborted and well-developed achenes between different treatments in the crossing experiment of tetraploids of *Ficaria* taxa under study.

Taxon	Ploidy	Treatment	Flow	Pop	n	Mean number of aborted achenes per collective fruit				n	Mean number of well-developed achenes per collective fruit				Germination rate [%]	
						Mean	± SD	Min.	Max.		Mean	± SD	Min.	Max.	In the 1. year	In the 2. year
F. verna subsp. *verna*	4x	apomixis	17	6	272	15.1	5.6	9	23	0	0.00	0.00	0	0	0.00	0.00
F. verna subsp. *verna* – western population	4x	sefing	17	6	317	18.65	5.94	11	13	0	0.00	0.00	0	0	0.00	0.00
F. verna subsp. *verna* – western population	4x	intrataxa (interpopulation) outcrosses with western population	11	6	181	12.9	8.64	8	26	16	1.14	3.21	0	12	0.00	0.00
F. verna subsp. *verna* – western population	4x	intrataxa (interpopulation) outcrosses with eastern population	16	6	219	13.70	5.30	6	25	6	0.38	1.03	0	4	0.00	0.00
F. verna subsp. *verna*	4x	apomixis	13	5	184	11.5	6.31	9	22	0	0.00	0.00	0.00	0.00	0.00	0.00
F. verna subsp. *verna* – eastern population	4x	sefing	15	5	225	14.06	5.95	7	24	0	0.00	0.00	0.00	0.00	0.00	0.00
F. verna subsp. *verna* – eastern population	4x	intrataxa (interpopulation) outcrosses with western population	12	5	150	10.71	5.66	5	19	3	0.21	0.58	0	2	0.00	0.00
F. verna subsp. *verna* – eastern population	4x	intrataxa (interpopulation) outcrosses with eastern population	13	5	171	11.4	6.57	5	22	5	0.31	0.87	0	3	0.00	0.00

n (count), **Flow**. (flowers), **Pop**. (populations), **SD** (standard deviation), **Min**. (minimum), **Max.** (maximum)

Table 5: Summary of genome size of offspring from the experimental crosses of selected *Ficaria* taxa under study.

				n		**2C DNA [pg]**			
Taxon	**Ploidy**	**Type of plant**	**Treatment**	**Ind.**	**Pop.**	**Mean**	**± SD**	**Min.**	**Max.**
F. verna subsp. *calthifolia*	2x	offspring	intrataxa (interpopulation) outcrosses	87	5	15.22	0.48	14.20	17.84
F. verna subsp. *calthifolia*	2x	maternal plant	intrataxa (interpopulation) outcrosses	7	4	14.9	0.19	14.63	15.15
F. verna subsp. *calthifolia*	2x	paternal plant	intrataxa (interpopulation) outcrosses	6	5	14.98	0.48	14.63	15.92
F. verna subsp. *calthifolia*	2x	offspring	intertaxa homoploid crosses with *F. verna* subsp. *fertilis*	38	3	17.38	0.27	16.85	17.92
F. verna subsp. *calthifolia*	2x	maternal plant	intertaxa homoploid crosses with *F. verna* subsp. *fertilis*	7	3	14.96	0.14	14.75	15.15
F. verna subsp. *calthifolia*	2x	paternal plant	intertaxa homoploid crosses with *F. verna* subsp. *fertilis*	5	4	20.34	0.17	20.09	20.51
F. verna subsp. *fertilis*	2x	offspring	intrataxa (interpopulation) outcrosses	26	4	20.27	0.74	18.25	21.37
F. verna subsp. *fertilis*	2x	maternal plant	intrataxa (interpopulation) outcrosses	5	3	20.8	0.12	20.60	20.90
F. verna subsp. *fertilis*	2x	paternal plant	intrataxa (interpopulation) outcrosses	4	2	20.27	0.13	20.09	20.38
F. verna subsp. *fertilis*	2x	offspring	intertaxa homoploid crosses with *F. verna* subsp. *calthifolia*	49	5	17.77	0.39	16.82	18.68
F. verna subsp. *fertilis*	2x	maternal plant	intertaxa homoploid crosses with *F. verna* subsp. *calthifolia*	8	5	20.56	0.27	20.16	20.92
F. verna subsp. *fertilis*	2x	paternal plant	intertaxa homoploid crosses with *F. verna* subsp. *calthifolia*	7	5	15.23	0.66	14.43	16.18

n (count), *Ind*. (Individual), **Pop**. (populations), **SD** (standard deviation), **Min**. (minimum), **Max.** (maximum)

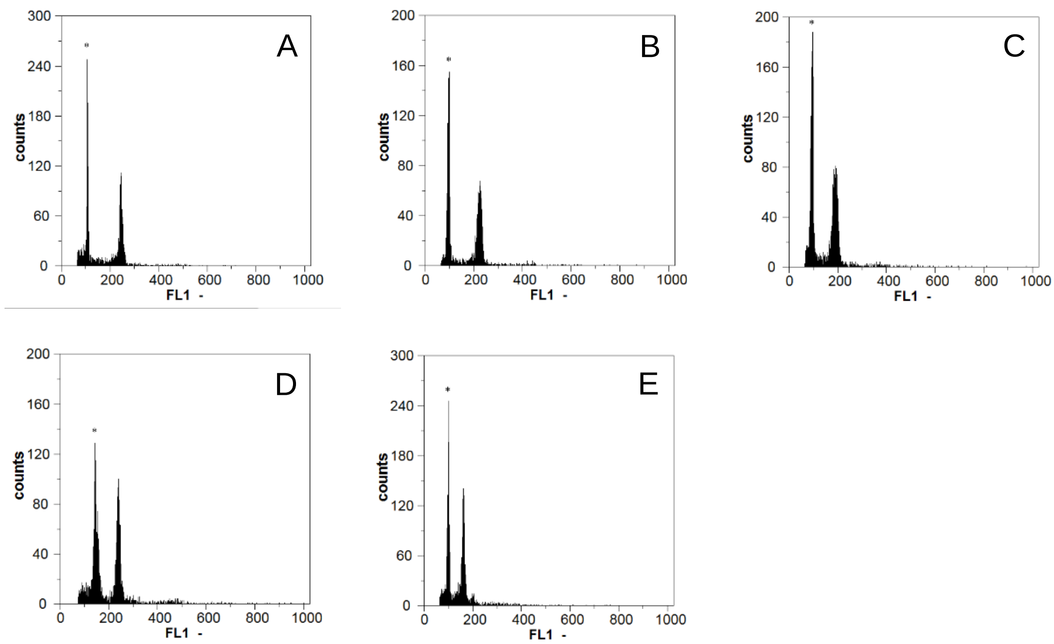

Figure 13: Examples of FCM histograms. **A** Maternal taxon of *F. verna* subsp. *fertilis*, **B** Offspring derived by intrataxa outcrossing of *F. verna* subsp. *fertilis*, **C** Offspring formed by intertaxa crossing of *F. verna* subsp. *fertilis* and *F. verna* subsp *calthifolia*, (maternal x paternal. taxon), **D** Maternal taxon of *F. verna* subsp. *calthifolia*, **E** Offspring formed by intrataxa outcrossing of *F. verna* subsp. *calthifolia*. *Pisum sativum* was used as an internal standard. * Indicates the position of peak of the standard.

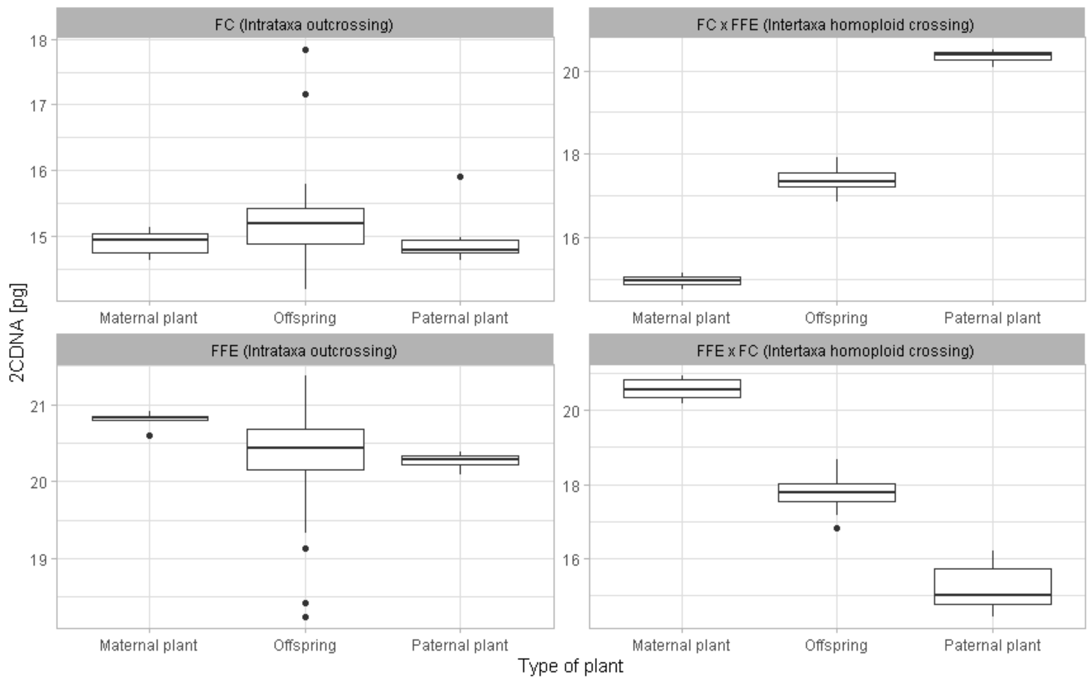

Figure 14: Comparison of variability of genome size (2C DNA, pg) of parental taxa and offspring derived by intrataxa (interpopulation) outcrossing (intrataxa xenogamy) of *F. verna* subsp. *calthifolia* (**FC**), *F. verna* subsp. *fertilis* (**FFE**), intertaxa homoploid crossing of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* (**FC x FFE**, maternal x paternal plant), and intertaxa homoploid crossing of *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (**FC x FFE**, maternal and paternal plant).

Table 6: Summary of pollen viability of offspring under the experimental crosses. Pollen viability was estimated by FDA.

			n		Viability [%]			
Taxon	**Ploidy**	**Treatment**	**Ind.**	**Pop.**	**Mean**	**± SD**	**Min.**	**Max.**
F. *verna* subsp. *calthifolia*	2x	Intrataxa outcrossing	5	2	30.37	13.33	10.14	43.76
F. *verna* subsp. *fertilis*	2x	Intrataxa outcrossing	2	2	25.61	9.19	19.11	32.11
F. *verna* subsp. *fertilis*	2x	Intertaxa outcrossing FFE x FC	3	1	20.96	14.83	4.29	32.69

n (count), **Ind**. (individual), **Pop**. (populations), **SD** (standard deviation), **Min**. (minimum), **Max.** (maximum), **FFE x FC** (*F*. *verna* subsp. *fertilis* x *F*. *verna* subsp. *calthifolia*, maternal plant x paternal plant).

Table 7: Summary of well-developed achenes formed by offspring under experimental crosses. Well-developed achenes were derived from spontaneous

			n		**Aborted achenes**				**Well-developed achenes**				**Well-developed achenes [%]**			
Taxon	Ploidy	Treatment	Ind.	Pop.	Mean	± SD	Min.	Max.	Mean	± SD	Min.	Max.	Mean	± SD	Min.	Max.
F. verna subsp. *calthifolia*	2x	Intrataxa outcrossing	5	2	165	6.78	8.00	27.00	8.00	1.10	0.00	3.00	5.47	5.01	0.00	12.50
F. verna subsp. *fertilis*	2x	Intrataxa outcrossing	2	2	55	5.69	12.00	23.00	4.00	2.31	0.00	4.00	6.25	8.84	0.00	12.50
F. verna subsp. *fertilis*	2x	Intertaxa outcrossing FC x FFE	3	1	162	6.27	11.00	32.00	3.00	1.06	0.00	3.00	2.17	3.77	0.00	6.52

n (count), **Ind**. (individual), **Pop**. (populations), **SD** (standard deviation), **Min**. (minimum), **Max.** (maximum), **FFE x FC** (*F. verna* subsp. *fertilis* x *F. verna* subsp. *calthifolia*, maternal plant x paternal plant).

5. Discussion

In the present study, the reproductive modes, pollen viability& length in most taxa of the genus *Ficaria* and intertaxa homoploid crossing and intrataxa (interpopulation) outcrossing between/within selected taxa of the genus *Ficaria* were assessed using a combination of pollen exclusion bags, pollen viability analysis, morphometric analysis of pollen length, genome size estimation and experimental crosses. Results of the present study indicated that autonomous apomixis and selfing are almost not present in the representatives of the genus *Ficaria*. Therefore, the high pollen viability, especially in diploids, enables sexual reproduction that provides potential for intertaxa hybridization. Potential for the generation of neopolyploids in the genus *Ficaria* are supported by the recorded subtle production of abnormally large and well-developed pollen by allotriploids of *F.* × *selli*, and by tetraploids, pentaploids, and hexaploids of *F. verna* subsp. *verna*. This study thus provides the first evidence for potential production of unreduced gametes in the genus *Ficaria*. The pollen length was found to increase with genome size, but it cannot be solely used for the delimitation of individual taxa/ploidy levels, because the pollen lengths were rather heterogenous within ploidy level, especially in odd ploidy levels.

The results also demonstrated that the absence of occurrence of autonomous apomixis and autonomous selfing and high pollen viability do not act as sufficient prezygotic barriers to prevent hybridization between most taxa of the genus *Ficaria*. This is supported by the recorded asymmetric hybridization between diploid cytotypes (*F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, and *F. verna* subsp. *verna* diploid plants, morphologically similar to the tetraploid cytotype of *F. verna* subsp. *verna*), which resulted in the formation of viable progeny, and experimental crosses between geographically distant lineages of the tetraploid cytotype (western and eastern populations of *F. verna* subsp. *verna*), which resulted in the formation of well-developed achenes. Seedlings produced by intertaxa homoploid crossing and intrataxa (interpopulation) outcrossing of diploids (*F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and diploid plants, morphologically similar to the tetraploid cytotype of *F. verna* subsp. *verna* were of the same ploidy as the parental taxa, and the genome sizes of those plants were intermediate between the genome sizes of the parental taxa. Recorded achenes (seeds) derived from homoploid crosses between diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, and *F. verna* subsp. *verna*, and within two (western and eastern) populations of the tetraploid cytotype of *F. verna* subsp. *verna*, supports the classification of those taxa as subspecies.

5.1 Reproductive modes do not depend on the ploidy level, but pollen viability affects the percentage of well-developed achenes

Reproductive mode plays a crucial role in reproductive outcome and on genetic within-taxa diversity (Hamrick & Godt 1996). In general, apomixis (agamospermy) as one possible mechanism to avoid hybrid sterility and establishment of hybrids, is almost exclusively associated with polyploidization (e.g., Asker & Jerling 1992). Only a few diploid taxa have been developed apomixis as a mechanism to prevent loss of genetic heterozygosity (Noirot et al. 1997), e. g. *Boechera* A. Löve and D. Löve (Böcher 1951). However, irrespective to the ploidy level, no well-developed achenes formed by autonomous apomixis in the studied *Ficaria* taxa were recorded in bagging experiments realised in two years. Presented results are not in line with those of Metcalfe (1939), who found the occurrence of autonomous apomixis in emasculated and unpollinated flowers of *F. verna* subsp. *fertilis* and the potential occurrence of pseudogamy in not emasculated and unpollinated flowers of *F. verna* subsp. *fertilis* and *F. verna* subsp. *verna* (Metcalfe 1939). However, these results need to be interpreted with caution, because the germination capacity of these seeds was not investigated. Moreover, Popelka et al. (2019a), in agreement with the present study, did not provide any evidence for the occurrence of autonomous apomixis in diploid *F. verna* subsp. *calthifolia* and tetraploid *F. verna* subsp. *verna* (Popelka et al. 2019a). Therefore, as was expected, the occurrence of autonomous apomixis within the genus *Ficaria* seems to be unlikely.

However, the occurrence of pseudogamy that requires pollen for proper endosperm development (Richards 1997) was not investigated in bagging experiment, since emybryo of *Ficaria* taxa is not fully developed at the end of the vegetation period. Therefore, the flow cytometric screening of seeds to reveal the mode of endosperm development cannot be applied. Thus, the possible achenes derived by pseudogamy within treat of spontaneous xenogamy (according to Uhlířová unpubl.) cannot be assessed. However, any evidence of the occurrence of pseudogamy in the intertaxa homoploid crosses between diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, and *F. verna* subsp. *verna* was not found, since hybrids with intermediate genome size were recorded. Similarly, Popelka et al. (2019a) in the experimental heteroploid crosses between diploids of *F. verna* subsp. *calthifolia* and tetraploids of *F. verna* subsp. *verna* found only triploid offspring (Popelka et al. 2019a). Therefore, the occurrence of pseudogamy such as autonomous apomixis seems to be unlikely, too.

