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Impact of interspecific hybridization within a polyploid agamic complex of *Pilosella* (*Asteraceae*, *Cichorieae*) in Bulgaria compared with Central Europe

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Abstract: The species-mixed *Pilosella* populations comprising diploid sexual and polyploid facultatively apomictic biotypes were studied in Bulgaria. Parentage of co-occurring recent hybrids was inferred from a combination of morphology and ploidy level that corresponded to simple/multiple crosses of basic species via either reduced or unreduced gametes. The flow cytometric seed screening illustrated the capacity for heteroploid hybridization both in open-pollinated plants in the mixed-ploidy populations and in crossing experiments. The diploid sexual species in Bulgaria have a limited impact on interspecific hybridization, and simple inter-cytotype hybrids are only sporadically formed. The origin of the most common hybrids in Bulgaria that are apomictic and retain the pentaploid/hexaploid ploidy level of a co-occurring putative apomictic parent remains unclear. The absence of stabilized hybridogeneous species and scarcity of commonly hybridizing polyploid sexual biotypes are crucial attributes that distinguish the *Pilosella* populations in Bulgaria from those in the Czech Republic and Germany. No recent high-polyploid hybrids of $2n + n$ origin that would potentially become drivers of ongoing hybridization in the mixed sexual-apomictic *Pilosella* populations similar to those in Central Europe have been recorded in Bulgaria. The pattern of co-occurring cytotypes in Bulgaria likely limits interspecific hybridization due to stronger ploidy barriers.

Key words: *Asteraceae*, Bulgaria, Central Europe, *Cichorieae*, *Compositae*, facultative apomixis, hybridization, *Hieracium* s.l., microevolution, mixed-ploidy populations, *Pilosella*, residual sexuality, unreduced gametes

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Introduction

The genus *Pilosella* Hill. has a complicated taxonomy due to polyploidy, ongoing hybridization (the crosses are both homoploid and across ploidy level), and diversity in modes of reproduction (Fehrer & al. 2007b). Both the fully sexual biotypes (diploid and polyploid) and facultatively apomictic biotypes (exclusively polyploid) form this agamic complex (Fehrer & al. 2007b). Apomixis of the aposporous type is autonomous in *Pilosella*; the development

of both embryo and endosperm occurs independently of pollination (Bicknell & Koltunow 2004). Taxonomic concepts in *Pilosella* define three groups of taxa according to their origin: the “basic” species, the stabilized “intermediate” species that have putatively originated from past hybridizations of the basic species, and the recent hybrids (Fehrer & al. 2007b). Population-based approaches reveal relationships among coexisting biotypes, i.e. the inter-cytotype mating interactions in a mixed-ploidy and exclusively sexual population of *P. echioides* (Lumn.) F. W.

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Schultz & Sch. Bip. (Trávníček & al. 2011), or affinity among allopatric taxa/sympatric cytotypes comprising the *P. alpicola* group (Šingliarová & al. 2011a, 2011b). However, many *Pilosella* populations in Central Europe are species-mixed, and large amounts of interspecific hybrids, both stabilized and recent, suggest rather weak hybridization barriers among species in this area (e.g. Chrtek 2004; Fehrer & al. 2007b; Bräutigam 2017).

There are several taxonomic approaches to solve an extremely complex structure in some apomictic genera (Majeský & al. 2017). A classic and still respected species concept in *Pilosella* is based on a combination of morphological characters that are shared by either the stabilized hybridogeneous taxa (intermediate species that have originated from past hybridizations) or the recent hybrids, and by their putative parents (two or more basic or intermediate species) (Zahn 1922–1930; Bräutigam & Greuter 2007–2009; Bräutigam 2017). This morphological classification of hybrids is supported by extensive experimental hybridizations performed in the past: in fact, many of the hybridogeneous taxa had been described just on the basis of experimental crosses (e.g. Peter 1884; Nägeli & Peter 1885). Some of the hybrids that were currently recovered from our own (yet unpublished) crossing experiments also perfectly correspond in morphology to hybridogeneous taxa from the wild. Evaluating the mature plants from the field and their field progenies, a morphometric and statistical analysis in a mixed hybridizing population of *P. officinarum* F. W. Schultz & Sch. Bip. and *P. bauhini* (Schult.) Arv.-Touv. in the Czech Republic illustrated a close relationship between hybridity and morphology (Urfus & al. 2014). A reliable genetic evidence of parentage in an overwhelming majority of hybridogeneous taxa of *Pilosella* that are polyploid and often closely related (Fehrer & al. 2007a) depends on availability of the still lacking species-specific molecular markers (Krahulec & al. 2012; Moffat & al. 2015). Nevertheless, analysis of the *trnT-trnL* intergenic spacer of chloroplast DNA, hereafter referred to as the haplotype, allowed identification of the maternal origin in many *Pilosella* hybrids in Central European populations (Fehrer & al. 2005, 2007a, 2007b; Krahulec & al. 2008; Krahulcová & al. 2009a, 2012, 2014; Rosenbaumová & Krahulec 2015). However, the diversification in specific haplotypes between both putative parents that is required for an unequivocal identification of maternal origin in the hybrids proved to be insufficient in populations in Bulgaria (Hand & al. 2015; Krahulec & al. unpublished data).

A complex study of the structure of species-mixed populations supplemented by evaluation of seed progenies revealed features that are commonly shared by hybridizing sexual-apomictic populations of *Pilosella* in the Czech Republic and Germany (Krahulec & al. 2004, 2008, 2014; Krahulcová & al. 2009a, 2012, 2013, 2014; Rosenbaumová & Krahulec 2015). Due to residual sexuality, facultatively apomictic plants play an important role as maternal parents in shaping the structure of these populations. The fertilization of an unreduced ($2n$) female

gamete allows the persistent formation of genomically unstable and high-polyploid $2n + n$ hybrids ($2n$ apomict $\times n$ sexual) that appear to be important drivers of microevolution in such mixed populations in Central Europe (Krahulec & al. 2008; Krahulcová & al. 2009a, 2014; Rosenbaumová & Krahulec 2015). Parallel field studies in south-eastern Europe, namely in Bulgaria, detected taxonomic diversity in *Pilosella* associated with variation in cytotypes and the reproductive system (Krahulcová & al. 2009b, 2016). However, the basic species that are potentially hybridizing in Bulgaria (Stojanov & al. 1967; Krahulcová & al. 2009b, 2016) are different from those in Central Europe (Chrtek 2004; Krahulec & al. 2004, 2008; Krahulcová & al. 2009a, 2014; Bräutigam 2017).

Unlike in Central Europe, diploid sexual *Pilosella* species are relatively common in Bulgaria. To date, six diploid species have been recorded in the country: *P. alpicola* (Hoppe) F. W. Schultz & Sch. Bip., *P. hoppeana* subsp. *testimonialis* (Peter) P. D. Sell & C. West, *P. onegensis* Norrl., *P. pavichii* (Heuff.) Arv.-Touv., *P. petraea* F. W. Schultz & Sch. Bip., and *P. pseudopilosella* (Ten.) Soják (Vladimirov 2000 and unpublished data; Šingliarová & al. 2011b). The diploid *P. hoppeana* subsp. *testimonialis* is the most common sexual species recorded in Bulgaria, whereas the rather common and diploid sexual *P. onegensis* is more specialized in ecological preferences similar to *P. pavichii*. The last species comprises both sexual and apomictic biotypes, and sexuality is confined to more common diploids and to part of the rather rare tetraploids (Krahulcová & al. 2009b, 2016). Nevertheless, free sexual mating of the diploid *P. pavichii* via the maternal lineage is likely reduced due to reduced seed fertility, whereas pollen maintains high viability that is typical of other diploid basic species of *Pilosella* (Krahulcová & al. 2009b; Rotreklová & Krahulcová 2016). The other sexual species have a limited distribution in Bulgaria, i.e. the tetraploid *P. cymosa* subsp. *sabina* (Sebast.) H. P. Fuchs, the diploid *P. pseudopilosella* and both diploid and triploid *P. alpicola* (Krahulcová & al. 2009b, 2016; Šingliarová & al. 2011a, 2011b under the name of *P. rhodopea* (Griseb.) Szeląg). On the other hand, the sexual tetraploid biotype of *P. officinarum*, which is widespread and easily hybridizes in Central Europe (e.g. Fehrer & al. 2007b; Suda & al. 2007; Mráz & al. 2008; Krahulcová & al. 2009a, 2014), is absent in Bulgaria, whereas the apomictic pentaploid and hexaploid biotypes of this species frequently occur there. In general, the tetraploid taxa common in Central Europe are rather rare in Bulgaria, where the pentaploids and hexaploids prevail among *Pilosella* biotypes (Krahulcová & al. 2009b, 2016).