The present study also showed that autonomous selfing does not occur in the studied taxa with except for tetraploids of *F. verna* subsp. *verna*, where a negligible number of well-developed achenes (seeds) formed by autonomous selfing were recorded in the bagging experiments. Present results confirm general assumption of the occurrence of the self-incompatibility in diploids, as selfing leads to loss of genetic heterozygosity, often reflected by inbreeding depression (Schemske & Lande 1985a, b). However, selfing may promote the likelihood of polyploid establishment (Ramsey & Schemske 1998) and the breaking of self-incompatibility is common in polyploids (Levin 1983; Thompson & Lumaret 1992; but see Mable 2004), as the high genetic diversity in polyploids masks any effects of inbreeding depression in short term period (Otto 2007). Therefore, negligible number of well-developed achenes formed by autonomous selfing in the tetraploids of *F. verna* subsp. *verna* might be explained by the breaking of self-incompatibility. The observed patterns of almost lacking ability of selfing contradict with Metcalfe (1939), who found the occurrence of autonomous selfing in unpollinated and not emasculasted flowers of *F. verna* subsp. *fertilis* and *F. verna* subsp. *verna* (Metcalfe 1939). However, such as in autonomous apomixis, the germination capacity of those seeds was not investigated by Metcalfe, too. Moreover, Pogan & Wcisło (1981a) did not provide any evidence for the occurrence of selfing in *F. verna* subsp. *calthifolia* and *F. verna* subsp. *verna* (Pogan & Wcisło 1981a). Furthermore, the flowers are slightly proterandric (Marsden-Jones 1933) and exhibit floral traits encouraging cross-pollination (Sell 1994; Veldkamp et al. 2015; Vázquez 2016). In addition, all taxa can reproduce vegetatively regardeless to the ploidy level (Marsden-Jones 1933, Sell 1994) and large local stands (Reisch & Scheitler 2009). Therefore, self-incompatibility can also evolve as consequence of natural selection, since many studies in self-compatible clonal plants declare the increased reduction of species fitness via geitonogamy (*Aconitum kusnezoffii* Reichenbach, Liao et al. 2009; *Pulsatilla vulgaris* Mill., DiLeo et al. 2018).

However, the induced autogamy, i.e., mentor effects has not been investigated in the present study. Mentor effects could lead to a break of self-incompatibility of outcrossing sexual species if pollen of another related species is present on the stigma (Richards 1997). Such a scenario was observed in close relatives of the genus *Ficaria*, e.g., in sexual diploids of *Ranunculus auricomus* L. complex (sect. Auricomus; Richards 1997; Horändl & Temsch 2009).

By contrast to autonomous apomixis and autonomous selfing, seeds and viable seedlings with the same ploidy level as parental taxa were formed by intrataxa (interpopulation) outcrossing of diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and tetraploids of *F. verna* subsp. *verna* in the present study. Observed numbers of well-developed achenes (seeds) formed by intrataxa (interpopulation) outcrossing in the present study are almost consistent with previous studies on the production of well-developed achenes by spontaneous outcrosses in the studied *Ficaria* taxa (diploids of *F. verna* subsp. *calthifolia*, Uhlířová 2019; Popelka et al. 2019a; diploids of *F. verna* subsp. *fertilis*, Uhlířová 2019; Marsden-Jones 1933; Veldkamp 2015; tetraploids of *F. verna* subsp. *verna*, Andreas 1966; Drenckhahn 2016; Uhlířová 2019; Popelka et al. 2019a). The number of well-developed achenes per collective fruit differed between parental taxa and ploidy levels. The number of well-developed achenes (seeds) per collective fruit was higher in diploids of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* in comparison with tetraploids of *F. verna* subsp. *verna*. Similarly, large number of well-developed achenes derived from the xenogamy and high germination of the diploids of *F. verna* subsp. *calthifolia* have been reported also by Drenckhahn (2016). Dominant importance of sexual reproduction for maintenance and dispersal of populations of *F. verna* subsp. *calthifolia* is also reported by Popelka et al. (2019a), who revealed that genotypic diversity in all three studied populations was 1.0, i.e., the highest possible. Prevalence of sexual reproduction is also expected in the diploids of *F. verna* subsp. *fertilis* (Uhlířová 2019; Marsden-Jones 1933; Veldkamp 2015), but the present study found that the number of well-developed achenes per collective fruit is slightly lower in comparison with that of the *F. verna* subsp. *calthifolia*. Similar pattern was observed also by Uhlířová (unpubl.). Lower pollen viability is likely to explain lower achene (seeds) production. However, recorded reduced number of well-developed achenes per collective fruit of *F. verna* subsp. *fertilis* might be an artefact of low number of the sampled populations through all distribution range, more extensive sampling would potentially reveal a distinct pattern.

In contrast to those diploid cytotypes, the tetraploids of *F. verna* subsp. *verna* produced a lower number of well-developed achenes per collective fruit. This is consistent with general assumption that the postzygotic genomic incompatibilities between parental taxa related to unbalanced chromosome pairing during meiosis are more common in polyploids (Ramsey & Schemske 1998). Observed pattern of production of well-developed achenes by *F. verna* subsp. *verna* is consistent with previous data that the *F. verna* subsp. *verna* is almost seed sterile taxon, spread by fragmentation of bellow ground tubers (Marsden-Jones 1933; Andreas 1966; Taylor & Markham 1978; Wcisło & Pogan 1981; but see Popelka et al. 2019a) and additionally also by axillary bulbils (Sell 1994). Vegetative reproduction is commonly prevalent in polyploids (Ramsey & Schemske 1998; Fawcett & Van de Peer 2010; Herben et al. 2017). However, Popelka et al. (2019a) in comparison to former studies (Marsden-Jones 1933; Andreas 1966; Taylor & Markham 1978; Wcisło & Pogan 1981) reported higher rates of sexual reproduction of *F. verna* subsp. *verna* and the relatively high genotypic diversity of these populations. Those contradictory results may be explained by the differenences of studied populations that were provided from different parts of distribution range of among authors and require further study. Polyphyletic origin of *F. verna* subsp. *verna* is also suggested by Drenckhahn et al. (2017). However, additional factors such as recurrent origin, variable selection pressure in the different parts of the distribution range could be responsible for the recorded variation in the numbers of well-developed achenes per individual. The high variation in numbers of well-developed achenes per collective fruit among individual plants of the tetraploids of *F. verna* subsp. *verna* has been actually observed by Uhlířová (unpubl.).

The observed intra-taxa variation in the numbers of well-developed achenes per collective fruit derived from spontaneous xenogamy at the large geographical range (according to Uhlířová unpubl.) could be partly explained by pollen viability of the pollen donor plants in the present study. The pollen viability is generally considered as the most important factor, that could contribute to the limitation of number of well-developed achenes per collective fruit (e.g., *Cirsium* (L.), Bureš et al. 2010). Although the limitation of number of well-developed achenes per collective fruit by resource availability and geitonogamy could be ruled out in the present study, the variation of numbers of well-developed achenes per collective fruit among the individual plants was still recorded. This variation (according to Uhlířová unpubl.) can be substantially affected by pollen limitation (Ramsey & Schemske 1998) or by interspecific pollen deposition (Briggs et al. 2015).

The interaction between intraspecific and interspecific pollen deposition generally results in lower seed set, as intraspecific pollen germination may be reduced by the high density of interspecific pollen on a stigma. This way of limitation of reproductive output was also reported in the family Ranunculaceae (e.g., *Delphinium barbeyi*; Briggs et al. 2015) and it could be expected also in the genus *Ficaria*. Considering the overlapping flowering periods, generalist-pollination and lack of the floral assurance among different *Ficaria* taxa/ploidy levels, the high interspecific pollen deposition in the common garden is probable.

5.2 Pollen viability relates to the ploidy level

Pollen viability is commonly influenced by the ploidy level and origin of taxa, where decreased pollen viability is usually detected in F1 homoploid hybrids (Ramsey & Schemske 1998; Ramsey & Schemske 2002). The pollen viability of diploids of *F. verna* subsp. *calthifolia* (mean 54.79 %, range 23.44 % – 78.49 %, n=69) was high, but in diploids of *F. verna* subsp. *fertilis* (mean 37.50 %, range 0 % – 70.53 %, n=25) was reduced. In contrast to the diploids of *F. verna* subsp. *calthifolia*, the pollen viability of tetraploids of *F. verna* subsp. *verna* (mean 36.20 %, range 0.58 % – 69.07 %, n=208) and *F. verna* subsp. *ficariiformis* (mean 21.12 %, range 0.3 % – 73.6 %, n=21). was reduced with the exception of tetraploid plants morphologically similar to the diploid cytotype of *F. verna* subsp. *calthifolia*, considered as tetraploids of the *F. verna* subsp. *calthifolia* (mean 51.36 %, range 27.81 % – 70.91 %, n=5). Poor pollen viability was detected in the odd ploidy levels, i.e., in the triploid cytotype of *F. ×sellii* (mean 11.55 %, range 1.23 % – 24.28 %, n=13), in the pentaploid cytotype of *F. verna* subsp. *ficariiformis* (mean 25.51 %, range 8.58 – 65.19, n=7), and *F. verna* subsp. *verna* (mean 8.8 %, range 0 % – 28.22, n=9). Observed patterns of pollen viability are almost consistent with previous studies on pollen viability in several *Ficaria* taxa (*F. verna* subsp. *calthifolia*, Pogan & Wcisło 1974; *F. verna* subsp. *fertilis*, Marchant & Brighton 1974; Nicholson 1983; tetraploid cytotype of *F. verna* subsp. *verna*, Neves 1942; Gill et al. 1972; Marchant & Brighton 1974; the triploid cytotype of *F. verna* subsp. *verna* (Marchant & Brighton 1974; Pogan & Wcisło 1974; Popelka et al. 2019b).

Findings of high pollen viability detected in diploids of *F. verna* subsp. *calthifolia* agreed with the general assumption that homologous chromosomes pair nonrandomly and are segregated independently and regularly in diploids (Ramsey & Schemske 2002). The lower mean pollen viability of *F. verna* subsp. *fertilis*, in comparison with mean pollen viability of *F. verna* subsp. *calthifolia* would be rather than to disorders in microsporogenesis closely matched to the existence of almost sterile plants of *F. verna* subsp. *fertilis* in the present study. Pollen sterility of *F. verna* subsp. *fertilis* might be explained by inappropriate conditions for this taxon in the common garden. Besides, more extensive and intensive sampling could reveal a different pattern, as a low rate of disorder during meiosis was actually observed in the diploids of *F. verna* subsp. *calthifolia* (Pogan & Wcisło 1983).

The observed larger reduction of pollen viability in tetraploids of *F. verna* subsp. *verna*, and *F. verna* subsp. *ficariiformis* in comparison with diploids of *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* could be interpreted as meiotic disturbances in microsporogenesis that are more common in taxa with hybrid or polyploid origin (Ramsey & Schemske 1998; Ramsey & Schemske 2002, and the references therein). In general, pollen viability of neoallopolyploids is higher than the pollen viability of neoautopolyploids, since the bivalent pairing of chromosomes during meiosis (disomic inheritance) and associated no complexes, bridges or fragments, and few univalents are observed in allopolyploids (Ramsey & Schemske 2002, and the references therein). Ramsey & Schemske (2002) reviewed that that the mean percent occurrence of multivalents (trivalents and quadrivalents) is significantly higher in autopolyploids (28.8 %) than in allopolyploids (8.0 %, Ramsey & Schemske 2002). However, the transition in meiotic behaviour from multivalent pairing to bivalent pairing was recorded in subsequent generations of autopolyploids (Sybenga 1996; Soltis et al. 2009). Therefore, pollen variability of tetraploids of *F. verna* subsp. *verna* that did not reflect any geographical pattern might be explained by different reccurent origin of individual plants in their distribution range.

In contrast to *F. verna* subsp. *verna* pollen viability of tetraploid of *F. verna* subsp. *ficariiformis*, measured on the lower number of populations was reduced, but populations examined on pollen viability covered the whole distribution range of this taxon. Therefore, reduced pollen viability of *F. verna* subsp. *ficariiformis* might be generally explained by recent origin (Ramsey & Schemske 2002, and the references therein).

Observed deviation patterns in pollen viability of the tetraploid plants, morphologically similar to diploid of *F. verna* subsp. *calthifolia*, where the autotetraploid origin with diploids of *F. verna* subsp. *calthifolia* as a parental taxon or allotetraploid origin with diploid ancestors of *F. verna* subsp. *calthifolia* as parental taxa is suggested (Popelka unpubl.), might be explained by the evolutionary divergence of the tetraploid of *F. verna* subsp. *calthifolia* in the past. Subsequent restoration of hybrid fertility in autopolyploids was observed several times (Ramsey & Schemske 2002, and the references therein).

The observed near full sterility of odd ploidy levels is caused by the absence of mechanism that could evenly divide the chromosomes of an odd-number configuration in meiosis (Ramsey & Schemske 1998). Consequently, aneuploid gametes are commonly produced by odd ploidy level plants, as, for instance, in triploids of either (a) two bivalents and one univalent; (b) one trivalent, or (c) three univalents are formed (Ramsey & Schemse 2002).

5.3 The degree of the variation in pollen length relates to ploidy level

In general, the size of pollen increases with the increase of genome size/ploidy level within related taxa (Bennett 1972, Knight et al. 2010, and the references therein). Differences in pollen length of the studied *Ficaria* taxa/ploidy levels support this assumption (Fig. 8). The mean pollen length increases from diploids to hexaploids (Fig. 8,9). However, it cannot be solely used for the delimitation of individual taxa/ploidy levels, since the differences in the genome size are small (Konečná 2018; Kobrlová unpubl.; Popelka unpubl.) and the heterogenous pollen lengths are produced by high ploidy levels. Degree of variability of pollen length differed between ploidy levels.

Variability of pollen length was lower in diploids of *F. verna* subsp. *calthifolia* (mean 42.82 µm, range 39.33 µm – 45.76 µm, n=67) and *F. verna* subsp. *fertilis* (mean 42.4 µm, range 39.31 µm – 45.49 µm, n=22) than in the tetraploids of *F. verna* subsp. *calthifolia* (mean 50.84 µm, range 39.31 µm – 52.34 µm, n=4), *F. verna* subsp. *verna* (mean 48.51 µm, range 43.16 µm – 54.44 µm, n=197) and *F. verna* subsp. *ficariiformis* (mean 49.58 µm, range 43.74 µm – 54.81 µm, n=13). The highest pollen viability was recorded in odd ploidy levels, i.e., triploids of *F.* ×*sellii* (mean 44.98 µm, range 39.79 µm – 51.92 µm, n=13), and the pentaploids of *F. verna* subsp. *ficariiformis* (mean 49.94 µm, range 48.60 µm – 51.09 µm, n=7), and *F. verna* subsp. *verna* (mean

49.21 μm, range 45.66 μm – 52.69 μm, n=5, Fig. 7). The observed pattern of pollen variability closely matches to the recorded higher homogeneity of pollen length in diploids than in even-ploidy and especially in the odd ploidy levels. In general, meiotic disturbances in microsporogenesis such as highly irregular chromosome pairing, lagging chromosomes and chromosome bridges, micronuclei, and multiple spindles are common in hybrids and polyploids, especially in neoautopolyploids and in odd ploidy levels (Ramsey & Schemske 1998; Ramsey & Schemske 2002; Henry et al. 2005; Wang et al. 2010, and the references therein). Frequent production of aneuploid gametes by a karyological approach was also documented in triploids of *F.* ×*sellii* (Pogan & Wcisło 1974) and tetraploids of *F. verna* subsp. *verna* (Pogan & Wcisło 1981a).

5.4 Abnormal pollen length suggesting a different amount of genome

Production of unreduced gametes with the full somatic chromosome number is generally representing a prevalent evolutionary mechanism contributing to the establishment of polyploids (Ramsey & Schemske 1998; Kreiner et al. 2017b) as unreduced gametes can serve as bridges between diploids and tetraploids ("triploid bridge", Ramsey & Schemske 1998). However, unreduced gamete production is generally rare in the field and unevenly produced across different individuals in dependence on the mating system and environmental stress (Bretagnolle & Thompson 1995; Ramsey & Schemske 1998; Mason & Pires 2015; Kreiner et al. 2017a, 2017b). Kreiner et al. (2017b), based on the analysis of 1696 individuals of 24 species of the family Brassicaceae by flow cytometry, revealed that most individuals (75.1 %) produced very low levels of unreduced gametes, from 0.1 % to 2 %, but a minority of individuals (6.7 %), produced substantial more unreduced gametes, which exceeded 5 %.