This study aims to describe the processes that affect the population structure of the agamic complex of *Pilosella* in Bulgaria. We chose our published data referring to those populations in Bulgaria that comprised two species distinct in morphology and their putative hybrids determined according to shared parental morphological characters (Krahulcová & al. 2009b, 2016). The poly-

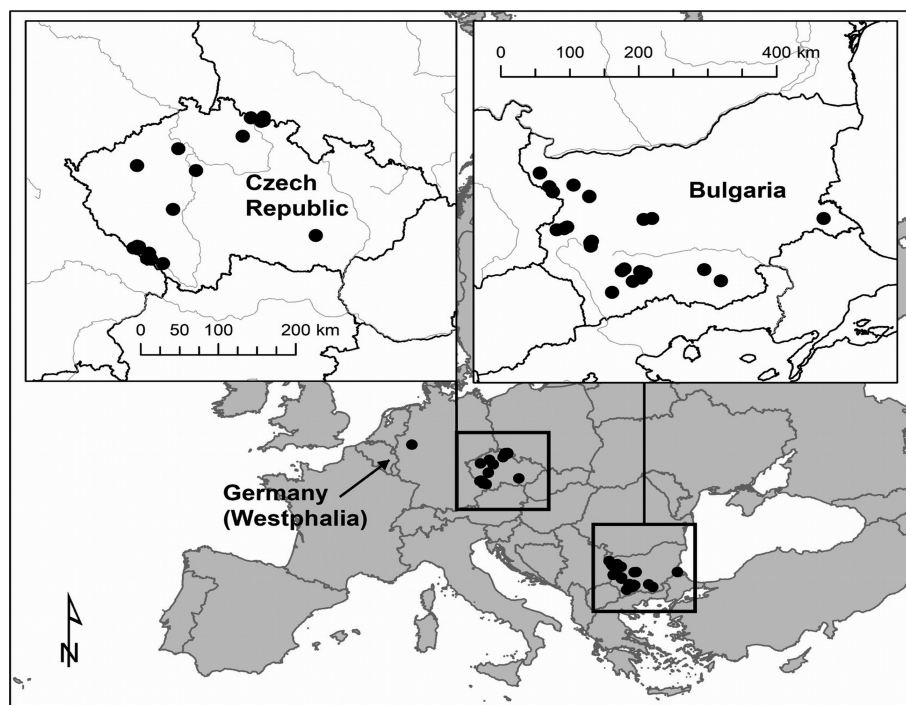


Fig. 1. Map of localities of the studied *Pilosella* species. For a detailed description of localities, see Table 4 in Supplementary material online.

ploid facultatively apomictic biotypes and their diploid sexual counterparts usually represented the parental species (Table 1, Table 4 in Supplementary material online). The only new data in this study refer to seed progenies generated in the field and to crossing experiments. According to a population-based approach that was used for studies in the species-mixed *Pilosella* populations in the Czech Republic and Germany (Krahulec & al. 2004, 2008, 2014; Krahulcová & al. 2009a, 2012, 2013, 2014; Rosenbaumová & Krahulec 2015), we compared the relevant processes at population level in two distant parts of the distribution area in Europe (Fig. 1). We analysed the populations encompassing the mature plants from the field and their progeny arrays in both regions. In Bulgaria, we particularly searched for new products of interspecific hybridization that, similar to the specific *Pilosella* hybrids described previously from Central Europe, might become drivers of microevolution in the mixed populations. We demonstrate how the different patterns of co-occurring species and cytotypes in the respective area influence the efficiency of interspecific hybridization.

Material and methods

In total, 289 plants that were sampled at 29 localities in Bulgaria from 2005 to 2010 were chosen for this study (Fig. 1; Krahulcová & al. 2009b, 2016). The plant collection comprised 160 accessions of six basic species (for plant collection data, see Table 4 in Supplementary material online), including *Pilosella bauhini* (67 accessions), *P. hoppeana* subsp. *testimonialis* (32 accessions,

hereafter referred to as “*P. *testimonialis**”), *P. officinarum* (32 accessions), *P. onegensis* (8 accessions), *P. pavichii* (18 accessions) and *P. pseudopilosella* (3 accessions), and 129 accessions of their hybrids (Table 1; for detailed data describing the hybrids, see Krahulcová & al. 2009b, 2016). After the revision of herbarium specimens by Siegfried Bräutigam, the taxonomic identity of parental taxa and hybrids was published previously (Krahulcová & al. 2009b, 2016). Using the same population-based approach as previously used for hybridizing *Pilosella* populations in Central Europe (for the details, see Krahulcová & al. 2009a, 2014), the population structure was studied in 19 mixed

populations (situated at 27 localities) in Bulgaria where the putative parents co-occurred with hybrids (Table 1). The plants sampled at closely situated localities were considered to form one population, whereas plants sampled at localities greater than 5 km apart were considered to represent separate populations.

All procedures used in this study follow the methods tried in previous papers that were aimed at hybridizing *Pilosella* populations in Central Europe (e.g. Krahulcová & al. 2009a, 2014). The nomenclature follows Bräutigam & Greuter (2007–2009) and Bräutigam (2017), and the herbarium specimens are deposited in the Herbarium of the Institute of Botany Průhonice (PRA).

Acquiring data extracted from previous publications

The plants were characterized according to taxonomic identity, status of origin (distinguishing the putative parental basic species and the hybrids), ploidy level and reproductive system. Plants sampled in the field were transferred to the Experimental Garden of the Institute of Botany in Průhonice, Czech Republic and cultivated to carry out subsequent analyses and experimental crosses. The DNA ploidy level was determined in most plants using flow cytometry (Krahulcová & al. 2004). In some problematic cases, the cytotype was determined/verified by chromosome counting (Krahulcová & Krahulec 1999). The hybrid origin of plants from the field was inferred from a combination of morphological characters of the respective putative parents (Stojanov & al. 1967; Sell & West 1976). The reproductive system was principally determined using the emasculation/open pollination ex-

Table 1. *Pilosella* species (sexual species underlined) and their co-occurring putative recent interspecific hybrids that were recorded in 19 populations (situated at 27 localities) in Bulgaria. Populations comprised two or more basic species and one or more different putative hybrids: plants sampled at closely situated localities (joined by a plus sign “+”) are considered to form one population; plants sampled at localities greater than 5 km apart (separated by a comma) are considered to form different populations. Locality symbols correspond to Table 4 in Supplementary material online, each locality label is specified by “Bu” plus a numeral and a superscript reference. Hybrid status of plants was inferred from their morphology combining characters of putative parental species that co-occurred in a respective population or in close vicinity. **Bold** cytotypes refer to those simple (primary) hybrids that might originate via an unreduced gamete from either parent ($2n + n$, $n + 2n$). A part of these hybrids (**dotted underlined bold**) might originate via an unreduced gamete from a sexual parent. Cytotypes considered to be multiple hybrids either had a ploidy that cannot be inferred from a simple hybridization of co-occurring parental cytotypes, or their morphology was markedly closer to either parent, suggesting a backcross. One of the closely related species, either the sexual diploid *P. *testimonialis* or the apomictic polyploid *P. officinarum*, could alternatively hybridize with *P. bauhini*, giving rise to hybrids (indicated “*”) : the morphology of these hybrids corresponded to the cross *P. bauhini* × *P. *testimonialis*/*P. officinarum*. Seed-sterile/semisterile hybrids are indicated “!”. Row numbers refer to individual pairs of parental biotypes of respective hybrids: species-rich populations/localities are therefore presented repeatedly in different rows.

Ploidy of hybrids		Putative parental species (ploidy)	Row	Locality ^{reference}
Simple	Multiple			
	2x, 6x	<i>P. bauhini</i> (6x), <u><i>P. onegensis</i></u> (2x)	1	Bu 19 ²
	6x	<u><i>P. pavichii</i></u> (2x), <i>P. *testimonialis</i> (2x)	2	Bu 15 ¹
	6x	<u><i>P. pavichii</i></u> (2x), <i>P. bauhini</i> (6x)	3	Bu 15 ¹ , Bu 4 ²
<u>5x</u>		<u><i>P. pavichii</i></u> (2x), <i>P. bauhini</i> (5x, 6x)	4	Bu 6 ² + Bu 7 ²
5x, 6x		<i>P. pavichii</i> (4x, 6x), <i>P. bauhini</i> (6x)	5	Bu 1 ² + Bu 2 ² + Bu 3 ²
3x!		<u><i>P. pavichii</i></u> (2x), unknown parent	6	Bu 15 ¹
<u>5x</u> , 5x, 6x		<i>P. bauhini</i> (6x), <u><i>P. pavichii</i></u> (2x) / <i>P. pavichii</i> (4x, 5x)	7	Bu 23 ² + Bu 24 ²
2x		<i>P. *testimonialis</i> (2x), <u><i>P. pseudopilosella</i></u> (2x)	8	Bu 11 ¹ + Bu 36 ²
4x		<u><i>P. pseudopilosella</i></u> (2x), <i>P. officinarum</i> (6x)	9	Bu 11 ¹ + Bu 36 ²
<u>5x</u>		<i>P. *testimonialis</i> (2x), <i>P. officinarum</i> (5x)	10	Bu 14 ² + Bu 15 ²
<u>5x</u>	6x	<i>P. *testimonialis</i> (2x), <i>P. officinarum</i> (6x)	11	Bu 11 ¹ + Bu 36 ²
	6x	<u><i>P. *testimonialis</i></u> (2x), <i>P. officinarum</i> (6x)	12	Bu 24 ²
<u>5x</u>		<u><i>P. *testimonialis</i></u> (2x), <i>P. officinarum</i> (not recorded)	13	Bu 13 ²
<u>5x</u>		<u><i>P. *testimonialis</i></u> (2x), <i>P. officinarum</i> (ploidy not determined)	14	Bu 18 ²
4x!, <u>5x</u> , 6x		<i>P. bauhini</i> (5x, 6x), <u><i>P. *testimonialis</i></u> (2x)	15	Bu 1 ¹
<u>5x</u>		<i>P. bauhini</i> (5x, 6x), <u><i>P. *testimonialis</i></u> (2x)	16	Bu 14 ² + Bu 15 ²
6x	6x	<i>P. bauhini</i> (6x), <i>P. officinarum</i> (6x)	17	Bu 19 ² , Bu 24 ² , Bu 26 ² + Bu 27 ²
6x	5x	<i>P. bauhini</i> (5x, 6x), <i>P. officinarum</i> (5x)	18	Bu 14 ² + Bu 15 ²
6x		<i>P. bauhini</i> (6x), <i>P. officinarum</i> (5x, 6x)	19	Bu 6 ¹
<u>5x</u> •, 6x•	5x•, 6x, 6x•	<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (6x)	20	Bu 15 ¹ , Bu 43 ²
6x•	6x•	<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (6x)	21	Bu 11 ¹ + Bu 36 ² , Bu 19 ² , Bu 24 ² , Bu 26 ² + Bu 27 ²
<u>5x</u> •, 5x•, 6x•		<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (5x, 6x)	22	Bu 2 ¹ + Bu 3 ¹
	6x•	<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (not recorded) / <i>P. officinarum</i> (5x)	23	Bu 32 ²
6x•, 6x•	6x•	<i>P. bauhini</i> (5x, 6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (5x)	24	Bu 14 ² + Bu 15 ²
<u>5x</u> •, 5x•, 6x•, 6x•		<i>P. bauhini</i> (5x, 6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (4x, 5x)	25	Bu 8 ¹
6x•	6x•	<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (not recorded)	26	Bu 16 ¹
<u>5x</u> •	5x•	<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (not recorded) / <i>P. officinarum</i> (6x)	27	Bu 7 ¹
<u>5x</u> •, 5x•		<i>P. bauhini</i> (6x), <u><i>P. *testimonialis</i></u> (2x) / <i>P. officinarum</i> (not recorded)	28	Bu 13 ²

References: ¹Krahulcová & al. (2009b); ²Krahulcová & al. (2016).

periments that sorted the parthenogenetic (assumed to be apomictic), sexual and seed-sterile plants (Gadella 1984; Krahulcová & al. 2004). The emasculation was carried out by cutting off the whole upper part of an unopened inflorescence (capitulum) before anthesis. This way, the anthers and stigmas were removed while the ovaries remained untouched. This method is routinely used in *Compositae* with an autonomous apomixis, for example, in *Hieracium* L. including *Pilosella* (Gadella 1984) and *Taraxacum* F. H. Wigg. (Richards 1986). Given that fertilization-independent seed formation is predominantly coupled with apomeiosis in wild-type apomicts in *Pilosella*, this test is an approximation for apomictically reproducing plants (Bicknell & Koltunow 2004; Hand & al. 2015). A noticeable reduction of seed-set in emasculated capitula compared with seed-set in open-pollinated capitula of the same *Pilosella* plant might indicate a significant participation of sexual reproduction related to a reduced penetrance of apomixis in a respective plant. An impact of residual sexuality in such a plant can be proved by open pollination/hybridization experiments and subsequent analysis of progeny (Krahulcová & al. 2009a, 2014).

Acquiring new data

Flow cytometric sorting of progeny origins and ploidy level

The seeds that were sampled from open-pollinated *Pilosella* plants in the field were analysed for way of origin using Flow Cytometric Seed Screening (FCSS; Matzk & al. 2000). The method was modified for *Pilosella* as follows. Fresh seeds (achenes) collected from fruiting plants were either analysed shortly after harvest or they were kept in a fridge. Cold storage preserves the endosperm tissue, allowing interpretable endosperm peaks in flow histograms up to several months after collection. Before the analysis, the filled (well-developed) seeds were screened using a stereomicroscope and an adequate pressure of tweezers on each seed. The filled seeds were pooled either two at a time or ten at a time (plus the rest of at least six seeds), so either the seed doublets or the groups comprising usually ten seeds each were prepared for flow cytometric analysis. The DAPI staining method (Otto 1990) was used, using the ice-cold nuclei-extracting buffer (Otto I) and the staining buffer (Otto II) supplemented by 2 $\mu\text{l ml}^{-1}$ mercaptoethanol as an antioxidant. The fluorescence intensity was determined by a Ploidy Analyser PA II (Partec GmbH, Münster, Germany) equipped with an HBO high-pressure mercury lamp for UV excitation. If possible, 5000 nuclei were scored in each sample.

Analysis of seed doublets (Krahulcová & al. 2009a, 2011) — Two achenes were chopped in nuclei-isolating buffer with a razor blade together with 0.05 cm^2 of leaf tissue of reference standard (*Bellis perennis*, diploid *Pilosella lactucella* or some of the polyploid *Pilosella* spe-

cies: the standard was chosen according to ploidy level of the maternal plant of analysed seed progeny). The suspension was filtered and incubated for 10 min at room temperature, stained and analysed. The histograms with CVs below 3.5% were considered.

Analysis of pooled samples comprising six to ten seeds (Krahulcová & Suda 2006; Krahulcová & al. 2011) — Pooled achenes were chopped in nuclei-isolating buffer with a razor blade, the suspension was filtered and incubated for 10 min at room temperature, stained and analysed. The histograms with CVs below 5% were considered. This threshold of accuracy of analyses was still acceptable because two simultaneous peaks in a histogram should distinguish the difference in DNA content among components within a sample that corresponds to double CV (Doležel & Göhde 1995). Quantification of the embryo ploidy classes was based on the proportion of nuclei in their respective embryo ploidy peaks, and the maternal ploidy peak was used as an internal ploidy standard. In most histograms the minor endosperm peak was evident, which was positioned either at 2 \times the distance of the embryo peak assigned to embryos sharing the ploidy of respective polyploid apomictic mothers, or at 1.5 \times the distance of the embryo peak assigned to embryos sharing the ploidy level of sexual mothers.

The FCSS procedures allowed detection of both ploidy level in progeny embryos (when analysing both the seed doublets and pooled samples of a maximum of ten seeds) and their reproductive origins (when analysing the seed doublets). The FCSS analyses of pooled samples comprising six to ten seeds revealed the most frequent embryos maintaining the maternal ploidy level; in addition to them, the analyses could distinguish and quantify the potential less frequent progeny embryos that had a ploidy level different from a maternal parent, namely, the products of (1) heteroploid crosses, (2) sexual mating via unreduced gametes and (3) haploid parthenogenesis (Krahulcová & Suda 2006). The seed-progenies, produced by both facultative apomicts and sexuals, were scored. Analysing the seed doublets, we distinguished the progeny generated by apospory versus sexual mating; analysing the pooled samples of six to ten seeds, we traced a potential diversity in embryo ploidy level (Krahulcová & Suda 2006; Krahulcová & al. 2009a, 2012, 2014). The results of these two procedures of seed screening were evaluated separately. Consequently, to illustrate a residual sexuality in facultative apomicts, the “off-type” progeny that did not replicate the maternal parent (Bicknell & Koltunow 2004) were detected and quantified. The capacity of facultative apomicts to avoid apomeiosis by forming a reduced female gamete was tested using emasculation experiments (Krahulcová & al. 2004). The seeds sampled from emasculated apomictic plants were also analysed using the FCSS analysis of pooled samples comprising six to ten seeds. This way, the embryos maintaining the maternal ploidy level (those generated from unreduced female gametes via apospory) were distinguished from

embryos having a half of maternal ploidy level (those generated from reduced female gametes via haploid parthenogenesis).

Crossing experiments

The capacity of selected pairs of parental accessions to hybridize was tested using hand crossing experiments. A restricted extent of crossing experiments was caused by availability of parental accessions in the experimental garden that flowered simultaneously in sufficient replicates. Namely, three hexaploid facultatively apomictic accessions of *Pilosella bauhini* were chosen as maternal parents and crossed with a sexual (mostly diploid) pollen parent. A high pollen stainability detected in pollinating species/cytotypes suggested a sufficient pollen viability of pollen donors (Rotreklová & Krahulcová 2016). The diploid sexual *P. *testimonialis* was used as a maternal parent exclusively in the reciprocal cross (Table 2). All inflorescences (capitula) of each parent were isolated in nylon bags before anthesis (prior to the outer ligular flowers of the capitulum opening). The capitula were crossed in the stage of stigma receptivity (when bifurcate stigmas protruded from the flowers), by rubbing the whole parental capitula together. As the flowers in the capitulum open successively from the margins to the centre, the maternal capitulum was usually pollinated 2 to 3 times a week, on each occasion with a new capitulum of the same pollen plant. The pollinated capitula were kept in nylon bags until the seeds ripened (Krahulcová & al. 2004).