Limited production of unreduced gametes is expected also in the studied *Ficaria* taxa here, as pollens with substantial length were only rarely recorded in triploids of *F.* × *sellii*, and tetraploids, pentaploids, and hexaploids of *F. verna* subsp. *verna* and it may therefore suggest the occurrence of unreduced gametes. However, the large size of those gametes can be also generally attributed to irregular pairing of chromosomes during meiosis that do not differ between auto and allopolyploids and subsequently cause a variable genome content ("aneuploidy", Ramsey & Schemske 2002; Henry et al. 2005) or to cytomixis (migration of chromosomes between meiocytes through cytoplasmic connections, Falistocco et al. 1995; Mursalimov et al. 2013). Moreover, triploid or hexaploid seedlings were not found in experimental homoploid crosses, and the pollen of the homoploid hybrids also did not show any evidence for unreduced gamete production. However, the non-occurrence of unreduced gametes in the experimental homoploid crosses may be simply an artefact caused by the low number of involved experimental plants that therefore resulted in a low number of achenes and less probability to detect the potential unreduced gametes.

Irrespective of the uncertainty related to the unreduced pollen production above, triploid (Drenckhahn 2016; Drenckhahn et al. 2017) and tetraploid plants (Popelka unpubl.) without knowledge of their origin, morphologically similar to diploids of *F. verna* subsp. *calthifolia* were reported from Greece (Drenckhahn et al. 2017; Popelka unpubl.). Furthermore, neoallotriploid origin of *F. ×sellii* was confirmed by a molecular approach (Popelka et al. 2019a). Moreover, the hexaploid plants morphologically similar to the tetraploids of *F. verna* subsp. *verna* with unclear origin have been recorded a few times in the past (Soó & Borhidi 1964; Anders-Gasser 1985). Moreover, nothing is known about the origin of other polyploid taxa such as tetraploids of *F. verna* subsp. *chrysocephala* and tetraploids and pentaploids of *F. verna* subsp. *ficariiformis*.

The establishment of polyploid of bulbil-producing *F. verna* subsp. *verna* by unreduced gametes was proposed by Drenckhahn et al. (2017) based on the genome size and geographical distribution (Drenckhahn et al. 2017). Drenckhahn et al. (2017) concluded that the tetraploid of *F. verna* subsp. *verna* contains two different lineages with divergent origin, western and eastern ones. For the western lineage/populations of the tetraploid cytotype of *F. verna* subsp. *verna* (a) allotetraploid origin with *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* as parental taxa or (b) autotetraploid origin with *F. verna* subsp. *fertilis* as a parental taxon is considered. Autotetraploid origin contradicts to Nicholson (1983), who revealed the absence of axillary bulbils in plants developed by experimental autotetraploidization of *F. verna* subsp. *fertilis*. For the eastern lineage of *F. verna* subsp. *verna* then Drenckhahn et al. (2017) suggested an autotetraploid origin of *F. verna* subsp. *verna* with *F. verna* subsp. *calthifolia* as a parental taxon. However, autotetraploid origin of the eastern lineage *F. verna* subsp. *verna* with *F. verna* subsp. *calthifolia* as a parental taxon contradicts to conclusions by Konečná (2018), who found that the monoploid genome size of *F. verna* subsp. *verna* is gradually decreasing along NW-SE direction in south-eastern Europe (Balkans), while that of *F. verna* subsp. *calthifolia* is increasing along the same direction. Therefore, Konečná (2018) was convinced that, if *F. verna* subsp. *verna* originated from *F. verna* subsp. *calthifolia*, the geographic patterns in the monoploid genome size of both taxa should be the same (Konečná 2018). However, unpublished research by Popelka (unpubl.) observed in the field and sampled *Ficaria* plants, morphologically similar to the tetraploids of *F. verna* subsp. *verna*, which were later identified to be diploids (Popelka unpubl.). The production of unreduced gametes by polyploids in the present study suggests the origin of auto(allo)polyploids by unreduced gametes, at least in the past.

5.5 Prezygotic barriers do not contribute to reduction of gene flow

Prezygotic barriers are usually recognized to be most important for reproductive isolation of parental species (Rieseberg & Willis 2007; Lowry et al. 2008; Widmer et al. 2009; Baack et al. 2015; Pickup et al. 2019; Yan et al. 2019) or for stabilization of once-established hybrids (Wissemann 2007; Koutecký et al. 2011; Barke et al. 2018) The prezygotic barries usually evolve faster than postzygotic reproductive isolation (Rieseberg & Willis 2007; Lowry et al. 2008; Widmer et al. 2009; Baack et al. 2015; Yan et al. 2019). Apomixis (Petit et al. 1999; Koutecký et al. 2011), and selfing (Petit et al. 1999; Widmer et al. 2009; Koutecký et al. 2011; Brys et al. 2016; Becher et al. 2020), which are among the strongest prezygotic barriers (Petit et al. 1999; Rieseberg & Willis 2007; Lowry et al. 2008), especially in established hybrids (Barke et al. 2018), do not seem to contribute to prevent homoploid mating between studied taxa of the genus *Ficaria*.

Besides, the lacking ability of autonomous apomixis and autonomous selfing, that was observed in the present study, generally strong prezygotic barriers such as different pollinators, diverged floral morphology and flowering periods (Schemske & Bradshaw 1999), were not recorded in the studied *Ficaria* taxa (Marsden-Jones 1933; Taylor & Markham 1978; Masters & Emery 2015; present study). Therefore, the absence of above-mentioned prezygotic reproductive barriers together enables opportunities for the occasional formation of homoploid and heteroploid hybrids between the studied taxa of the genus *Ficaria*. The formation of viable seedlings in homoploid crosses between diploids (*F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and diploid plants morphologically similar to the tetraploid cytotype of *F. verna* subsp. *verna*) and in heteroploid crosses between diploids (*F. verna* subsp. *calthifolia*) and tetraploids (*F. verna* subsp. *verna*) were demonstrated (present study, Popelka et al. 2019a, respectively).Therefore, pollen-stigma incompatibility as another postpollination prezygotic barrier is weak or absent in the studied *Ficaria* taxa.

However, another prezygotic barrier such as complete ecogeographical differentiation, generally may promote complete reproductive isolation (Vallejo-Marín & Hiscock 2016). However, the ecogeographical differentiation between recognized taxa of the genus *Ficaria* is not well known (e.g., Gill et al. 1972; Taylor & Markham 1978; Kästner & Fischer 2006; Post et al. 2009; Veldkamp 2015; Popelka et al. 2019b), but mixed populations consisting of more taxa/ploidy levels were found to be rare (the total number of sites sampled were 443, Popelka unpubl.). Therefore, the recent intertaxa hybridization between *Ficaria* taxa probably could be extremely reduced in the field. However, a possible hybrid could be maintained considering the ability of vegetative reproduction in most *Ficaria* taxa (Marsden-Jones 1933; Sell 1994). Therefore, one established hybrid could persist and spread in the field such as an almost seed sterile allotriploid of *F. verna* subsp. ×*selli* (Popelka et al. 2019b).

5.6 Reproductive output of experimental crosses depends on the ploidy level and direction of crosses: consequences on hybrid fitness

Reproductive outputs by homoploid crosses between different taxa (species) are usually constrained by postzygotic barriers, i. e., endosperm failure (Lafon-Placette & Köhler 2016), hybrid inviability, hybrid sterility (Rieseberg & Carney 1998; Rieseberg et al. 1999; Lowry et al. 2008; Abbott et al. 2013; Baack et al. 2015; Vallejo-Marín & Hiscock 2016). However, these postzygotic barriers could be lacking/overcome, and homoploid hybridization results in the formation of hybrid swamps/hybrid zones by introgression (Barton & Hewitt 1985; Rieseberg et al. 1999; Abbott 2017). Investigation of intertaxa compatibility demonstrated that the postzygotic barrier via endosperm failure did not contribute to the prevent of intertaxa homoploid crosses in studied *Ficaria* taxa.

Intertaxa homoploid crosses between diploid cytotypes (*F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* and *F. verna* subsp. *verna*) and within one tetraploid cytotype (western and eastern lineages of *F. verna* subsp. *verna*), resulted in the formation of viable achenes (seeds) and in diploids also in the formation of viable seedlings. However, viable achene (seed) production per collective fruit considerably varied between different taxa/ploidy levels and pollination treatments. Different levels of reproductive isolation between the studied taxa that can have different evolutionary impact are suggested.

Intertaxa homoploid crosses of *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal x paternal taxon) produced a lower number of viable achenes (seeds) than the intrataxa (interpopulation) outcrosses of those taxa. In general, genome incompatibility increases with increasing differences in genomes between parental taxa (Ramsey & Schemske 1998) and depends on the direction of crosses (Städler et al. 2021, and the references therein).

The maternal excess crosses were found to be more successful, since endosperm failure is less common in maternal excess crosses (retrieved by Städler et al. 2021 and references therein). Asymmetric hybridization is well known from many homoploid crosses (Tiffin 2001; Lowry et al. 2008). Asymmetric hybridization was also observed in the present study, where maternal excess intertaxa homoploid crosses of *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal x paternal taxon) was more successful than the reciprocal crosses.

However, no asymmetry was observed in homoploid crosses of diploids of *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal taxa) with diploids of *F. verna* subsp. *verna* (paternal taxon). The homoploid crosses of *F. verna* subsp. *fertilis* and *F. verna* subsp. *calthifolia* (maternal taxa) and the of *F. verna* subsp. *verna* (paternal taxon) produced a comparable number of viable achenes (seeds), but these numbers were lower than in homoploid reciprocal crosses between *F. verna* subsp. *calthifolia* and *F. verna* subsp. *fertilis* and than intrataxa (interpopulation) outcrosses. The obtained pattern can be caused by the geographical distance between the studied taxa. The diploid of *F. verna* subsp. *verna* was provided from Russia, i.e., the distinct part of the distribution range of *Ficaria* taxa. In general, the genomic incompatibilities are reflected by geographic distance of the studied taxa (”Dobzhansky-Müller model”, Dobzhansky 1936). However, simply artefact cannot be also excluded, since just one plant was involved in experimental homoploid crosses in the present study.

The present study also showed that asymmetric hybridization was also not recorded in the intrataxa (interpopulation) crosses between the western and eastern populations and within the western and eastern ones of the tetraploid cytotype of *F. verna* subsp. *verna*. The yield of viable achenes (seeds) from intrataxa (interpopulation) crosses between the western and eastern populations and within western and eastern ones of *F. verna* subsp. *verna* was comparable but scarce. The obtained pattern of limited production of achenes (seeds) did not reflect the geographical distance of the involved plants. Therefore, it supports the low degree of genome differentiation between western and eastern populations of the tetraploids of *F. verna* subsp. *verna*. Observed patterns contradict with Drenckhahn et al. (2017), who proposed the existence of two separated lineages, eastern and western ones, within *F. verna* subsp. *verna* (Drenckhahn et al. 2017).

However, the observed patterns of achene (seed) production in intertaxa homoploid crosses of diploids of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, and *F. verna* subsp. *verna* and intrataxa (interpopulation) crosses of the western and eastern populations of the tetraploid cytotype of *F. verna* subsp. *verna* in the present study could be distinct from the patterns under natural conditions. In general, two possible constraints of experimental hybridization should be considered. First, in experimental conditions, the hybridization may be completely different from hybridization in natural conditions owing to the absence of natural selection and competition pressure (e.g., Popelka et al. 2019a, 2019b). Secondly, mixed pollination by intrataxa and intertaxa pollen was not realized. Therefore, we cannot rule out the possible role of pollen competition (Rieseberg et al. 1995; Baack et al. 2015; Alonso-Marcos et al. 2018) and mentor effect (Richards 1997). Experimental crosses based on mix pollination combined with revealing the origin of progeny by molecular markers would be crucial for the elucidation of the mechanism of pollen competition between *Ficaria* taxa.

Irrespective of the constraints associated with differences between expeimental and field conditions and mixed pollination, germination have strongest effect on the postzygotic barrier. Offspring produced by intertaxa homoploid, reciprocal crosses of diploids (*F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis*, *F. verna* subsp. *verna*) and outcrosses weakly differed in their germination capacity. Reduced germination capacity of seeds from intertaxa homoploid crosses of *F. verna* subsp. *calthifolia*, *F. verna* subsp. *fertilis* was probably caused by genomic incompatiblities in developing seeds, as a large amount of seemingly developed seeds are unable to germinate in the *Ficaria* taxa (Andreas 1954, Metcalfe 1939). Furthermore, seeds of *Ficaria* taxa are dormant (Salisbury 1925 cit. in Taylor& Markham 1978) and germinate gradually in several consecutive years. Sterckx observed that achenes sown in summer mostly germinate after two years (Sterckx 1900 cit. in Metcalfe 1936).

Unfortunately, the fitness of hybrids and thus the role of the potential backcrossing of hybrids with parental taxa and the production of unreduced pollen by homoploid hybrids cannot be fully assessed by the present study. *Ficaria* seedlings usually form one single cotyledon leaf and one assimilation leaf in the first year (Taylor & Markham 1978), and no offspring usually flower in the first year, almost all offspring flower in the third year (Marsden-Jones 1933). The period of this study was too short to obtain enough flowering hybrids, allowing experimental backcrossing with parents to reveal the potential of gene flow between taxa via their (homoploid) hybrids. There was just limited number of measures of pollen viability of F1 hybrids to allow statistical comparisons, but findings of pollen viability seem to be in agreement with general assumption that the pollen viability of new hybrid is lower than is their parental taxa (Ramsey & S Schemske 2002). However, persistence of once-formed hybrid in the genus *Ficaria* is suggested by the capacity of vegetative reproduction (Marsden-Jones 1933; Sell 1994), and comparable habitat requirements of hybridizing taxa that generally lead to the mitigated reduction of hybrid fitness in parental habitats (reviewed by Baack et al. 2015). Consequently, the introgressive hybridization, which is the prevailing outcome of hybridization (Gross & Rieseberg 2005), cannot be excluded, since the restoration of hybrid fertility often occurs in subsequent generations (Rieseberg 1995; Rieseberg et al. 1999; Seehausen 2004; Abbott 2017). Molecular evidence are needed to provide proof of recent gene flow between *Ficaria* taxa.

5.7 Genome size and morphology of hybrids seem to be intermediate between parental taxa

Homoploid hybridization is usually accompanied with changes in genome size, including chromosome rearrangements, amplifications of tandem repeats, activation of mobile repetitive elements, and gene expression modifications (reviewed by Glombik et al. 2020). It is hypostatized that the genome size of homoploid hybrids is intermediate between parental taxa (e. g., *Hieracium* s. str., Mráz & Paule 2006; *Cyanus* Mill., Olšavská & Löser 2013). Here, the genome size of offspring from the first filial generation formed by intertaxa homoploid crosses between diploids of *F. verna* subsp. *calthifolia*, and *F. verna* subsp. *fertilis* in reciprocal crosses almost agreed with this general assumption. The intermediate genome size of hybrids was in the genus *Ficaria,* also observed for *F. verna* subsp. ×*selli* derived from inter-ploidy hybridization between *F. verna* subsp. *verna* and *F. verna* subsp. *calthifolia* (Popelka et al. 2019b). However, shifts from the intermediate genome size of fertile hybrids would be expected in the subsequent generations by backcrossing of hybrids with parental taxa (Baack et al. 2005).

Few deviating cases in genome size of offspring derived from intrataxa (interpopulation) crosses of diploids of *F. verna* subsp. *calthifolia*, and *F. verna* subsp. *fertilis* with either slightly higher genome size or lower genome size were observed, respectively. Increasing in genome size might be explained by differences in intron size and transposon copy number between merging parental genomes. Decreasing in genome size may be generally caused by elimination of retrotransposons (Baack et al. 2005).

Hybridization is also reflected by the changes of the morphology. In general, hybrids display a mosaic parent-like, and novel trait rather than intermediate ones in the first filial and especially in the subsequent generations (Rieseberg & Ellstrand 1993, Rieseberg 1995; Rieseberg et al. 1999; Mallet 2005, Abbott et al. 2013). In contrast to this general assumption, intermediate character of homoploid hybrids was based on the visual elucidation suggested in the present study. This contradictory result might be explained by rarely occurred coherence of parental traits that is reflected by the intermediate character of the hybrids (Rieseberg 1995 and references therein). Intermediate character is furthermore common in the first filial generation, since the backcrossing and transgressive hybridization (Rieseberg 1995 and references therein), that are resulted in reinforcements associated with phenotypic divergence are occurr in subsequent generations (Ramsey & Schemske 2002). Similar intermediate character of hybrids was documented in an almost sterile allotriploid hybrid between *F. verna* subsp. *calthifolia*, and *F. verna* subsp. *verna* (*F.* ×*selli*, Pogan & Wcisło 1974, 1986; Popelka et al. 2019b) and between *F. verna* subsp. *verna* and *F. verna* subsp. *fertilis* (Mardsen-Jones & Turrill 1952).

5.8 Taxonomic implications

Hybridization may have made taxonomic difficulties in the genus *Ficaria* resulting in considerable nomenclatural chaos (Veldkamp 2015), but it also may strengthen reproductive barriers between taxa that occasionally came into contact and contribute to speciation (e. g., *Euphrasia* L., Becher et al. 2020). Previous classifications based on morphological approach (Sell 1994; Post et al. 2009; Stace 2010) and the combined geographical and morphological approach (Veldkamp et al. 2015) have delimited all recognized taxa within the genus *Ficaria* as subspecies (Sell 1994; Post et al. 2009; Stace 2010; Veldkamp et al. 2015). In contrast to delimitation as subspecies, several recently published studies based on the cytogenetic approach with knowledge of the genome size and ploidy level (Zonneveld et al. 2015), and the morphological approach (Drenckhahn 2016; Vázquez 2016) suggest an infrageneric classification of all recognized taxa as species (Zonneveld et al. 2015; Drenckhahn 2016; Vázquez 2016).

Various taxonomic classifications are caused by the existence of morphologically intermediate phenotypes, overlapping values of morphological traits between taxa, strong morphological plasticity (Sell 1994; Post et al. 2009; Veldkamp et al. 2015; Drenckhahn 2016; Popelka et al. 2019b; Uhlířová 2019), shared haplotypes in different taxa (Sochor unpul.) and observed genome size continuum (Konečná 2018). However, the present study suggests that the hybridization between taxa with the same ploidy level potentially can occur at sites where different taxa co-occur. In general, this ability of gene flow might be explained by the low degree of the genomic divergence between parental taxa and close evolutionary relationships (Paun et al. 2009). Therefore, I expect that the recent divergence of *Ficaria* taxa is not enough for the creation of sufficient reproductive isolation barriers. This mechanism at the microevolutionary level is supported by Yakimowski & Reiseberg (2014), who found that the creation of effective reproductive isolation barriers, particularly within homoploid hybrids, is long-term process. Thus, successful hybridization and backcrossing may threaten the existence of closely related parental taxa of the genus *Ficaria* because of the demographic replacement of parental taxa or the breakdown of the genetic integrity of parental taxa. Blurring species borders is a quite widespread phenomenon in the field (e.g., Rhymer & Simberloff 1996). Therefore, in line with Sell (1994), Post et al. (2009), Stace (2010), and Veldkamp et al. (2015), I proposed classification of diploids of *F. verna* subsp. *caltihifolia* and *F. verna* subsp. *fertilis* and tetraploids of *F. verna* subsp.

verna in the genus *Ficaria* as subspecies. In addition, rare production of unreduced gametes may lead to the (auto)hexaploid origin of the hexaploid cytotype of *F*. *verna* subsp. *verna*. Additional, detailed studies on nuclear and chloroplast markers, chromosome number, and morphology can be useful to the comprehensive taxonomic classification of the *Ficaria* taxa.

6. Conclusion

By combining a quantitative and qualitative study of autonomous apomixis, autonomous selfing, and pollen viability & length of most *Ficaria* taxa, and experimental intertaxa homoploid crosses between selected taxa, the present study demonstrates that the prezygotic and postzygotic reproductive isolation mechanisms between selected taxa of the genus *Ficaria* are weak. Autonomous apomixis and autonomous selfing seem to not contribute to reproductive isolation or to maintain the genetic integrity of established hybrids. Even pollen viability does not act as barrier against gene flow. Hence, the potential hybridization, and persistence of hybrids via vegetative reproduction, especially in polyploids, could threaten the taxa integrity of the genus *Ficaria* at sites where they co-occur.

Nevertheless, further studies to fill the gaps in the knowledge of prezygotic and postzygotic reproductive barriers are needed, together with additional studies on unreduced gamete formation, germination capacity of progeny and experiments on gene flow from hybrids to parental taxa, are necessary to understand the strength of reproductive isolation barriers and the role of hybridization and backcrossing in the genus *Ficaria*. Additional studies upon using molecular markers (e.g., AFLP, cp DNA, microsatelites) are required for understanding the phylogenetic structure of the genus *Ficaria*.

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8. Appendices

Appendix 1: Detailed summary of population of *Ficaria* taxa included in this study.

Population	Taxon	Ploidy	Country	Locality	Latitude, longitude	Altitude (m a.s.l.)	Description of the locality	Collector	Treatment
11_01	FC;FS; FV	2x, 3x, 4x	Czech Republic	Plumlov	49°27'17.000"N, 17°1'19.000"E	280	edge of Wet scrubs at the stream in the natural reservation Kněží Hora	Trávníček B.	pollen viability, pollen length, genome size (according to Konečná 2018)
11_02	FV	4x	Austria	Nagelberg near Gmünd	48°50'28.000"N, 14°59'46.000"E	510	grasslands in central part of the village	Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
11_03	FC	2x	Czech Republic	Havraníky	48°48'40.000"N, 16°0'30.000"E	300	grasslands at the church in the village	Trávníček B.	pollen viability, pollen length
12_01	FV;FFE	2x, 4x	Great Britain	Aberdeen	57°8'16.000"N, 2°3'2.160"W	11	road edge in Walker Park	Mládek J.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment, genome size (according to Konečná 2018)
12_02	FV	4x	Great Britain	Aberdeen	57°7'42.000"N, 2°7'31.000"W	22	grasslands in central part of the town	Mládek J.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
12_03	FFE	2x	Great Britain	Newtonhill	57°2'12.000"N, 2°8'49.000"W	47	grasslands in the NE edge of the town	Mládek J.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment, genome size (according to Konečná 2018)
12_04	FV	4x	Great Britain	Newtonhill	57°1'57.000"N, 2°8'55.000"W	59	grasslands in central part of the town	Mládek J.	pollen viability, pollen length, genome size (according to Konečná 2018)
12_10	FC	2x	Czech Republic	Lužec	50°12'45.000"N, 15°24'58.000"E	230	wet grasslands in central part of the village	Šiková P.	pollen viability, pollen length

12_12	FC	2x	Czech Republic	Vinary	50°17'13.000"N, 15 °26'1.000"E	268	wet grasslands at the gate of communal farming	Šiková P.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
12_14	FV	4x	Czech Republic	Domanice	49°54'22.199"N, 16 °3'23.567"E	355	wet meadows in edge part of the village	Duchoslav M.	pollen viability, pollen length, genome size (according to Konečná 2018)
12_16	FC	2x	Czech Republic	Horky u Milotic	48°56'37.000"N, 17 °8'5.000"E	225	grasslands in the amphitheatre	Duchoslav M., Šiková P., Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
12_17	FC;FS	2x, 3x	Czech Republic	Dobelice	49°1'1.000"N, 16°1 6'19.000"E	256	*Robinia* groves at the road in the west enge of the village	Duchoslav M., Šiková P., Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
13_04	FV	4x	Hungary	Sajkod	46°54'42.000"N, 17 °51'19.000"E	220	wet canopy gap in the forests	Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
14_01	FC	2x	Hungary	Tiszasas	46°49'52.000"N, 20 °5'31.000"E	90	dry grasslands at the road	Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
14_03	FC	2x	Slovakia	Nitra	48°17'55.000"N, 18 °5'28.000"E	500	tree stands in the the S edge of the town	Trávníček B.	pollen viability, pollen length, genome size (according to Konečná 2018)
14_05	FV	4x	Slovakia	Kozárovce	48°18'33.000"N, 18 °31'34.000"E	300	edge of the field at the forest	Trávníček B.	pollen viability, pollen length
14_13	FV	4x	Bulgaria	Sofia	42°37'12.720"N, 23 °17'58.260"E	1010	oak forests along the road at Dragalevtsi Monastery	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)

15_2	FC	2x	Slovakia	Vinosady	48°18'36.961"N, 17°17'36.065"E	175	grasslands in central part of the village	Trávníček B.	pollen viability, pollen length
15_3	FV	4x	Slovakia	Nitra	48°20'42.7"N, 18°05'36.9"E	328	forests at the medical resort Zobor: Zobor 2	Trávníček B.	pollen viability, pollen length
15_4	FC;FS;FV	2x,3x,4x	Slovakia	Svatý Jur	48°14'15.6"N, 17°12'10.8"E	145	meadows with randomly placed trees at chapel Svatý Jur	Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
15_8	FV	4x	Serbia	Bačevci	44° 08' 59,73"N, 19° 54' 42,28"E	417	mesic alder groves and scubs at the stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
15_10	FV	4x	Serbia	Nova Varoš	43°27,903"N, 019°43,974"E	710	mesic and wet alder groves on the bank of the river	Popelka O.	pollen viability, pollen length
15_11	FV	4x	Montenegro	Kosanica	43°09,909"N, 019°17,179"E	905	mesic *Carpinus* forests	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment, genome size (according to Konečná 2018)
15_12	FV	4x	Montenegro	Šljivansko	43°07,806"N, 019°18,614"E	674	mesic and wet alder forests at confluence of stream and .	Popelka O.	pollen viability, pollen length, crossing experiment
15_14	FV	4x	Montenegro	Mojkovac	42°54,966"N, 019°34,541"E	860	masic scrubs on the bank of the river	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
15_17	FC	2x	Montenegro	Vladni	42°20,262"N, 019°17,516"E	30	dry, ocassionaly mesic meadows verge on dry grasslands	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
15_18	FV	4x	Montenegro	Bukovik	42°12,675"N, 019°00,254"E	628	tree stands	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment, genome size (according to Konečná 2018)
15_19	FC	2x	Montenegro	Cetinje	42°23,742"N, 018°50,528"E	1466	sunny and dry grasslands with limestone outcrops	Popelka O.	reproductive modes (apomixis, autogamy)

15_22	FC	2x	Bosnia and Herzegovina	Neum	42°54'43.260"N, 17°37'41.940"E	27	verge of semi sunny and sunny scrubs at the meadow road	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment, genome size (according to Konečná 2018)
15_23	FV	4x	Croatia	Murvica	44°09,141"N, 015°20,134"E	63	shady scrubs edge on the bank of canal filled by water	Popelka O.	reproductive modes (apomixis, autogamy), crossing experiment
15_24A,B	FC?;FS?;FV?	2x,3x,4x	Croatia	Zagraj	45°33'33,63"N, 015°36'35,96"E	116	semi-sunny edge od oak forests with developed scrubs	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
15_31	FV	4x	Poland	Nowy Żmigród	49°36'42"N, 21°31'54"E	285	grasslands at the Jewish cemetery (at the road towards Jasło town)	Trávníček B.	pollen viability, pollen length
15_37	FC	2x	Czech Republic	Hradčany	50° 09,450"N, 15° 17,7	243	old overgrown orchard to the edge of an oat meadow	Popelka O.	pollen viability, pollen length
15_39	FC	2x	Czech Republic	Milá	50° 26' 08,9"N, 013° 45' 37,1"E	414	*Fraxinus* forests at the foot of the hill Milá	Popelka O.	pollen viability, pollen length
15_41	FC	2x	Czech Republic	Klapý	50° 26' 03.6"N, 14° 00' 54.0"E	386	mixed deciduos board- leaved and coniferous forests at the road on the top of hill with castle Hazmburk	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
15_42	FC	2x	Bulgaria	Vlahi	41°44'25.7"N, 23°13'40.2"E	520	a recently developed *Ostrya* scrubs in the edge of a cementary in the E part of the village	Kalous R.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
16_01	FV	4x	Germany	Ammerndorf	49°25'01"N, 10°51'15"E	300	wet bank of the water canal	Trávníček B.	pollen viability, pollen length
16_03	FV	4x	Germany	Altenbamberg	49°47'39.000"N, 7°49'18.000"E	210	wet path verge at the stream Grasbach	Trávníček B.	pollen viability, pollen length, genome size (according to Konečná 2018)

16_04	FC	2x	Croatia	Paklenica	44°17'37.48"N, 15° 27'28.66"E	22	lower parking place, at the main gate to the canyon Velké Paklenice, left bank of the river	Sochor M.	pollen viability, pollen length
16_05	FC	2x	Croatia	Starigrad	44°17'12.22"N, 15° 27'8.10"E	11	garden grassland in the camp Marco in the SE edge of the town	Sochor M.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_06	FC	2x	Croatia	Paklenica - Jurline	44°19'12.78"N, 15° 29'20.14"E	625	thermophilous oak forests in the national park Paklenica, approx. 50 m SE from the disappearead village Škiljići at path to Jurline	Sochor M.	pollen viability, pollen length
16_08	FS?	3x	Switzerland	Brione	46°17'18.649"N, 8°47'49.904"E	705	forests at the path next to the river	Předotová M.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_10	FV	4x	Finland	Meriehamn	60°2'48"N, 19°58'28"E		meadow (former cultivated field) at the parking place of the Nato Biological Station.	Hæggström C. A.	pollen viability, pollen length
16_12	FV	4x	Slovenia	Gaberje	45°49'57"N, 13°52'29"E	150	mesic meadow, SW from the village	Trávníček B.	pollen viability, pollen length
16_16	FV	4x	Germany	Neudorf	49°50'42.540"N, 10°23'46.464"E	275	alder groves at the stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_21	FV	4x	Germany	Würmersheim	48°56'19.536"N, 8°14'30.840"E	100	wet and shady ditch in the deciduos board-leaved forests	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_22	FV	4x	France	Saint-Bernard	47°39'38.232"N, 7°13'3.072"E	270	wet and sunny pastures	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment

16_23	FV	4x	France	Aibre	47°33'1.224"N, 6°41'37.572"E	350	sunny road edge at the meadows	Popelka O.	pollen viability, pollen length
16_24	FV	4x	France	Chaudefontanie	47°20'20.904"N, 6°9'29.808"E	290	semi-sunny ecoton between parking place and forests	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment,
16_25	FV	4x	France	Monay	46°49'58.692"N, 5°36'16.776"E	260	sunny and wet road ditch	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
16_27	FFE	2x	France	Chuzelles	45°35'35.304"N, 4°51'25.128"E	260	shady Carpinus forests on the slope at resting place	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
16_30	FFE	2x	France	Mercurol	45°5'40.236"N, 4°53'36.996"E	266	semi-sunny road edge at *Aesculus* stands	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
16_32	FV?	4x	France	Lapalud	44°18'40.428"N, 4°41'3.840"E	45	sunny and wet marsh vegetation	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_34	FCH	4x-5x	France	Signes	43°17'8.304"N, 5°53'31.272"E	311	grasslands on the bank of a stream at *Platanus* alley	Popelka O.	reproductive modes (apomixis, autogamy)
16_36	FV	4x	France	Hyeres	43°9'3.924"N, 6°8'16.116"E	8	shady poplar groves at the stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_37	FFE	2x	France	Gassin	43°13'28.776"N, 6°34'17.184"E	28	shady alder groves on the bank at the stream .	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size
16_38	FFE	2x	France	Draguignam	43°32'54.060"N, 6°34'22.944"E	157	periodic bed of the stream edged by scrubs and adjacent grasslands, sandy soil	Popelka O.	pollen viability, pollen length

16_39	FFI	4x	France	Saint-Laurent-du-Var	43°41'34.620"N, 7°11'6.144"E	25	wet and sunny ditch with standing water with adjacent marsh vegetation	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
16_40	FFI?	4x	France	Carros	43°46'29.064"N, 7°12'57.780"E	70	wet and sunny canals at the road	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_42	FFI	4x	France	Sospel	43°52'15.312"N, 7°24'18.396"E	893	semi-shady shrubs along a mountain stream	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
16_43	FV	4x	Italy	Tetti Mecci	44°11'0.456"N, 7°33'45.972"E	1131	mixed deciduous board-leaved forests at a mountain stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_45	FV	4x	Italy	Varazze	44°23'5.244"N, 8°33'16.776"E	75	shady deciduous board-leaved forests at a stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_47	FV	4x	Italy	Mocones	44°24'43.344"N, 9°13'24.168"E	95	shady, gravel-sandy bar in the forests at the stream	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_49	FV	4x	Italy	Baverino Castello	44°12'12.132"N, 9°48'17.568"E	65	sunny and grassy slope above the road at the vineyard	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_50	FFI	4x	Italy	Massa	43°58'58.584"N, 10 °8'14.352"E		shady alder-poplar groves in the wet depression	Popelka O.	pollen viability, pollen length
16_51	FV	4x	Italy	Gualdo	43°54'13.860"N, 10 °21'15.444"E	176	wet and grassy road verge at the stream	Popelka O.	pollen viability, pollen length, crossing experiment
16_56	FV	4x	Italy	San Michele Al Tagliamento	45°46'10.776"N, 12 °59'7.728"E	2	wet and sunny ditch at the road	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)