The seed-progeny gained from crosses was evaluated using the FCSS method in the same manner used for those progeny generated by open-pollinated plants in the field. Because the FCSS method destroys the seed, a portion of seeds gained from crossed maternal plants with an abundant seed-set was used for seedling recovery. The ploidy level of these cultivated seedlings was determined using flow cytometry with the DAPI staining method (Krahulcová & al. 2004). Approximately 0.3 cm² of fresh leaf tissue from the seedling was chopped together with the same amount of leaf tissue from the maternal plant (internal standard) in nuclei-extracting buffer, then the same method used for analysis of seeds (described above) was followed.

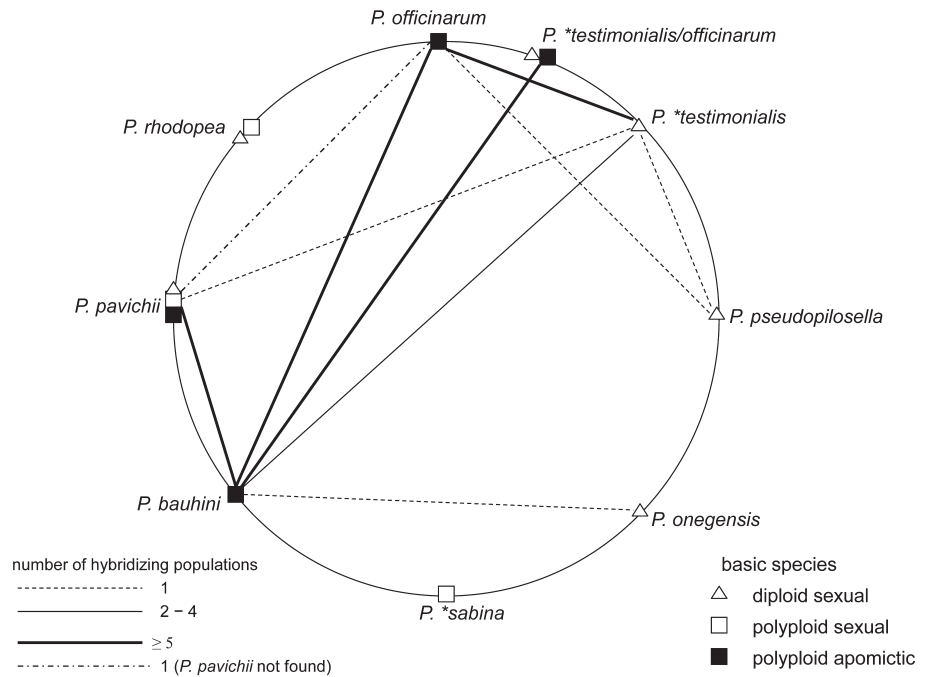


Fig. 2. Schematic drawing illustrating putative spontaneous crosses among *Pilosella* species in Bulgaria. Selection of taxa is based on systematic field studies by Krahulcová & al. (2009b, 2016). Except for *P. cymosa* subsp. *sabina* (shown as “*P. *sabina*”) and *P. rhodopea*, the parental basic species co-occurred with respective recent hybrids in 19 populations examined (Table 1). Position on circle does not exactly reflect relationships among individual taxa. Number of hybridizing populations corresponds to entries in Table 1.

Results – populations in Bulgaria

Origin of hybrids

The hybrids that co-occurred with putative parental species were recorded in 19 populations in Bulgaria (Table 1). The origin of these hybrids, as inferred from a combination of parental morphological characters (Stojanov & al. 1967; Sell & West 1976) and ploidy level, corresponded to 28 pairs of parental species/cytotypes (see the numbered rows in Table 1). Irrespective of ploidy level, the most frequent putative parental combination, *Pilosella bauhini* × *P. *testimonialis*/*P. officinarum*, was recorded in thirteen populations (Table 1, Fig. 2). Similarly, other less frequent hybrid morphotypes that were recorded in five populations each putatively originated from the following crosses: *P. pavichii* × *P. bauhini*, *P. *testimonialis* × *P. officinarum* and *P. bauhini* × *P. officinarum* (Table 1, Fig. 2). The other five hybrid morphotypes involving those that putatively originated from hybridization of *P. onegensis* were rarely recorded, mostly in one population each (Table 1, Fig. 2). Only two plants of hybrid morphotype that would correspond to an interspecific cross of two diploid sexual parents were recorded in 19 populations studied (rows 2 and 8 in Table 1).

Comparing the ploidy level of parental taxa with ploidy level of hybrids in respective species-mixed populations, the origin of seven hybrid cytotypes can be inferred from both simple and multiple crosses of co-

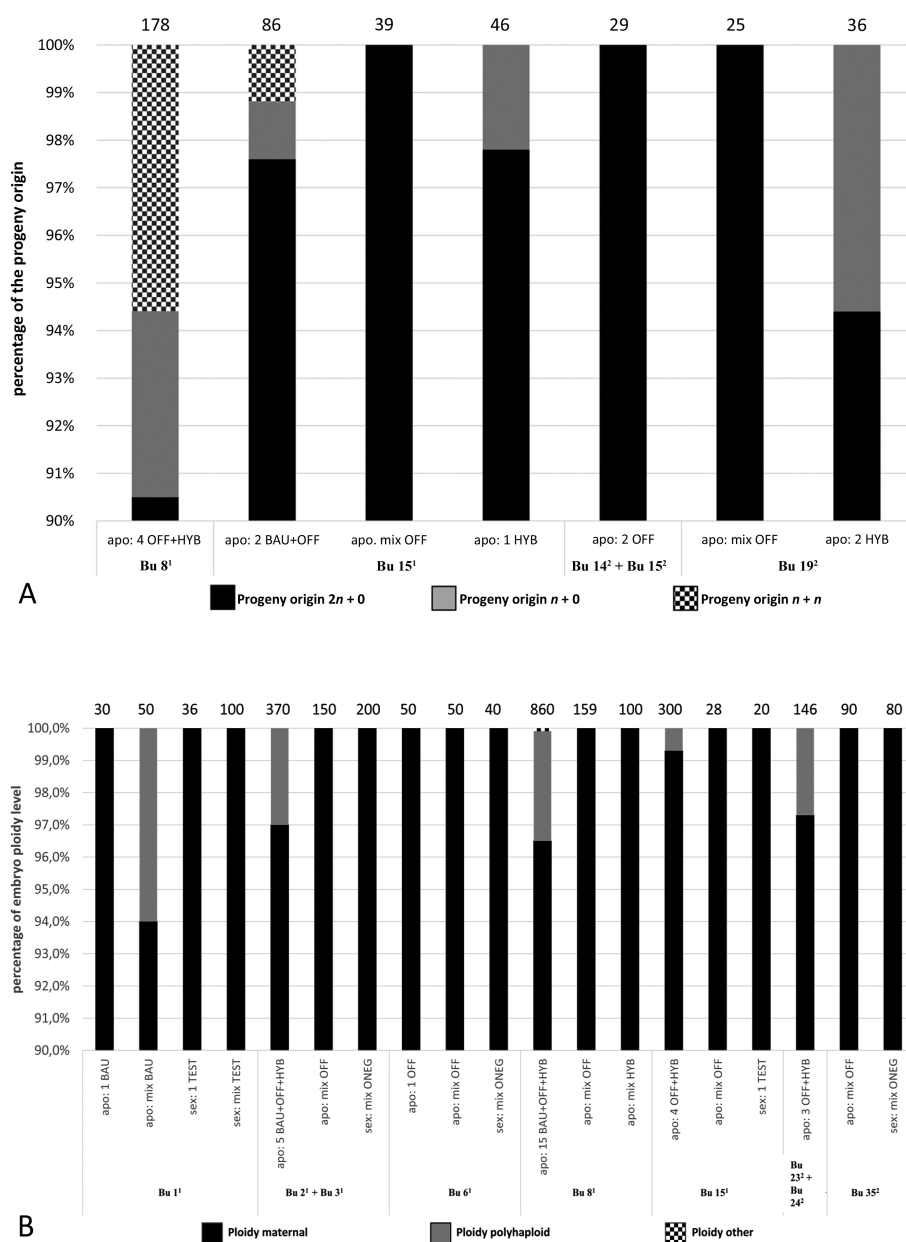


Fig. 3. A: frequency of reproductive pathways operating in apomictic maternal plants (terminology follows Harlan & deWet 1975) and B: ploidy variation in progeny embryos generated in the field based on the reproductive system (RS) of maternal plants. Seeds were sampled in nine selected populations in Bulgaria (locality labels are below x -axis) where diploid sexual and polyploid apomictic species coexisted (Table 1, Table 4 in Supplementary material online). Seeds of sexual taxa were available in only five out of these nine populations studied. The remaining progeny origins/embryo ploidies completing the bars from zero to 90% were exclusively $2n + 0$ in A (black) and maintaining the maternal cytotype in B (black), respectively. A minor section in the bar depicting “other embryo ploidy” (B: population at locality Bu 8¹) corresponded approximately to a ploidy of $6.7x$, detected in a single embryo formed along with hexaploid and trihaploid embryos by a hexaploid apomictic maternal plant. Number of source maternal plants is indicated below x -axis next to symbol of maternal RS; mix = indefinite number of maternal plants representing same taxon. Number of seeds analysed (A: FCSS analysis of seed doublets; B: FCSS analysis of pooled samples comprising 6–10 seeds) is presented in upper line above respective bar. Maternal RS: apo = facultatively apomictic; sex = sexual. Symbols of maternal taxa: BAU = *Pilosella bauhini*; HYB = hybrid *P. bauhini* × *P. *testimonialis*/*P. officinarum*; OFF = *P. officinarum*; ONEG = *P. onegensis*; TEST = *P. hoppeana* subsp. *testimonialis*. Locality labels correspond to those assigned in Table 1 and Table 4. See Material and methods for a description of methods used.

occurring parental cytotypes (Table 1). An unreduced gamete of a diploid sexual parent was potentially involved in 13 simple crosses that produced pentaploid recent hybrids (Table 1, dotted underlined bold). An ambiguous origin of the hexaploid hybrid from a simple cross was possible in two cases via the fertilization of either a reduced (n) or unreduced ($2n$) gamete from an apomictic pentaploid parent of *Pilosella bauhini* (rows 24 and 25 in Table 1). The only hexaploid hybrid could unambiguously originate from a simple cross via fertilization of an unreduced ($2n$) pentaploid gamete from an apomictic parent *P. bauhini* by a reduced (n) gamete from a sexual diploid parent of *P. *testimonialis* (row 15 in Table 1). Most of the hybrid cytotypes potentially originated from either simple or multiple crosses via reduced ($n + n$) parental gametes. Nevertheless, only three $n + n$ hybrids were recorded that definitely originated from a cross of a diploid sexual and a polyploid facultatively apomictic parent. Namely, the seed-sterile tetraploid hybrid coexisted at a single locality with both parents, i.e. a diploid *P. *testimonialis* and a pentaploid/hexaploid *P. bauhini* (row 15 in Table 1). Another unique tetraploid $n + n$ hybrid likely originated from a cross of a diploid *P. pseudopilosella* and a hexaploid *P. officinarum* (row 9 in Table 1). The third triploid and seed-sterile hybrid corresponds to the cross of a diploid *P. pavichii* and an unknown parent, most likely a tetraploid or pentaploid (row 6 in Table 1).

Progeny generated in natural mixed populations

Almost 3300 progeny embryos that originated in altogether nine mixed sexual diploid-apomictic polyploid populations were analysed for either reproductive origins (Fig. 3A) or ploidy variation (Fig. 3B). All 476 embryos in seeds that were sampled of diploid sexual maternal plants, namely *Pilosella *testimonialis* and *P. onegensis*, were exclusively diploid, retaining the maternal ploidy level (Fig. 3B, black bars referring to sexual maternal plants). Therefore, this progeny of sexual mothers must be of $n + n$ origin. Similarly, no hybrids corresponding to polyploid \times diploid crosses were identified among 2822 progeny embryos that were formed by open-pollinated polyploid facultatively apomictic maternal plants. These maternal polyploids that co-occurred in all nine mixed populations with diploid sexual counterparts (Table 1, Table 4 in Supplementary material online), were represented by *P. bauhini*, *P. officinarum* and the hybrids *P. bauhini* \times *P. *testimonialis*/*P. officinarum* (Fig. 3A, 3B). These open-pollinated facultatively apomictic plants generated predominantly clonal apomictic progeny ($2n + 0$, formed via apospory, Fig. 3A, black bars) and thus retained the maternal ploidy level (Fig. 3B, black bars), along with rare sexual progeny ($n + n$, formed via sexual mating, Fig. 3A, chequered bars) and polyhaploid progeny ($n + 0$, formed via haploid parthenogenesis) (Fig. 3A, 3B, grey bars). The frequency of clonal apomictic progeny exceeded 90% in all facultatively apomictic polyploid plants that were open-pollinated in the field (Fig. 3A, black bars). Those facultatively apomictic plants that exceptionally formed $n + n$ progeny via sexual mating were recorded in two populations only (Fig. 3A, populations Bu 8¹ and Bu 15¹). These parents included one plant each of *P. officinarum* (frequency of $n + n$ progeny 1%), *P. bauhini* (frequency of $n + n$ progeny 3%) and a single hybrid (frequency of $n + n$ progeny up to 18%). However, this minority sexual $n + n$ progeny always retained the maternal ploidy level. In addition, analysing the embryo ploidy variation, a single embryo of which the ploidy corresponded to $6.7x$ was recorded among 30 hexaploid embryos formed by a hexaploid facultatively apomictic hybrid plant at locality Bu 8¹. This embryo, not retaining a maternal hexaploid ploidy level (Fig. 3B, a chequered bar), was likely of sexual $n + n$ origin. The polyhaploid progeny produced by polyploid apomictic plants were detected in six out of nine populations studied (Fig. 3A, 3B, grey bars). The maximum rate of polyhaploid embryos generated by an open-pollinated plant in the field was 14%, namely in one hexaploid hybrid.

Summarizing the progeny variation observed at the embryonic stage in the field, we detected no hybridization of a diploid sexual and a polyploid apomictic parent that could occur in any mixed-ploidy population. The sexual diploids exclusively produced diploid ($n + n$) progeny, whereas the polyploid facultative apomicts reproduced predominantly via clonal seeds retaining the

maternal ploidy level ($2n + 0$ progeny). The rare off-type progeny of the facultative apomicts that did not replicate the maternal genotype was mainly represented by polyhaploids ($n + 0$ progeny, Fig. 3A, 3B, grey bars).

Degree of residual sexuality in apomicts

The off-type progeny embryos, comprising both the products of sexual mating and the polyhaploids, reflect the degree of the residual sexuality in a facultatively apomictic parent. If the individual maternal plants from each population were grouped together irrespective of taxonomic identity and ploidy level, the rate of residual sexuality in polyploid apomicts that were either open-pollinated in the field or emasculated in the garden did not exceed 10% in any population. Specifically, the off-type progeny embryos were produced in these populations at a maximum frequency of 9.5% and 6% by open-pollinated (Fig. 3A) and emasculated plants, respectively. Evaluating the individual open-pollinated maternal plants in the field, the maximum frequency of the off-type progeny embryos was scored as 32%, i.e. in a hexaploid hybrid *Pilosella bauhini* \times *P. *testimonialis*/*P. officinarum* that formed an exceptionally high proportion of both sexual progeny and polyhaploids. The highest capacity to form polyhaploid embryos (i.e. 16%) was observed in an emasculated plant of the hexaploid *P. bauhini*. Although the trihaploid progeny of $n + 0$ origin was produced by hexaploid apomictic plants whether emasculated in the garden or open pollinated in the field, this progeny was not recorded among mature plants that were established in the field. The only triploid plant recorded in the field was most likely a diploid \times tetraploid/pentaploid hybrid (Table 1, row 6).

Degree of polyploid \times diploid hybridization

Limited polyploid \times diploid hybridization was recorded in garden experiments using the parental plants from populations in Bulgaria. When a hexaploid facultatively apomictic maternal parent (*Pilosella bauhini*) was pollinated by a diploid sexual parent (*P. *testimonialis*, *P. onegensis*, *P. pavichii*), hybrid embryos rarely resulted with a maximum frequency of 7.5% embryos per progeny array considering individual progenies of either parentage (Table 2). Most of the off-type progeny were trihaploids ($n + 0$ progeny) that were generated by the maternal parent autonomously via haploid parthenogenesis (Table 2). The maternal hexaploid accession 963 of *P. bauhini* displayed some variation in the frequency of apomictically derived ($2n + 0$) progeny (Table 2). Depending on the pollen parent, the rate of $2n + 0$ progeny ranged from 78.4% to 93.3% for embryos and 82.4% to 93.9% for seedlings. Evaluating the crosses of *P. bauhini* from Bulgaria as a whole, the “off-type” progeny embryos (i.e. those not replicating the maternal parent sensu Bicknell & Koltunow 2004) represented a total frequency of 10%. Of these embryos, the most frequent class was rep-

Table 2. Experimental interspecific crosses in *Pilosella*. Each parental accession of *P. bauhini* represents a specific genotype (clone), as inferred from a unique pattern of isozyme phenotypes (Krahulec & al. 2004). Ten trihaploid ($n + 0$) seedlings (all derived of accession 963), as well as two hybrid seedlings of $n + n$ and $2n + 2n$ origin, respectively, perished before they achieved maturity. For data referring to taxonomy, ploidy, reproduction system and source localities of parental accessions, see Table 4 in Supplementary material online. Abbreviations: apo = apomictic; Bu = Bulgaria; CZ = Czech Republic; RS = reproduction system; sex = sexual. Developmental phase of examined progeny individuals: ^e = embryos, ^s = seedlings (total number of available seeds used for seedling is given in parentheses). The symbols of progeny origin follow Harlan & deWet (1975).