16_59	FFI	4x-5x	Slovenia	Izola	45°32'24.432"N, 13 °40'54.912"E	9	wet and sunny ditch at the vineyard	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_67	FV	4x	Austria	Schillingsdorf	47°6'11.340"N, 15° 33'50.328"E	519	alder forests on the slope at the wet canal and adjacent *Fagus* forests	Popelka O.	pollen viability, pollen length
16_69	FV	4x	Austria	Markt Sankt Martin	47°33'40.248"N, 16 °25'1.344"E	313	grassy bank of the river lined by alder, willow and poplar	Popelka O.	pollen viability, pollen length
16_71	FV	4x	Germany	Weilheim in Obernbayern	47°50'21"N, 11°11' 43"E	620	scrubs at the stream	Trávníček B.	pollen viability, pollen length
16_72	FV	4x	Germany	Ettal	47°34'23"N, 11°04' 50"E	840	meadow at the road towards Oberammergau village	Trávníček B.	pollen viability, pollen length
16_73	FV	4x	Finland	Helsinki	60°10'31" N, 24°56'40" E	10	garden grassland under deciduous and coniferous trees in the W part of the garden	Hæggström C. A.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_74	FV	4x	Slovakia	Hlohovec	48°23'48"N, 17°51' 03"E	185	N from the village, woodland (*Quercus*, *Robinia*)	Trávníček B.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), genome size (according to Konečná 2018)
16_75	FC	2x	Slovakia	Kamenín	47°52'46"N, 18°38' 35"E	120	S from the village, roadsides and adjacent *Robinia* groves	Trávníček B.	pollen viability, pollen length
16_76	FC	2x	Slovakia	ChĺaBa	47°50'05"N, 18°49' 35"E	115	grasslands and bushes at NE village margin	Trávníček B.	pollen viability, pollen length
16_88	FV	4x	Romania	Cisnadie	45°40'12.7"N, 24°0 7'24.6"E	941	forests	Kobrlová L., Hroneš M.	pollen viability, pollen length, genome size (according to Konečná 2018)

16_89A,B	FV	4x	Finland	Salo	Hlikko: 60°23'41.892"N, 23 °4'31.082"E, Häävälä: 60°26'25.330"N, 22 °59'9.500"E	16	Ab (Regio Aboënsis), Salo, Halikko, Häävälä, clayey meadow on the eastern bank of River Halikonjoki about 20 m N and 170 m S of the brook Rainoja. Uniform Coordinate System (UCS): Grid 27 °E 671083:328070 and 671064:328066. Helena Åström & Carl-Adam Hæggström, May 20, 2016. (Ab, Salo, Halikko, Häävälä: larger plants (larger package) are from the first place (first mentioned coordinates), and smaller ones from the second place (671064: 328066). The places are situated near each other so this sample could be regarded as one population.)	Åström H. & Hæggström C. A.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_92	FV	4x	Poland	Brzeźnica	49°58'03.8"N, 19°3 6'16.4"E	240	ditch at the deciduous board-leaved forests	Kalous R.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_93	FV	4x	Sweden	Kågeröd	55°59'47.983"N, 13 °5'23.491"E	50	luxuriant deciduous wood in the ENE part of Kågerödslund	Hæggström C. A.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_94	FV	4x	Denmark	Gentofte	55°45'0.000"N, 12° 32'0.000"E	20	foot path between Fiskebakken and the E shore of Lake Gentofte	Hæggström C. A.	pollen viability, pollen length, genome size
16_95	FV	4x	Poland	Radojewo	52°29'44.2" N, 16°57'19.7"E	113	mesic deciduous board-leaved floodplain forests on the slope, aluminous-sandy substrate	Kalous R.	pollen viability, pollen length, genome size (according to Konečná 2018)

16_96	FV	4x	Lithuania	Plokščiai	55°4'27.000"N, 23° 10'32.000"E	60	alder forests at streamlet	Karpavičienė B.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_97	FV	4x	Lithuania	Kretkampis	55°2'9.000"N, 23°3 1'15.000"E	40	alder forests at streamlet	Karpavičienė B.	pollen viability, pollen length, genome size (according to Konečná 2018)
16_98	FV	4x	Lithuania	Pravieniškés	54°56'24.000"N, 24 °14'59.000"E	40	Ass. *Circaeo-Alnetum* Oberdorfer 1953, along	Karpavičienė B.	pollen viability, pollen length
16_99	FV	4x	Lithuania	Vilnius	54°41'45.000"N, 25 °18'25.000"E	120	Vilnius city park dominated by *Acer platanoides* and *Tilia cordata*	Karpavičienė B.	pollen viability
17_01	FV	4x	Germany	Bonn	50°42'39.776"N, 7°8'48.355"E	52	forest in the Freizeitpark Rheinaue	Kobrlová L.	pollen viability, pollen length
17_03	FV	4x	Poland	Żmigród	51°28'50.135"N, 16 °54'51.437"E	100	forest in the adjacent gas station Parkowa 2	Horák D.	pollen viability, pollen length
17_04	FV	4x	Germany	Memleben	51°16'39.000"N, 11 °30'49.000"E	145	scrubs at the Wangen	Trávníček B.	pollen viability, pollen length
17_08	FV	4x	Great Britain	Bradford	53°50'10.462"N, 1° 48'17.312"W	90	low lying *Alnus glutinosa* woodland; also sparse *Acer pseudoplatanus*, *Ilex aquifolium* and occasional *Salix x fragilis*, ground flora very patchy	Wilcox M.	pollen viability, pollen length
17_12	FV	4x	Luxembourg	Noertzange	49°30'04.3"N, 6°03'14.2"E	278	bank with trees at regulated stream	Popelka O.	pollen viability, pollen length
17_14	FV	4x	France	Wignicourt	49°34'46.5"N, 4°35'26.9"E	178	grassland at the road adjacent by deciduos board-leaved forest	Popelka O.	crossing experiment
17_15	FV	4x	France	Saint-Aignan	49°38'46.5"N, 4°49'26.8"E	170	wet meadow at the stream	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment

17_17	FV	4x	Netherlands	Urmond	50°59'01.3"N, 5°45'38.2"E	40	shady poplar grove	Popelka O.	pollen viability, pollen length
17_19	FV	4x	Belgium	De Kolonie	51°15'50.8"N, 5°21'44.7"E	38	shady, mixed deciduous board-leaved forest dominated by *Prunus*, *Tilia* and *Alnus*	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_20	FV	4x	Netherlands	Waardenburg	51°49'58.7"N, 5°15'54.5"E	4	shady, mixed deciduous board-leaved forest at canal	Popelka O.	pollen viability, pollen length
17_22	FV	4x	Netherlands	Egmond aan Zee	52°36'45.569"N, 4°37'30.832"E	10	wet depression with trees in the dunes	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_24	FV	4x	Netherlands	Wageningen	51°57'52.6"N, 5°41'15.1"E	20	shady, mixed, deciduous board-leaved forest on the slope at arboretum	Popelka O.	pollen viability, pollen length
17_28	FV	4x	Netherlands	Harderwijk	52°21'01.9"N, 5°37'01.2"E	7	sunny, grassland at the stream adjacent by deciduous board-leaved forest	Popelka O.	pollen viability, pollen length
17_30	FV	4x	Germany	Innenstadt-Weststadt	52°15'59.9"N, 8°00'35.2"E	74	semi- shady grassland between foot path and scrubs in the town	Popelka O.	pollen viability, pollen length
17_32	FV	4x	Germany	Emmerthal	52°03'17.7"N, 9°22'03.9"E	73	mixed, deciduous board-leaved grove	Popelka O.	pollen viability, pollen length
17_33	FV	4x	Germany	Volpriehausen	51°39'34.8"N, 9°45'24.5"E		wet scrubs at meadow	Popelka O.	pollen viability, pollen length
17_41	FC	2x	Austria	Pyhra	48°35'34.5"N, 16°2 2'54.0"E	300	gap in the canopy of *Robinia* groves at the margin of the village	Duchoslav M.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
17_43	FC	2x	Austria	Guntersdorf	48°38'56.0"N, 16°2' 58.7"E	250	mowed grassland along the road in the centre of the	Duchoslav M.	pollen viability, pollen length
17_46	FV	4x	France	Torcy	50°29'7.622"N, 2°1' 19.224"E	100	deciduous board-leaved grove at the road	Roussel J.J.	pollen viability, pollen length
17_50	FS;FV	3x,4x	Croatia	Gradec Pokupski	45°33'10"N, 15°51'2 8"E	105	deciduous board-leaved forests at the parking place	Trávníček B.	pollen viability, pollen length

17_55	FV	4x	Germany	Konstanz	47°40'43"N, 09°09'36"E	400	grassland at the road	Trávníček B.	pollen viability, pollen length
17_56	FFE	2x	France	Lamothe	45°18'52.092"N, 3°25'32.343"E	450	tree stands in the N part of the town, lamothe 43	Tort M.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_57	FFE	2x	France	Le Pont de Lamothe	45°18'14.769"N, 3°24'18.070"E	420	deciduous board-leaved forests at bank of the river	Tort M.	pollen viability, pollen length
17_63	FCH	4x	Greece-Krete	SPILI	35°13'8.862"N, 24° 32'7.590"E	425	grassland in the centre of the town	Dančák M.	reproductive modes (apomixis, autogamy)
17_64	FCH	5x	Greece-Krete	OMALOS	35°20'37.978"N, 23 °54'16.325"E	1050	under randomly occurred trees	Dančák M.	reproductive modes (apomixis, autogamy)
17_69	FFE	2x	Great Britain	Preston, Bamber Bridge	53°43'10.4"N 2°39' 35.3"W	50	grassland in the St Saviour's Churchyard	Earl D.P., Earl J.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
17_70	FV;FFE	2x,4x	Great Britain	Preston, Bamber Bridge	53°43'10.4"N 2°39' 35.3"W	50	grassland in the St Saviour's Churchyard	Earl D.P., Earl J.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
17_71	FFE	2x	France	Maumusson	47°29'7.715"N, 1°6' 46.315"W	50	grassland at the road edge	Geslin J.	pollen viability, pollen length
17_77	FC	2x	Serbia	Basaid	45°38'13.0"N, 20°2 4'2.6"E	84	grassland at the road, sparse *Prunus* scrubs	Popelka O.	pollen viability, reproductive modes (apomixis, autogamy)
17_81	FV	4x	Macedonia	Katlanovo	41°52'54.9"N, 21°4 1'18.0"E	230	scrubs at the ditch between fields	Popelka O.	pollen viability, pollen length
17_83	FFI	4x	Greece	Lefkadia	40°39'51.4"N, 22°0 8'02.2"E	61	shady riparian forests at the river	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_85A	FC	4x	Greece	Kato Milia	40°14'10.4"N, 22°1 9'46.2"E	198	shady *Ostrya* grove	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
17_85B	FC	4x	Greece	Kato Milia	40°14'10.304"N, 22 °19'43.294"E	190	sparse pasture woodland around the stream	Popelka O.	pollen viability, pollen length

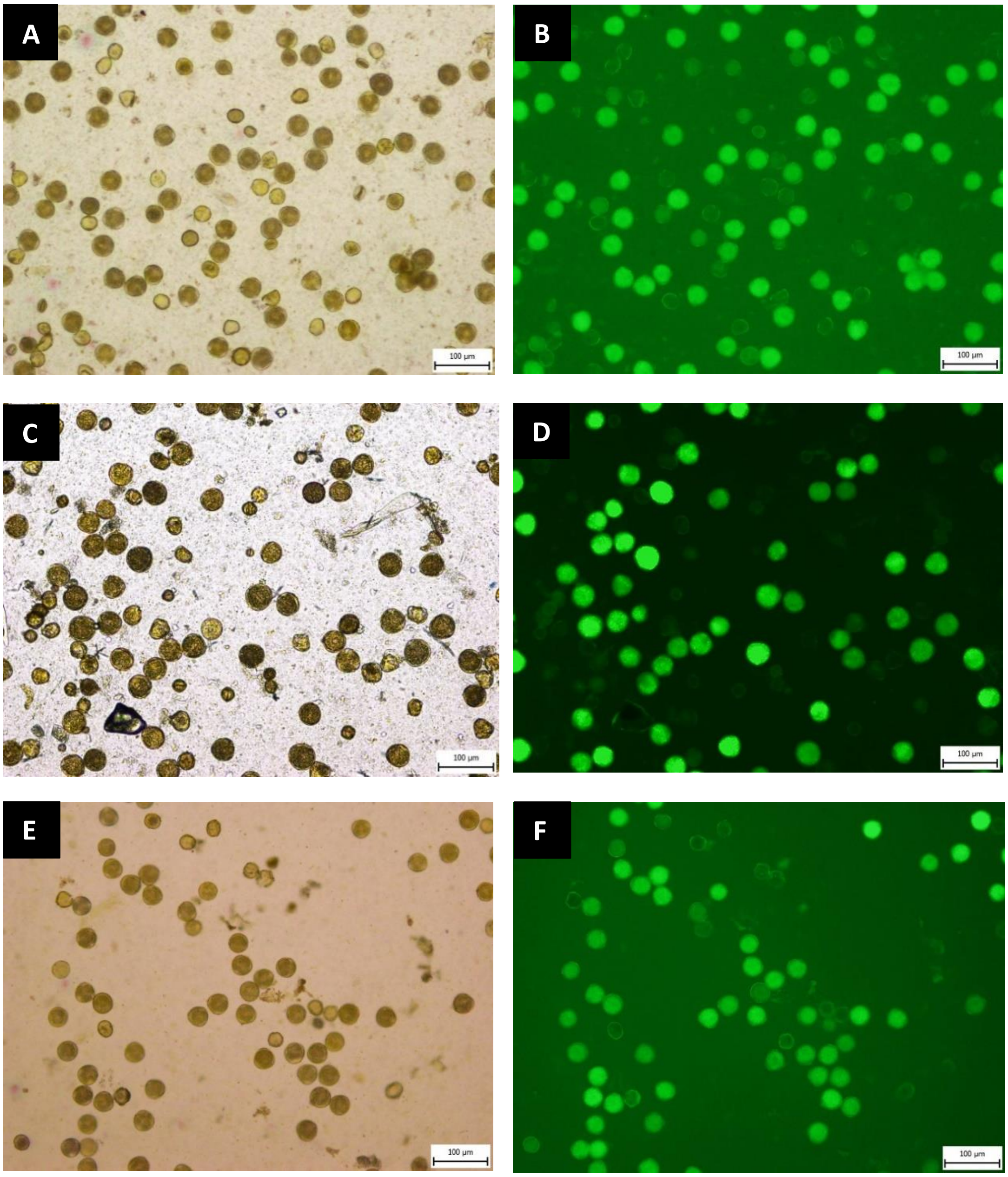
17_86	FCH	5x	Greece	Agios Stefanos	39°01'31.7"N, 22°13'56.1"E	507	sunny, semi-shady edge of the field and scrubs, centre of the field, grove at the stream	Popelka O.	reproductive modes (apomixis, autogamy)
17_91	FV	6x	Greece	Karpenisi	38°56'29.062"N, 21°45'30.176"E	1423	wet, probably grazed grassland at the edge of *Picea* woodland with *Juniperus*	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_93	FFO	2x	Greece	Kato Lousi	38°0'15.733"N, 22° 11'46.393"E	1720	sunny, stony and wet places at the ski resort	Popelka O.	pollen viability, pollen length
17_96	FC	4x	Greece	Chalkianika	38°02'07.2"N, 22°15'11.8"E	940	semi-shady orchard and adjacent meadow	Popelka O.	pollen viability, pollen length, genome size (according to Konečná 2018)
17_97	FCH	4x	Greece	Velo	37°59'40.5"N, 22°45'51.5"E	3	grassy stand at the water canal	Popelka O.	pollen viability, reproductive modes (apomixis, autogamy)
17_98	FV	4x	Greece	Stathmos Afidnon	38°10'38.712"N, 23°51'47.736"E	248	riparian valley with deciduous forest and meadow with sparse trees	Popelka O.	pollen viability, pollen length
17_99	FV	5x	Greece	Pyrgetos	39°54'26.100"N, 22°37'6.960"E	23	shady, clay bank above the path in the forest (floodplain forest)	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_100	FV	5x	Greece	Neos Panteleimonas	40°0'23.364"N, 22° 35'53.160"E	100	semi-shady, clay bank of the road on the edge of shrubs, vegetation 3 x 3 m (under the castle wall)	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_101	FV	4x	Macedonia	Demir Kapija	41°23'54.852"N, 22°18'38.628"E	8	riparian valley with deciduous broad-leaved f…	Popelka O.	pollen viability, pollen length
17_105	FV	4x	Romania	Stoenesti	44°07'32.1"N, 24°30'27.7"E	74	shady floodplain forest	Popelka O.	pollen viability, pollen length
17_108	FFI	4x,5x	Bulgaria	Provadia	43°08'51.5"N, 27°27'23.6"E	36	*Carpinus* forests on the slope	Popelka O.	pollen viability, pollen length