Female parent		Male parent		Progeny origin		Number of progeny individuals (ploidy)			Total no. of progeny	% of apomictic ($2n + 0$) progeny		
Species & accession	Ploidy/RS	Locality	Species & accession	Ploidy/RS	Locality	$2n + 0$	$n + 0$	$n + n$	$2n + n$	$2n + 2n$		
<i>P. bauhini</i> 1244	6x/apo	Bu 15 ¹	<i>P. pavichii</i> 1237	2x/sex	Bu 15 ¹	183 (6x)	2 (3x)	14 (4x)	1 (7x)	0	200 ^e	91.5
						4 (6x)	0	0	0	0	4 ^s (53)	
<i>P. bauhini</i> 963	6x/apo	Bu 3 ¹	<i>P. *testimonialis</i> 962/1	2x/sex	Bu 3 ¹	40 (6x)	7 (3x)	1 (4x)	0	0	48 ^e	83.3
						31 (6x)	2 (3x)	0	0	0	33 ^s (59)	93.9
			<i>P. onegensis</i> 946 + 975	2x/sex	Bu 2 ¹ , Bu 4 ¹	40 (6x)	8 (3x)	3 (4x)	0	0	51 ^e	78.4
				4x, sex	CZ, Valov	42 (6x)	8 (3x)	1 (4x)	0	0	51 ^s (85)	82.4
			<i>P. officinarum</i> 629	2x/sex	CZ, Kvilida	182 (6x)	9 (3x)	8 (5x)	1 (8x)	0	200 ^e	91.0
			<i>P. lactucella</i> 269	2x/sex	Bu 3 ¹	56 (6x)	2 (3x)	1 (4x)	1 (7x)	0	60 ^e	93.3
<i>P. bauhini</i> 1081/2	6x/apo	Bu 3 ¹	<i>P. *testimonialis</i> 962/1 + 969	2x/sex	Bu 3 ¹ , Bu 1 ¹	48 (6x)	0	0	0	0	48 ^e	100.0
			<i>P. *testimonialis</i> 962/1	2x/sex	Bu 3 ¹	15 (6x)	0	0	0	1 (8x)	16 ^s (40)	93.8
<i>P. *testimonialis</i> 969	2x/sex	Bu 1 ¹	<i>P. bauhini</i> 1081/2	6x/apo	Bu 3 ¹	0	0	0	0	0	0 ^s (7)	

Reference: ¹Krahulcová & al. (2009b).

represented by polyhaploids (5%) followed by $n + n$ progeny (4%), whereas the least frequent class included the progeny formed via an unreduced gamete (1%) from either a maternal parent ($2n + n$) or from both parents ($2n + 2n$). The destructive FCSS method did not allow scoring of survival to maturity in most of the hybrids that were rarely detected among embryos generated from experimental crosses. Only two hybrid seedlings were recorded (Table 2), but they both perished early after their ploidy level had been determined. Only one reciprocal cross was performed, i.e. the diploid *P. *testimonialis* × the hexaploid *P. bauhini*, but no progeny were produced (Table 2).

Discussion

Sexual species have a limited effect on interspecific hybridization in Bulgaria

The putative products of hybridization of two diploid sexual species were sporadically recorded during our detailed six-year field studies in Bulgaria (Krahulcová & al. 2009b, 2016). The combination of specific morphological characters in only two putatively hybrid plants suggested the parentage of co-occurring sexual diploids, namely, *Pilosella pavichii* × *P. *testimonialis* and *P. *testimonialis* × *P. pseudopilosella*, respectively (Table 1; Krahulcová & al. 2009b, 2016). In comparison with occasional coexistence of two sexual species in a population, the mixed populations consisting of a sexual diploid species and polyploid apomicts are much more common in Bulgaria. Sexual diploids, as for example the most common *P. *testimonialis*, *P. onegensis* or *P. pavichii*, often co-occur with widespread polyploid apomictic species that are primarily represented by *P. bauhini* and *P. officinarum* (Kra-

hulcová & al. 2009b, 2016). Nevertheless, the simple interspecific $n + n$ hybrids that potentially originated from crosses of coexisting diploid sexual and pentaploid/hexaploid apomictic parental species were only rarely recorded among mature plants established in the field (Table 1). In fact, numerous polyploid hybrids that were recorded in the field, for example, the pentaploids, might theoretically be derived from a diploid \times polyploid cross via an unreduced gamete from a sexual diploid parent (Table 1). However, this eventuality contradicts the results of experimental crosses that were previously performed in *Pilosella* species from other regions in Europe because these crosses revealed a relatively low frequency of unreduced gametes produced by sexual biotypes compared with apomictic biotypes (Gadella 1988; Rosenbaumová & Krahulec 2015; Krahulcová & al., unpublished data).

An almost uniform cytotype structure was observed in the progenies of embryos that were formed by open-pollinated plants in the mixed populations in the field. Specifically, the progenies of both sexual diploids and facultatively apomictic polyploids mostly retained the maternal ploidy level, which confirms the rarity of potentially emerging diploid \times polyploid hybrids in Bulgaria. The rare hybrid embryos generated from experimental crosses of a polyploid apomictic maternal parent and a diploid pollen parent (Table 2) also suggests a low chance of such hybrids originating. Furthermore, their germination and survival may be poor under field conditions. A limited spontaneous reciprocal hybridization of the diploid and polyploid parental species may be attributed to several reasons. For example, the absence of the diploid \times polyploid hybrids may be caused by cytological (ploidy) barriers that may be combined with a different timing of flowering in parental species in the respective area. The impact of flowering phenology on hybridization rate may exhibit year-on-year variation in the field. Nevertheless, a regularly delayed start of flowering in spring was obvious for several years in the diploid *Pilosella* **testimonialis* compared with other *Pilosella* species that were cultivated together in lowlands under garden conditions. Another factor potentially limiting the heteroploid crosses of diploid mothers may be male competition. For example, the fertile pollen of diploid sexuals conquers the pollen of triploid apomicts in the mixed-cytotype populations of *Taraxacum* sect. *Taraxacum*, thus preventing the crosses between diploid sexual mothers and triploid apomictic pollen donors (Mártonfióvá 2015). Consequently, the diploid *Taraxacum* plants produce exclusively the diploid seed-progeny (Mártonfióvá 2015).

Interpreting origin in recent polyploid hybrids in Bulgaria

A limited diversity in the progeny of apomictic plants generated in the field and the rarity of hybrids generated from inter-ploidy crossing experiments suggest a rather low level of residual sexuality in populations of facultative

apomicts in Bulgaria. The recent polyploid hybrid morphotypes, which are apomictic and often coexist in mixed populations with both putative parents, are common in Bulgaria (Krahulcová & al. 2009b, 2016; Table 1). The ploidy level is typically identical with the ploidy level of an apomictic putative parent in these mostly pentaploid and hexaploid hybrids. Considering the obvious combination of parental morphological characters, the origin of these hybrids mostly corresponds to the following cross: *Pilosella bauhini* (always apomictic and polyploid) \times *P. *testimonialis* (always sexual and diploid)/*P. officinarum* (always apomictic and polyploid). Identification of the second parent is possible only in those hybrids in which the morphological characters of either *P. *testimonialis* or *P. officinarum* are clearly manifested (Krahulcová & al. 2009b, 2016). In fact, these two putative parental species are closely related. These species share some morphological characters and often co-occur with other morphologically distinct *Pilosella* species in the mixed populations in Bulgaria. This fact complicates the definition of the parental contribution of *P. officinarum*/*P. *testimonialis* in co-occurring hybrids. The most commonly hybridizing parents were found among the species *P. bauhini*, *P. officinarum*, *P. *testimonialis* and *P. pavichii* (Table 1, Fig. 2). However, some of the hybrids detected in Bulgaria are generally rarely reported, i.e. the pentaploid and hexaploid hybrid of *P. bauhini* and *P. pavichii* [*P. xgeorgieffiana* (Zahn) Soják] or the diploid and hexaploid hybrid of *P. bauhini* and *P. onegensis* (Krahulcová & al. 2016).