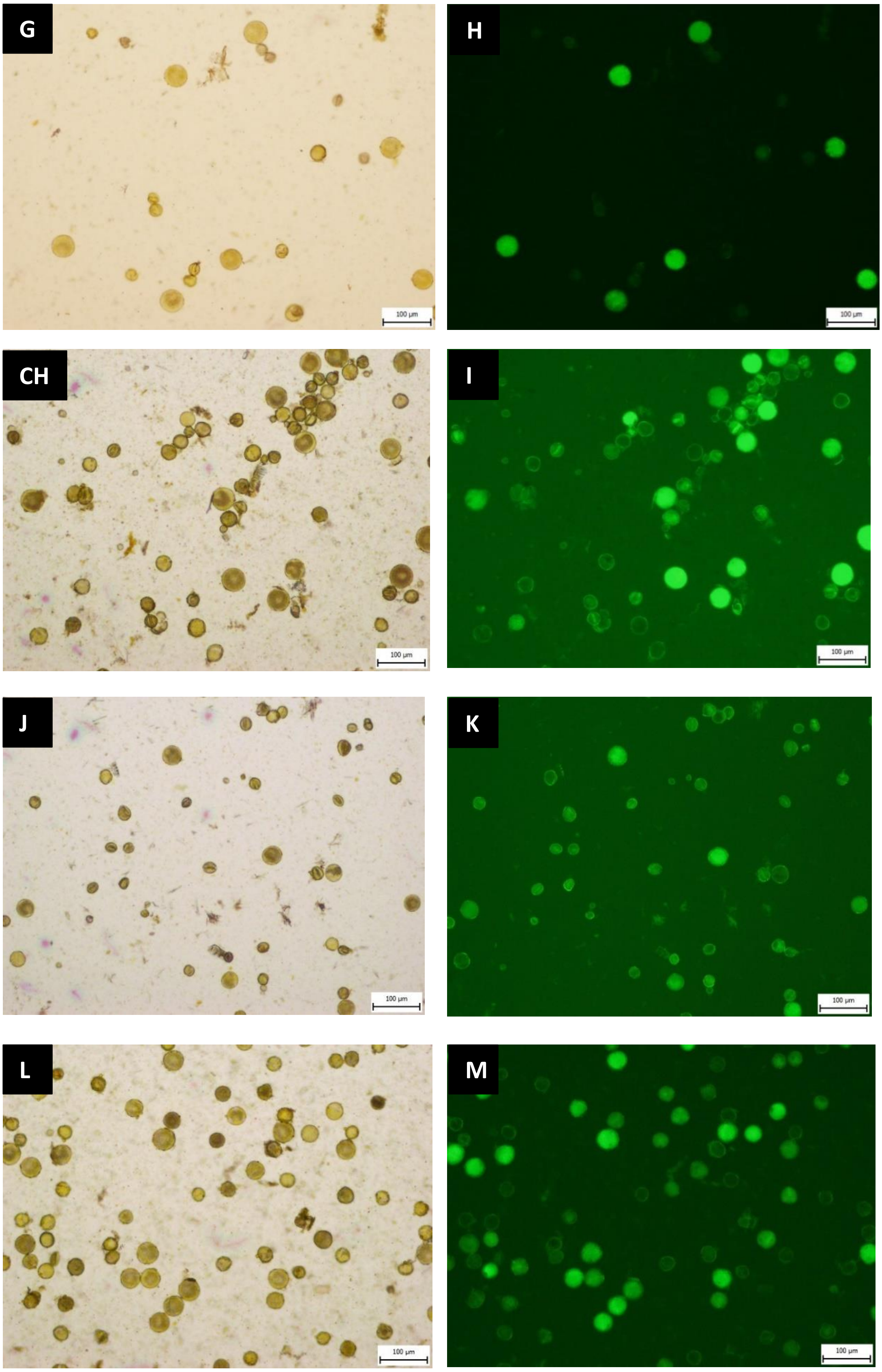
17_109	FFI	5x	Bulgaria	Venets	42°38'55.6"N, 26°51'27.2"E	195	deciduous broad-leaved forests along the stream	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_110	FFI	4x	Bulgaria	Chukarovo	42°09'38.9"N, 26°21'12.3"E	154	*Carpinus* forests along the stream	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_111	FV	4x	Bulgaria	Nadejden	41°53'49.2"N, 25°57'27.7"E	65	deciduous broad-leaved forests with trampled paths	Popelka O.	pollen viability, pollen length
17_112	FV	4x	Bulgaria	Plovdiv	42°09'21.5"N, 24°45'55.3"E	160	mesic and wet deciduous broad-leaved forests in the	Popelka O.	pollen viability, pollen length
17_113	FV	4x	Bulgaria	Ustina	42°02'51.9"N, 24°31'38.6"E	246	semi sunny grasslands under trees along the road	Popelka O.	pollen viability, pollen length
17_117	FV	5x	Serbia	Vrelo	43°22'19.920"N, 22°3'5.067"E	440	wet and mesic grassy path along the stream under forests	Popelka O.	pollen viability, pollen length, reproductive modes (apomixis, autogamy)
17_118	FV	4x	Romania	Svatá Helena	44°40'41.777"N, 21°42'43.383"E	310	deciduous broad-leaved forests on the edge of the	Popelka O.	pollen viability, pollen length
17_119	FFE	2x	Great Britain	North Dell	58°28'41.652"N, 6°18'25.941"W	20	Airnistean, sea chii (influenced by blown sand), Lewis, Outer Hebrides	Smith P.	pollen viability, pollen length
17_125	FV	4x	Ukraine	Khlibodarivka	46°23'40.320"N, 33°48'43.200"E	26	irrigated field	Moysiyenko I.	pollen viability, pollen length
18_03	FFE	2x	France	Angers	47°28'49.6"N, 0°36'25.8"W	50	grasslands in the area of Agrocampus Ouest in the W part of the town	Smýkal P.	reproductive modes (apomixis, autogamy), crossing experiment
18_04	FFE	2x	Great Britain	Rottingdean	50°48'18.446"N, 0°3'47.376"W	35	pasture 70 m S of the Rottingdean Windmill	Brantová P.	reproductive modes (apomixis, autogamy), crossing experiment
18_05	FFE	2x	Great Britain	East Dean	50°45'29.812"N, 0°12'31.025"E	50	pasture near the centre of the village	Brantová P.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment

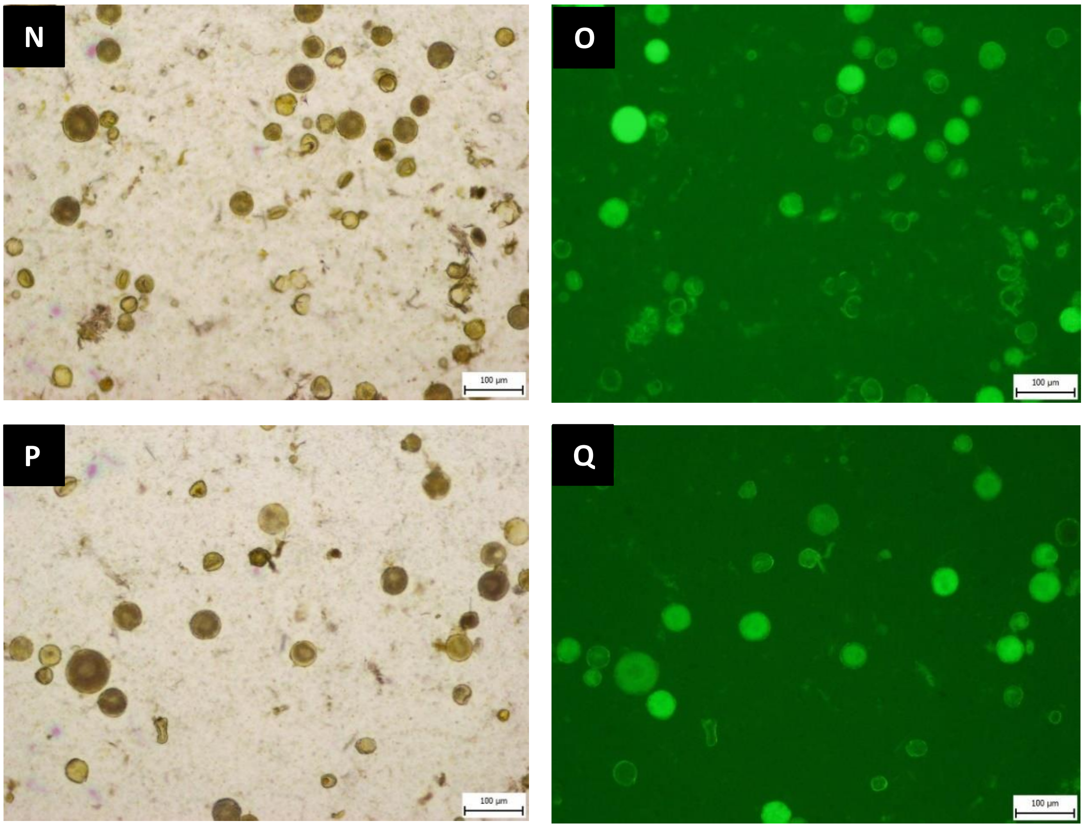
18_12	FV	4x	Ukraine	Svaliavka	48°40'31.9"N, 22°4 8'38.4"E	280	road ditch	Hroneš M., Kobrlová L.	pollen viability, pollen length
18_14	FC	2x	Hungary	Parád	47°55'32.000"N, 20 °3'28.000"E	223	road ditch in the centre of village	Trávníček B.	pollen viability, pollen length, crossing experiment
18_17	FV	4x	Croatia	Podstena	45°25'16.3"N 14°54 '13.6"E	650	edge of forests with dominance of *Abies* and *Fagus*, at the road to the	Sochor M.	pollen viability, pollen length, reproductive modes (apomixis, autogamy), crossing experiment
18_19	FC	2x	Bosnia and Herzegovi na	Šuica	43°49'9.780"N, 17° 10'13.740"E	1090	forests in distance of 300 m W from the road in direction to Zagoričani	Hroneš M., Kobrlová L.	pollen viability, pollen length
19_12	FFI	4x	Portugal	Castelo	39888760, - 8562333	230	grassland along the filed road N of the village	Loureiro J.	pollen viability, reproductive modes (apomixis, autogamy)
19_34	FV	4x	Germany	Urloffen	48°33'38.000"N, 7°56'40.000"E	150	wet shrubs near road in the W part of the village	Trávníček B.	pollen viability, pollen length
19_36	FFE	2x	Great Britain	Pendleton	53°51'5.837"N, 2°2 2'6.172"W	140	All Saints Churchyard	Earl D.P., Earl J.	pollen viability, pollen length
19_38	FV	4x	Croatia	Baške Oštarije	44°32'19.194"N, 15 °9'40.860"E	971	Stupačinovo settlement NW of the village, *Fagus* forest and adjacent pasture/meadow	Sochor M.	pollen viability
19_56	FV?	2x	Russia	Serpukhov	54°54'21.5"N 37°25'58.2"E	150	private garden on the street Ulitsa Lermonova	Shovkun M.	pollen viability, pollen length
19_59	FV	4x	Denmark	Rønde	56°16'57.929"N, 10 °28'50.304"E	1	grasslands along the seacoast, Grevens Skanse	Koch W.	pollen viability, pollen length
19_60	FV	4x	Denmark	Ebeltoft	56°13'34.957"N, 10 °34'27.869"E	50	tree stands around the road near the village Strandkaer	Koch W.	pollen viability, pollen length

FC *F. verna* subsp. *calthifolia*, **FFE** *F. verna* subsp. *fertilis*, **FFI** *F. verna* subsp. *ficariiformis*, **FFO** *F. verna* subsp. *ficaroides*, **FCH** *F. verna* subsp. *chrysocephala*, **FS** *F. ×sellii (F. verna subsp. calthifolia × F. verna subsp. verna)*, **FV** *F. verna* subsp. *verna*

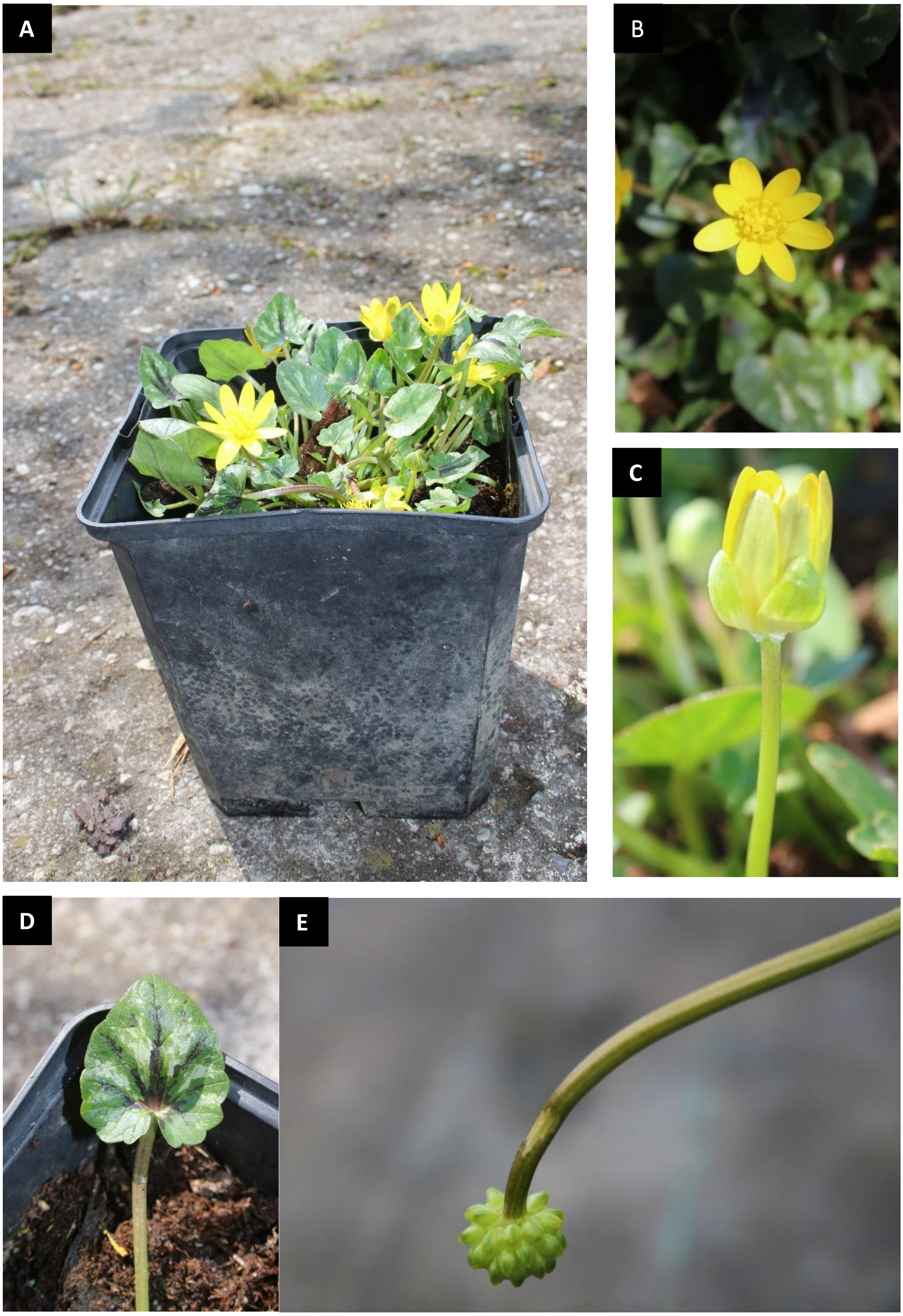
Appendix 2: Examples of graphical output from a microscope Olympus BX60: observing of pollen viability, shape and length, pollen viability estimated by fluorescein diacetate, (A, B) diploid cytotype of *F. verna* subsp. *calthifolia*, (C,D) tetraploid cytotype of *F. verna* subsp. *calthifolia*, (E,F) diploid cytotype of *F. verna* subsp. *fertilis*, (G,H) tetraploid cytotype of *F. verna* subsp. *ficariiformis*, (CH,I) pentaploid cytotype of *F. verna* subsp. *ficariiformis*, (J,K) triploid cytotype of *F. verna* subsp. *sellii*, (L,M) tetraploid cytotype of *F. verna* subsp. *verna*, (N,O) pentaploid cytotype of *F. verna* subsp. *verna*, (P,Q) hexaploid cytotype of *F. verna* subsp. *verna*, all pollen grains are on the left, fluorescently detected viable pollen grains are on the right