The residual sexuality of a facultatively apomictic maternal parent certainly contributed to the origin of the polyploid (mostly pentaploid and hexaploid) apomictic hybrids of *Pilosella* in Bulgaria, regardless of whether they were formed via simple or multiple hybridizations (Table 1). The rate of the sexual mating recorded in the facultative apomicts in the mixed populations is rather low (Fig. 3A, 3B), although it might be slightly underestimated because of a possible impact of spontaneous mentor effects in the field (this possibility may concern those progeny of apomicts that retain the maternal ploidy, see Fig. 3B). Indeed, *Pilosella* formed the selfed progeny in crossing experiments due to an effect of heterospecific pollen (Krahulcová & al. 1999; Bicknell & al. 2003). Namely, the frequency of seedlings of autogamous origin was recorded up to 7.6% and 25% in two sexual species of *P. lactucella* (Wallr.) P. D. Sell & C. West and *P. officinarum*, respectively (Krahulcová & al. 1999), whereas the facultatively apomictic *P. aurantiaca* (L.) F. W. Schultz & Sch. Bip. produced the selfed progeny in an order of 10^{-4} (Bicknell & al. 2003). Consequently, the contribution of mentor effects on the rate of sexual mating in facultative apomicts seems to be many times lower than in sexual biotypes. Furthermore, the level of residual sexuality in apomictic mothers, which in Bulgaria are primarily represented by the most common species *P. bauhini* and *P. officinarum*, may show a year-on-year variation depending on environmental conditions. There-

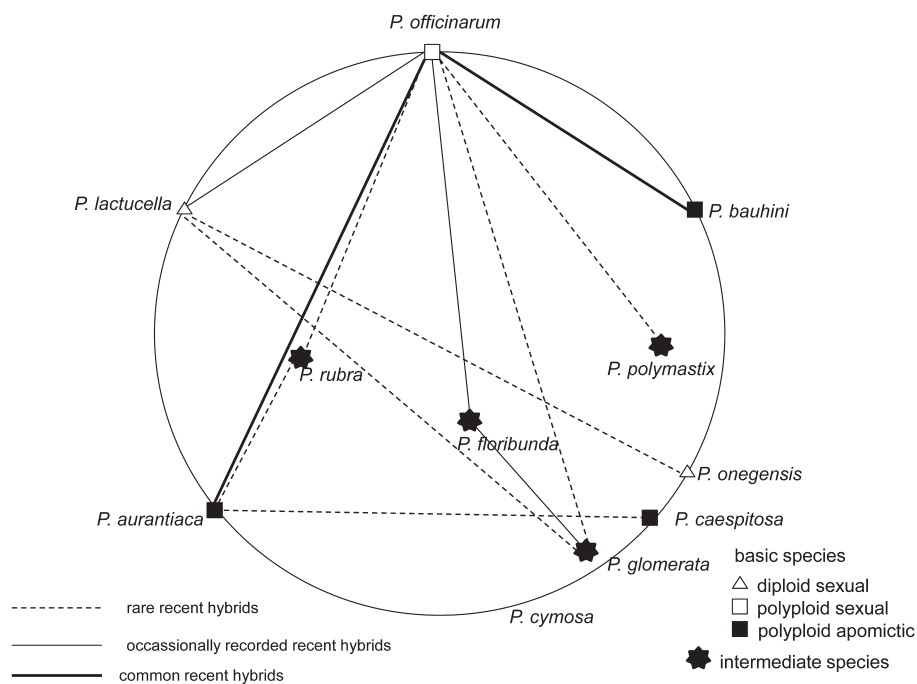


Fig. 4. Schematic drawing illustrating putative spontaneous crosses among *Pilosella* species in 18 hybridizing populations in the Czech Republic and Germany. Selection of taxa is based on nine published population studies excerpted in Table 3. Position on circle does not exactly reflect relationships among individual taxa. *Pilosella cymosa* (out of circle, no recent hybrids found in excerpted population studies) and *P. caespitosa* are considered parental species of a stabilized intermediate species *P. glomerata* (e.g. Fehrer & al. 2007b). Indication of intensity of particular hybridizations corresponds to published population studies excerpted in Table 3 and additional unpublished data from Central European regions.

fore, an influence of external environmental factors on reproduction mode, as was recorded in, e.g., *Rubus* L. subg. *Rubus* (Šarhanová & al. 2012), may increase the chance that apomictic mothers will hybridize. However, an actual way of origin still remains unclear in the most common apomictic pentaploid and hexaploid recent hybrids in Bulgaria, whereas the origin of rare recent hybrids at lower ploidy levels can be interpreted as a simple cross, either homoploid or heteroploid (Table 1).

Traits of hybridizing populations in Bulgaria compared with Central Europe

Attributes and distribution of species occurring in both regions

Present studies regarding recently hybridizing populations of *Pilosella* cover three parental basic species that occur in both Central Europe and Bulgaria, namely, *P. bauhini*, *P. officinarum* and *P. oenegensis* (Fig. 2, Fig. 4). The first two species, which vary in ploidy level, are widespread in both regions. However, *P. officinarum* exhibits a specific pattern of cytotype variation in Central and SE Europe (Mráz & al. 2008). The diploid sexual *P. oenegensis* is rather common in Bulgaria but rare in Central Europe. Despite this difference, recent hybrids of this species were only sporadically found in both regions

(Fig. 2, Fig. 4). Facultatively apomictic biotypes of *P. bauhini* (both pentaploid and hexaploid) form interspecific hybrids in both regions in a similar manner. However, *P. bauhini* exhibits major ploidy variation in Central Europe, ranging from tetraploid to heptaploid ploidy levels. The tetraploids are predominantly sexual and less frequently apomictic, whereas the other cytotypes are apomictic (Rotreklová 2004; Krahulcová & al. 2009a, 2014). Interestingly, the pentaploid and hexaploid apomictic biotypes of *P. bauhini* exhibit substantial genotypic diversity in Central Europe both within and among populations (Krahulcová & al. 2009a, 2014 and unpublished data; Rosenbaumová & Krahulec 2015).

The hybrids of two basic species mentioned above, *Pilosella bauhini* and *P. officinarum*, are common both in Bulgaria and Central Eu-

rope (Fig. 2, Fig. 4). The species *P. officinarum* generates interspecific hybrids with other co-occurring species in both regions (Fig. 2, Fig. 4). Nevertheless, the biotypes of *P. officinarum* that commonly hybridize in Bulgaria differ from those in Central Europe, primarily with respect to ploidy level and reproduction mode. Namely, *P. officinarum*, which was frequently recorded as a putative parental species in hybridizing populations in Central Europe, is tetraploid and sexual (Fig. 4). This biotype of *P. officinarum* is absent in Bulgaria and is substituted by facultatively apomictic pentaploids and hexaploids that are considered to be putative parents of interspecific hybrids in this region (Fig. 2; Krahulcová & al. 2009b, 2016). Function of the sexual tetraploid *P. officinarum* in shaping of the genus *Pilosella* in Central Europe may be comparable to the role of sexual tetraploids in *Rubus* subg. *Rubus* (Sochor & al. 2015; Šarhanová & al. 2017). These taxa with a high level of sexuality that are represented by both *R. ser. Glandulosi* (Wimm. & Grab.) Focke and an obligately sexual *R. caesius* L., force the evolution and speciation of *R. subg. Rubus* in Central Europe (Sochor & al. 2015; Šarhanová & al. 2017).

Capacity for sexual $2n + n$ mating in apomicts

A strongly different capacity for sexual mating via unreduced gametes predominantly from an apomictic par-

ent was confirmed in Bulgaria and Central Europe. The seed progeny that originated in the field in this manner was recorded in six out of eight mixed apomictic-sexual populations in Central Europe (Krahulcová & al. 2014; Rosenbaumová & Krahulec 2015), reaching the maximum frequency of 7% out of all progeny embryos that were formed by apomictic mothers in a population (Fig. 3 in Krahulcová & al. 2014). By contrast, no such progenies were recorded in nine analogous populations studied in Bulgaria (Fig. 3A, 3B).

The experimental crosses simulating the spontaneous hybridizations in the field also provided specific results in both regions. Two polyploid facultatively apomictic species from Central Europe, *Pilosella bauhini* and *P. aurantiaca*, were crossed with the sexual counterpart *P. officinarum* from this region. When either the pentaploid *P. bauhini* or the tetraploid *P. aurantiaca* were pollinated by the tetraploid *P. officinarum*, no polyhaploid ($n + 0$) progeny seedlings were formed, and a substantial part of the sexually derived progeny was of $2n + n$ origin (Table 2 in Krahulcová & al. 2014). On the other hand, the off-type progeny seedlings recovered from a cross of the hexaploid *P. bauhini* from Central Europe and another tetraploid clone of *P. officinarum* comprised the prevailing $n + n$ hybrids as well as the rarer trihaploids ($n + 0$) and the hybrids that originated via an unreduced gamete of either parent ($2n + n$, $n + 2n$) (Rosenbaumová & Krahulec 2015). Parallel crosses of the apomictic hexaploid *P. bauhini* from Bulgaria and a sexual parent yielded various proportions of the off-type progeny, probably reflecting the taxonomic/ploidal affinity of the parents (Table 2). Nevertheless, sexual mating via unreduced gamete(s) was rarely recorded, and a substantial part of the off-type progeny included trihaploids of $n + 0$ origin (Table 2). Consequently, the specific parental ploidy relations that are attributed to the two compared regions might primarily influence the structure of progenies potentially generated from interspecific crosses.