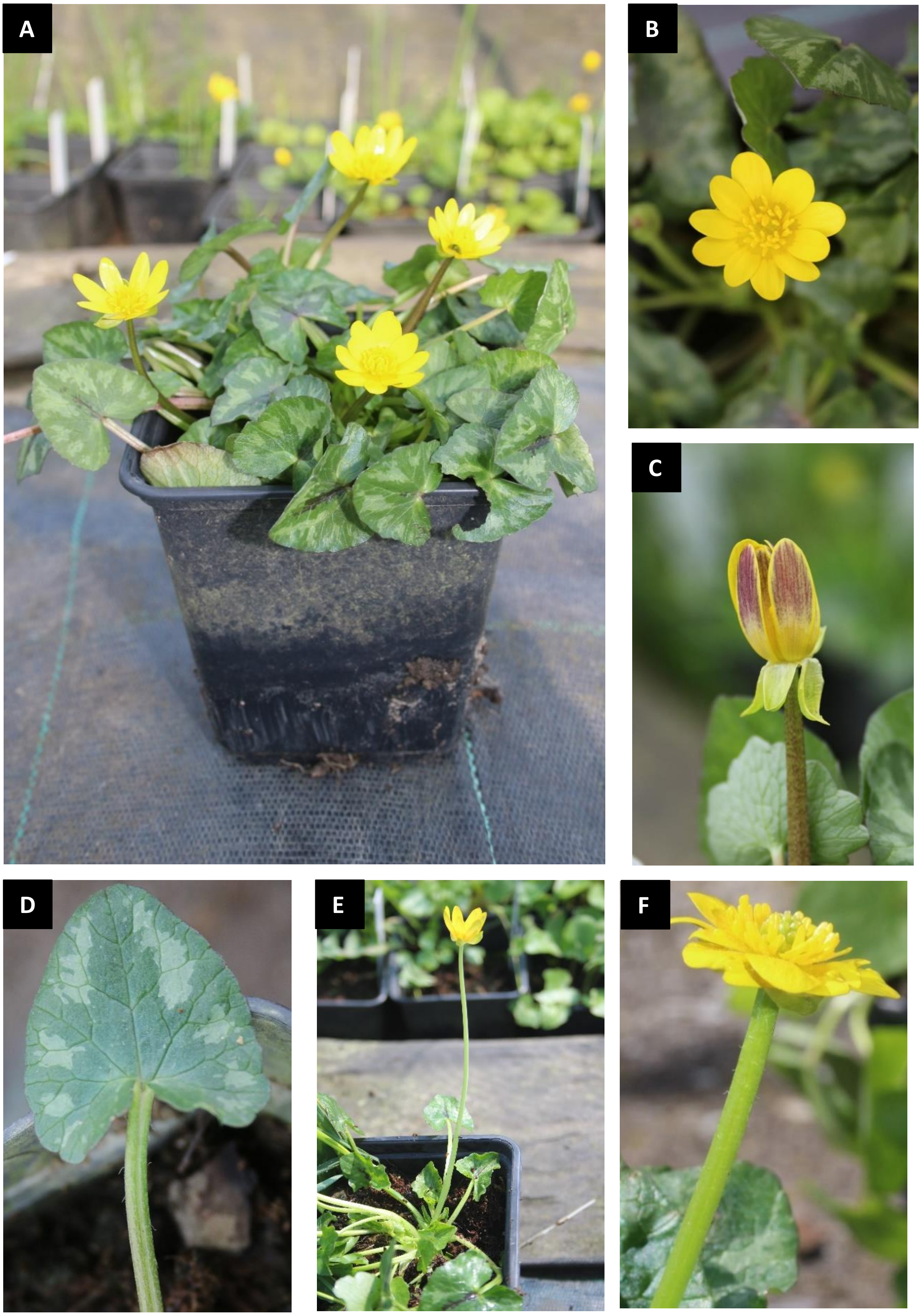

G
H
CH
I
J
K
L
M
100 µm


N
O
P
Q
100 µm
100 µm
100 µm
100 µm

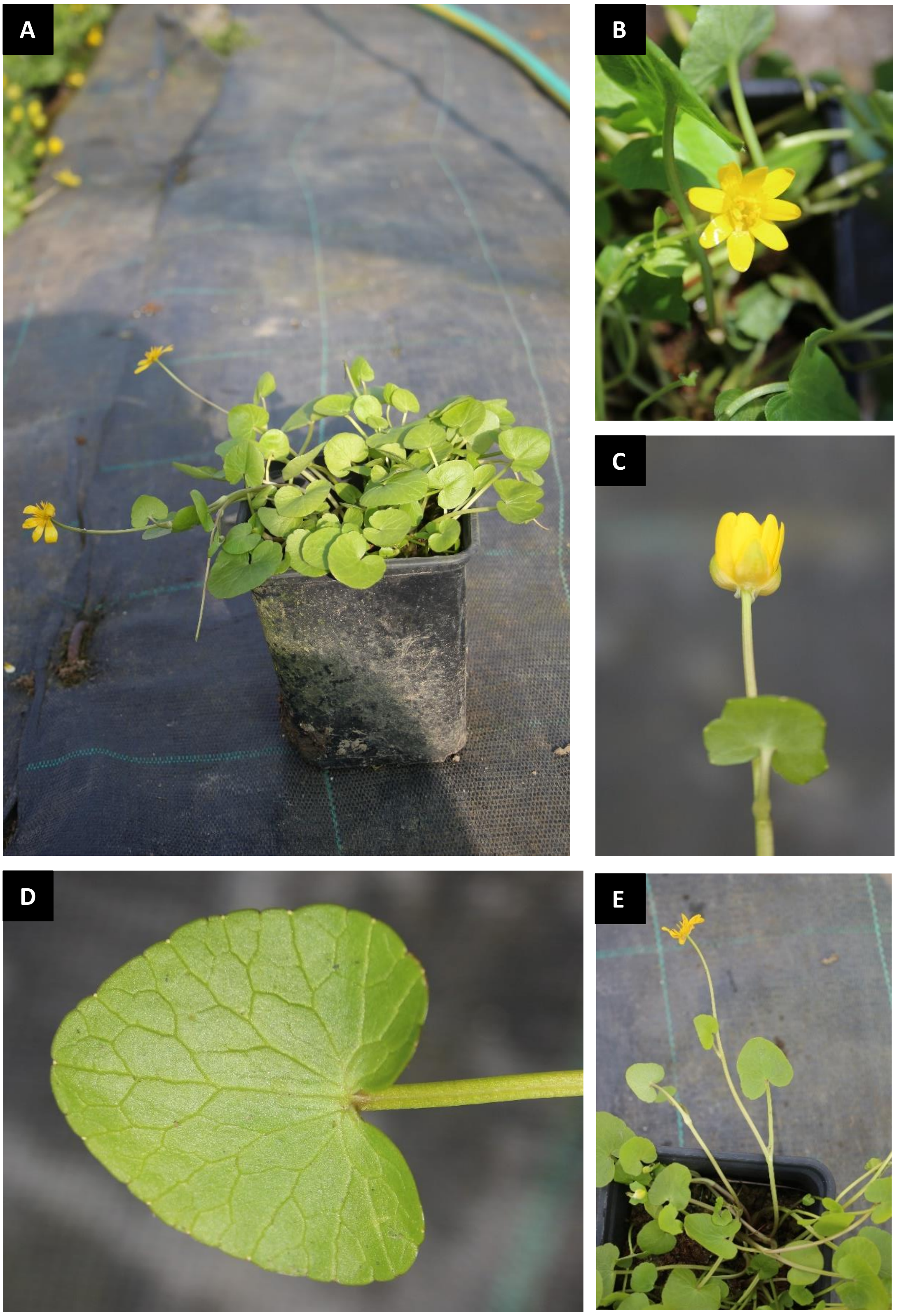
Appendix 3: Diploid cytotype of *F. verna* subsp. *calthifolia*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) achenes, ID: 15_22_8.



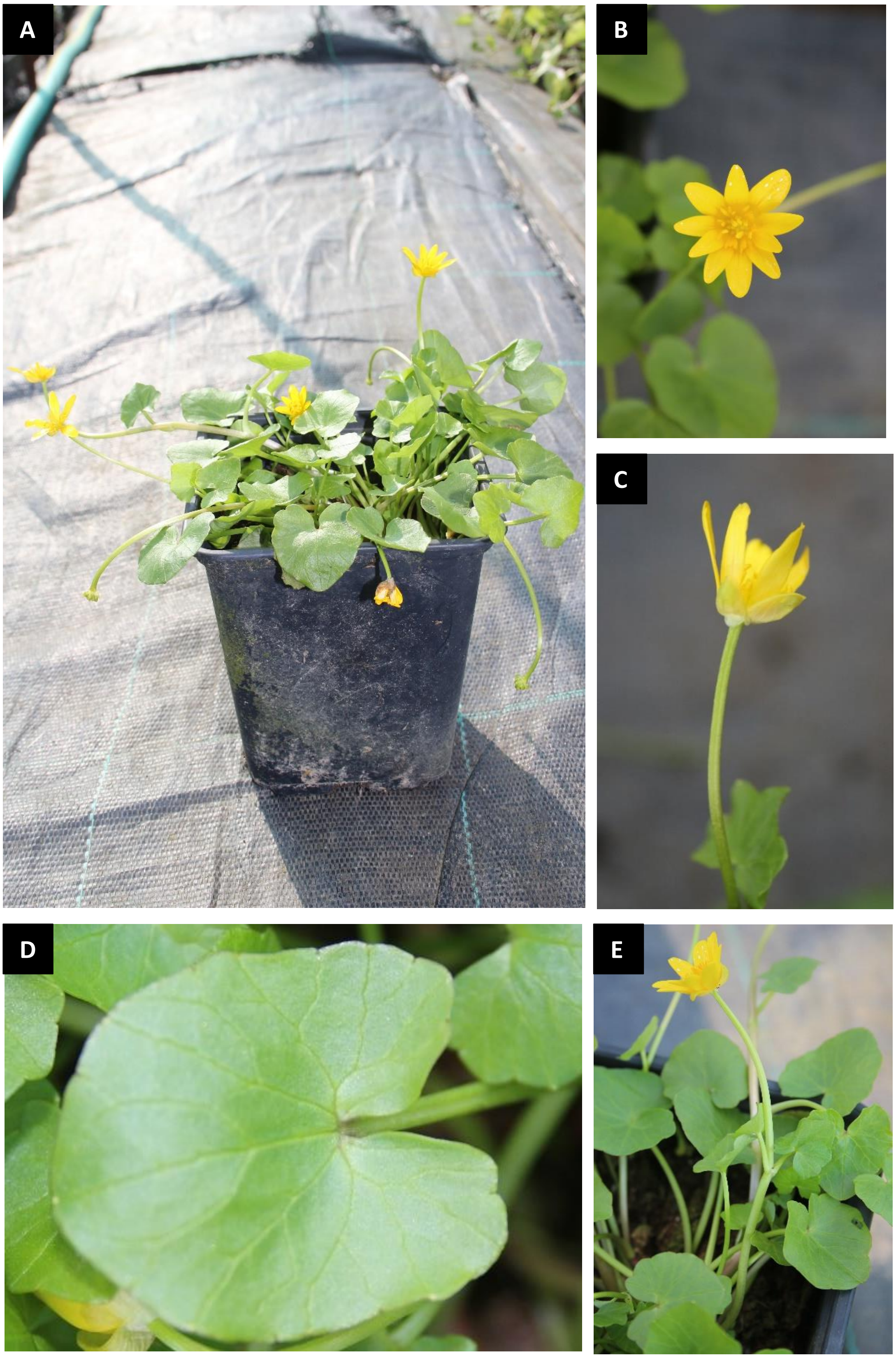
Appendix 4: Diploid cytotype of *F. verna* subsp. *fertilis*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, (F) flower, ID: 17_56_5 (A, B, C, E), and 19_36_4. (D, F)



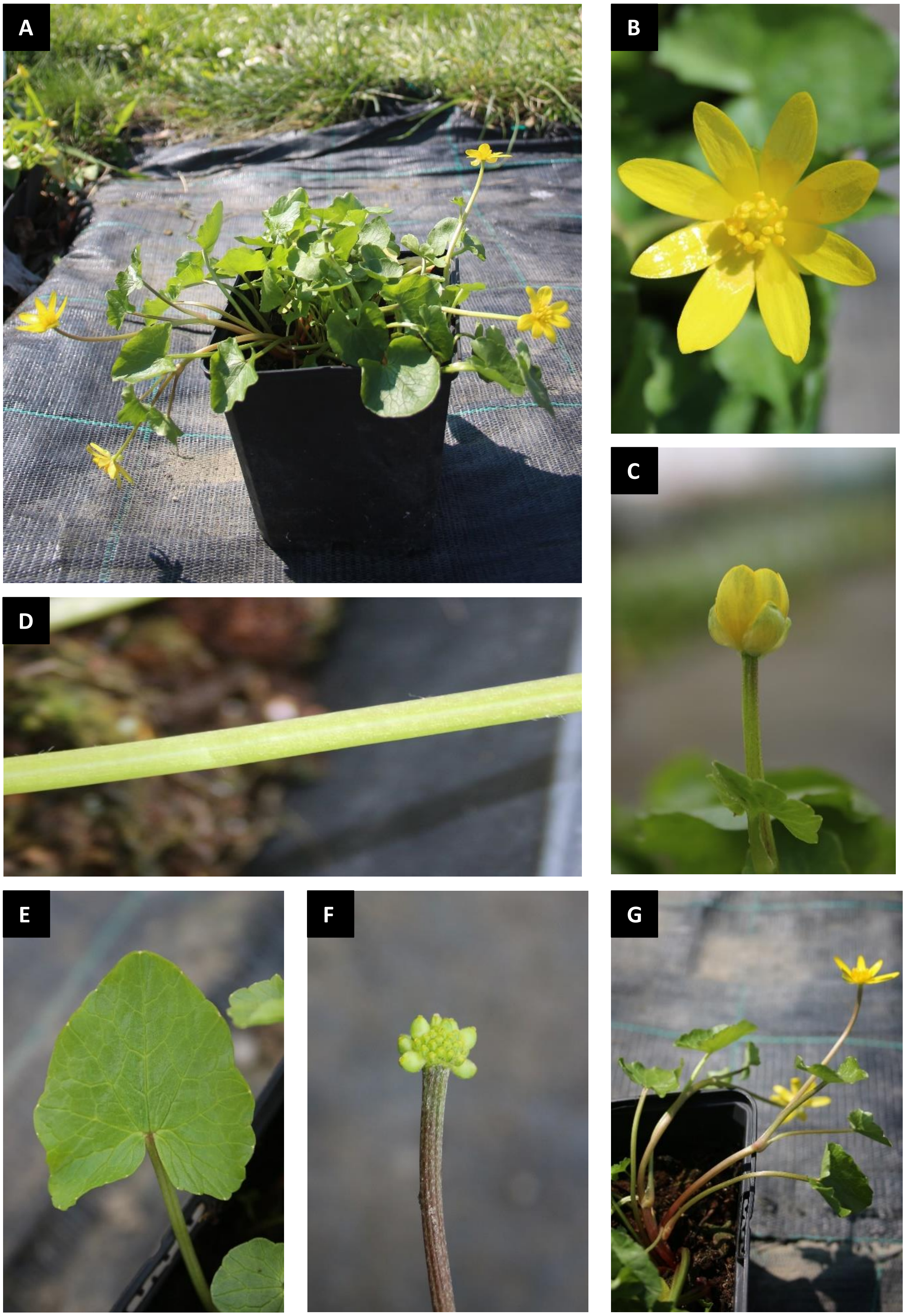
Appendix 5: Diploid cytotype of *F. verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 15_24A_13



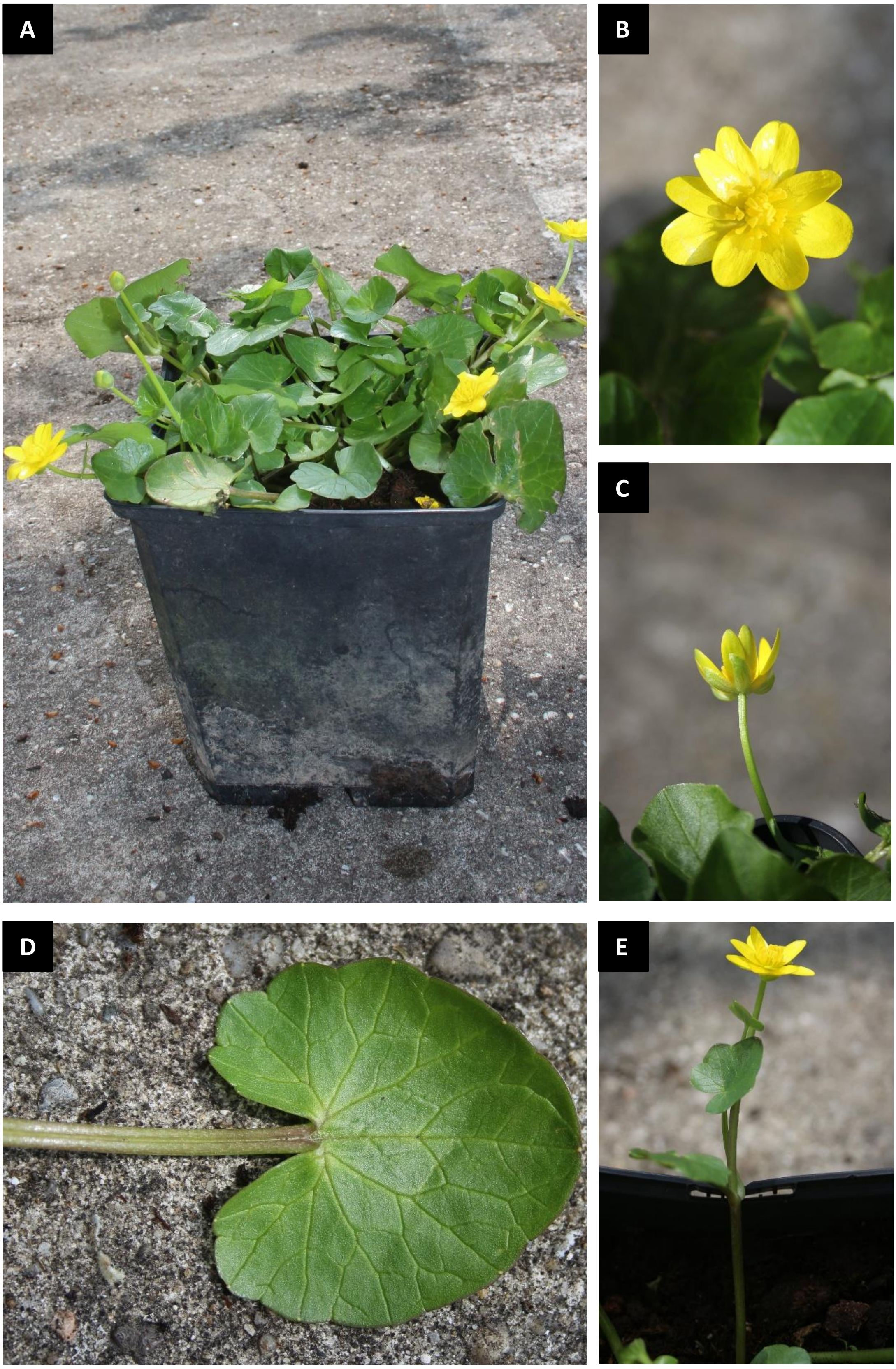
Appendix 6: Triplod cytotype of *F. verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 15_24A_10.



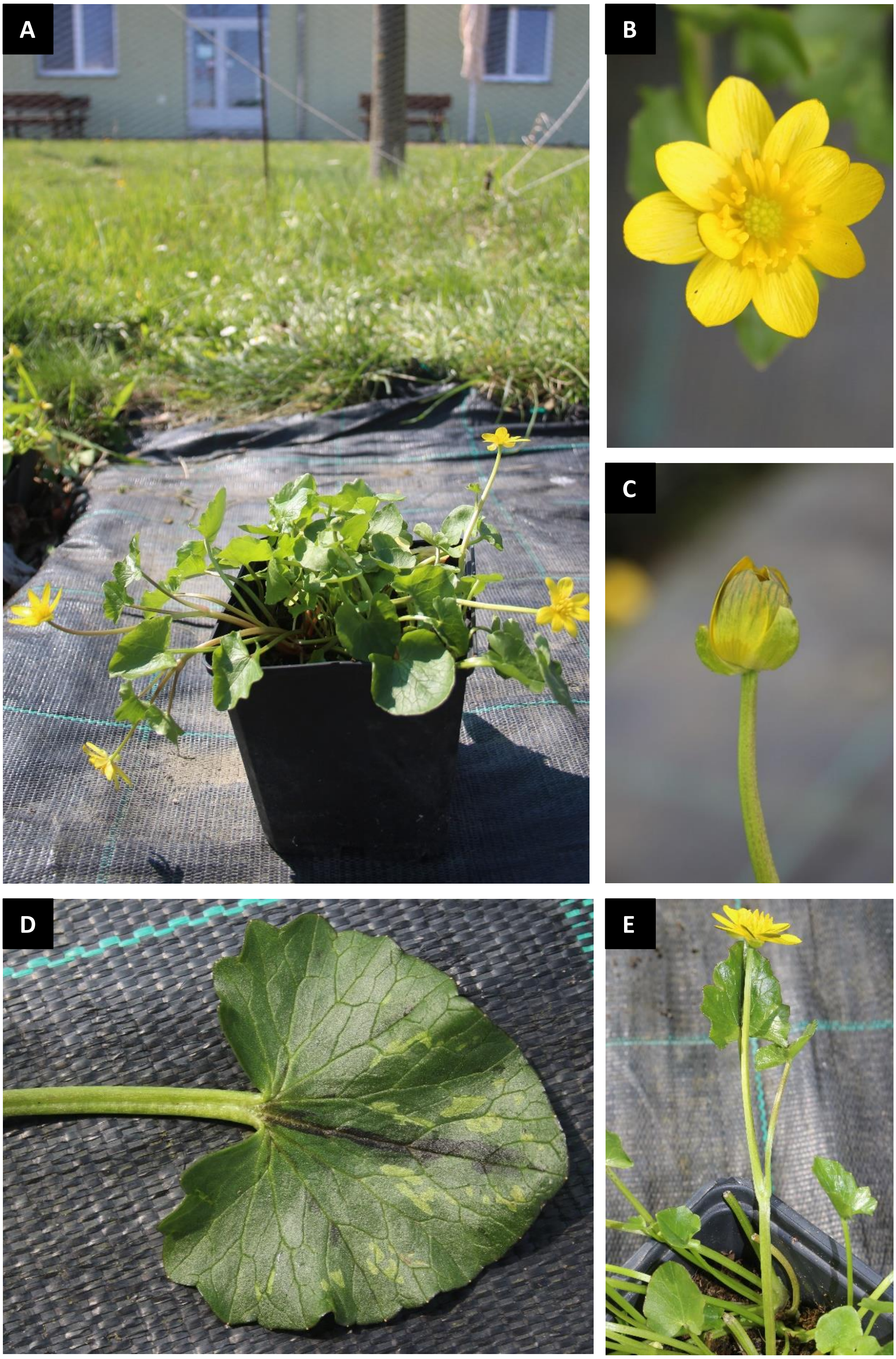
Appendix 7: Tetraploid cytotype of western lineage of *F. verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, d) petiole, (E) leaf, (F) achenes, (G) flower stalk, ID: 16_32_2 (A, B, C, D, F, G)and 16_49_1. (E)



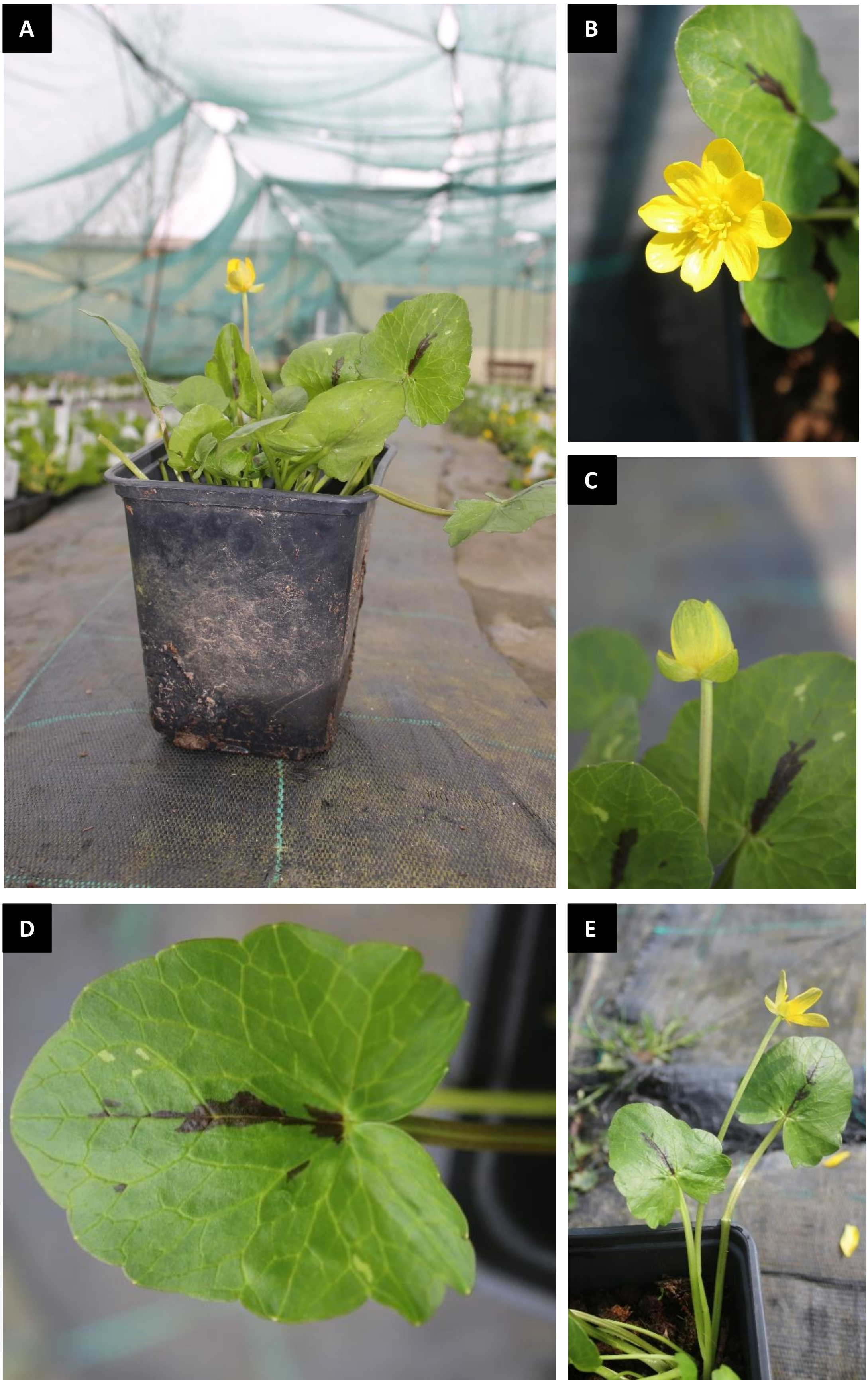
Appendix 8: Tetraploid cytotype of eastern lineage of *F*. *verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 15_12_7.



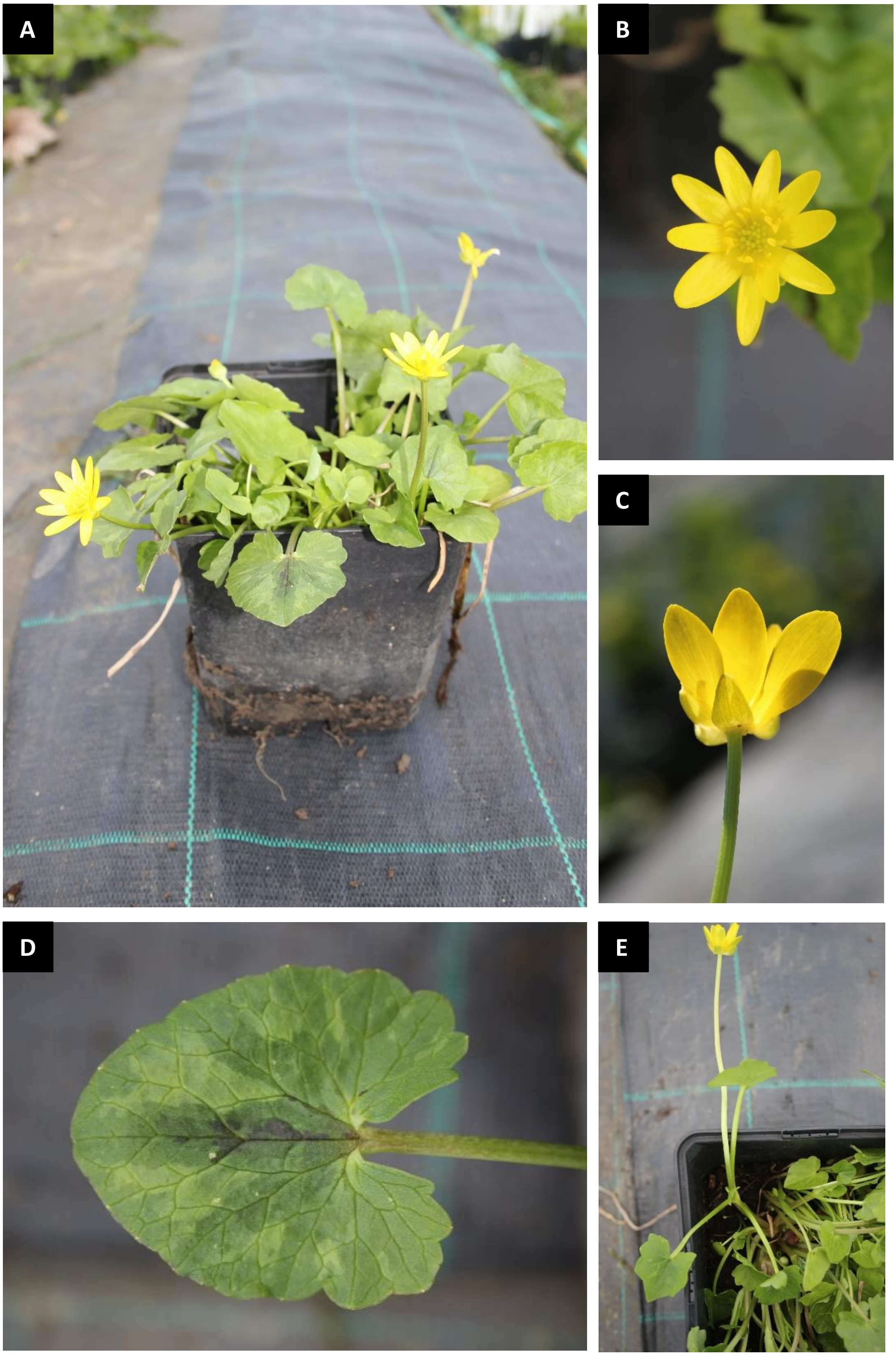
Appendix 9: Pentaploid cytotype of *F. verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 17_99_3.



Appendix 10: Hexaploid cytotype of *F. verna* subsp. *verna*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 17_91_1.



Appendix **11:** Tetraploid cytotype of *F. verna* subsp. *ficariiformis*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 16_72_4.



Appendix 12: Pentaploid cytotype of *F. verna* subsp. *ficariiformis*, (A) habitus, (B) flower, (C) anthokyan in the petals, (D) leaf, (E) flower stalk, ID: 17_109_8.