Composition of biotypes affects specific microevolution in mixed populations in both regions

All putative hybrids sampled in the field in Bulgaria are considered recent because they were not spread outside the hybridizing populations and mostly coexisted with both putative parents as individual plants (Table 1). Unlike in Central Europe, no widespread stabilized hybridogeneous taxa that occur independently of their parental species were found in Bulgaria except *Pilosella guthnikiana* (Hegetschw.) Soják (Vladimirov & Szélag 2001). The presence of hybridogeneous species, both of $n + n$ and $2n + n$ origin (e.g. Fehrer & al. 2007), leads to a more complicated structure of *Pilosella* populations in Central Europe in comparison with Bulgaria. In Central Europe, the hybridogeneous species behave independently of parents and often hybridize with other co-

curring taxa thereby forming new combinations. It is not rare to find there multiple hybrids where the morphological characters indicate an involvement of three or rarely four parental basic species (e.g. Křišťálová & al. 2010; Bräutigam 2017; and Fig. 4 in this paper). By contrast, we did not record any such complex multiple hybrids during our six-year population studies in Bulgaria.

The fundamental difference in both areas involves the cytotype structure of (potentially) hybridizing taxa: (1) whereas the sexual diploid and apomictic polyploid taxa often co-occur in hybridizing populations in Bulgaria (Table 1), the diploid sexual putative parents were rather rarely recorded in hybrid swarms in Central Europe (Table 3). (2) Unlike in Bulgaria, sexual taxa exceeding the tetraploid ploidy level occur in Central Europe (Rotreklová & al. 2002; Mráz & al. 2008; Trávníček & al. 2011). Similarly, the tetraploid sexual biotype of *Pilosella officinarum*, which is the most common sexual parent of both recent (Table 3) and stabilized hybrids in Central Europe (e.g. Suda & al. 2007; Bräutigam 2017), is absent in Bulgaria. (3) In addition to *P. officinarum*, numerous other tetraploid hybridizing taxa occur in Central Europe, whereas tetraploids are rather rare among *Pilosella* species in Bulgaria. Therefore, the interspecific crosses occurring in the Central European populations often involve at least one tetraploid parent (Table 3). Consequently, the difference in parental ploidy level is reduced in most of the hybridizing species in Central Europe (Table 3) compared with Bulgaria (Table 1), suggesting the impact of stronger inter-ploidy hybridization barriers in Bulgaria compared with Central Europe.

Many recent hybrids established in the Central European populations were formed via an unreduced gamete. Six out of 14 examples of such recent $2n + n$ hybrids (Table 3) originated via fertilization of an unreduced gamete from an apomictic maternal parent ($2n + n$ hybrids), whereas the sexual parent formed an unreduced gamete most likely in two crosses only (Table 3). The determination of the maternal lineage in respective hybrids in Central Europe was possible due to a specific haplotype shared by the maternal parent and the hybrid (Table 3). The apomictic $2n + n$ hybrids that repeatedly originate from an apomictic and a sexual parent and exceed the parental ploidy level play an important role as sources of population diversity in Central Europe (summarized in Krahulcová & al. 2014). Although their genome is unstable due to a high level of residual sexuality, such hybrids stimulate the origin of entirely new hybridogeneous types because they produce an extremely variable progeny. On the other hand, in 19 Bulgarian populations, we detected only a single recent hexaploid hybrid *Pilosella bauhini* × *P. *testimoniales* (row 15 in Table 1) that might have unambiguously originated from a simple cross via fertilization of an unreduced gamete from an apomictic pentaploid parent *P. bauhini*.

Table 3. *Pilosella* species (sexual species underlined) and their putative recent interspecific hybrids recorded at 18 localities in Central Europe (for data referring to taxonomy, ploidy level and description of localities, see references below table). Hybrid status was inferred from a combination of morphology and ploidy level of both putative parental species that co-occurred with the hybrids at the respective locality. **Bold** cytotypes refer to 14 simple (primary) hybrids that originated via an unreduced gamete from either parent ($2n + n$, $n + 2n$). Out of them, only two hybrids (**dotted underlined bold**) might originate via an unreduced gamete from a sexual parent. The 18 hybrid cytotypes that shared the maternal cp-haplotype (double underlined) originated verifiably via fertilization of the maternal facultatively apomictic parent, suggesting either an unreduced (six hybrids) or a reduced female gamete (12 hybrids). Cytotypes considered as multiple hybrids either had a ploidy that cannot be inferred from a simple hybridization of co-occurring parental cytotypes, or their morphology was markedly closer to either parent, suggesting a backcross. Seed-sterile/semisterile hybrids are indicated “!”. Abbreviations: CZ = Czech Republic; D = Germany. Parental species comprise five basic species (*P. aurantiaca*, *P. bauhini*, *P. lactucella*, *P. officinarum*, *P. onegensis*) and four intermediate species (*P. floribunda*, *P. glomerata*, *P. polymastix*, *P. rubra*).

Ploidy of hybrids	Putative parental species co-occurring at locality (ploidy)	Country, region (number of populations): locality ^{reference}
6x	<i>P. aurantiaca</i> (4x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (2): Slučí Tah, Zhůří near Železná Ruda ^{3,7}
<u>4x</u> , 4x	<i>P. aurantiaca</i> (4x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (3): Hadí vrch, Zhůří near Železná Ruda, Zhůří near Kašperské Hory ^{3,7}
<u>4x</u> , 4x	<i>P. floribunda</i> (4x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (5): Jelení vrchy, Slučí Tah, Zhůří near Kašperské Hory, Zhůří near Železná Ruda, Hadí vrch ³
6x	<i>P. floribunda</i> (4x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (1): Hadí vrch ³
3x ¹ , 4x	<i>P. lactucella</i> (2x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (1): Jelení vrchy ³
<u>2n = 38</u>	<i>P. glomerata</i> (5x), <i>P. officinarum</i> (4x)	CZ, Šumava Mts (1): Filipova Hut ³
4x, <u>4x</u> , 5x, <u>5x</u> , 6x, <u>7x</u> , 8x , 8x	<i>P. bauhini</i> (4x), <u>5x</u> , 6x, <u>7x</u> , <i>P. officinarum</i> (4x, 5x)	CZ, C Bohemia (1): Praha – Vysočany ^{4,7}
aneuploid, <u>aneuploid</u>		
5x, 7x	<i>P. bauhini</i> (5x), <i>P. officinarum</i> (4x)	CZ, C Bohemia (1): Přestavilky ⁷
4x, <u>7x</u>	<i>P. bauhini</i> (5x), <i>P. officinarum</i> (4x)	CZ, NE Bohemia (1): Plouznice ⁷
5x, <u>5x</u> ¹ , <u>7x</u> , 8x	<i>P. bauhini</i> (<u>5x</u> , 6x), <i>P. officinarum</i> (4x)	CZ, NW Bohemia (1): Valov ⁹
5x ¹ , 6x, 6x ¹ , 8x	<i>P. bauhini</i> (5x, 6x), <i>P. officinarum</i> (6x) / <i>P. officinarum</i> (6x)	CZ, S Moravia (1): Brno ^{4,7}
4x, <u>4x</u> , <u>6x</u>	<i>P. aurantiaca</i> (4x), <i>P. officinarum</i> (4x)	D, North Rhine–Westphalia (1): Hagen ^{5,7}
7x	<i>P. aurantiaca</i> (4x), <i>P. rubra</i> (6x)	CZ, Krkonoše Mts (1): Horní Mísečky ⁶
2x	<i>P. lactucella</i> (2x), <i>P. onegensis</i> (2x)	CZ, Krkonoše Mts (1): Janovy Boudy ^{1,2}
5x	<i>P. rubra</i> (6x), <i>P. officinarum</i> (4x)	CZ, Krkonoše Mts (1): Pomezní Boudy ^{1,2}
4x ...	<i>P. glomerata</i> (5x), <i>P. lactucella</i> (2x)	CZ, Krkonoše Mts (1): Janovy Boudy ^{1,2}
<u>4x</u>	<i>P. glomerata</i> (5x), <i>P. floribunda</i> (4x)	CZ, Krkonoše Mts (1): Javoří Boudy ^{2,6}
<u>4x</u>	<i>P. glomerata</i> (5x), <i>P. floribunda</i> (4x)	CZ, Šumava Mts (2): Jelení vrchy, Zhůří near Kašperské Hory ³
4x	<i>P. glomerata</i> (4x), <i>P. floribunda</i> (4x)	CZ, Šumava Mts (1): Gerlova Hut ³
<u>4x</u> ¹	<i>P. aurantiaca</i> (4x), <i>P. caespitosa</i> (4x)	CZ, Krkonoše Mts (1): Pomezní Boudy ^{1,2}
4x, 6x	<i>P. polymastix</i> (4x), <i>P. officinarum</i> (4x)	CZ, C Bohemia (1): Mlín ⁸

References: ¹Krahulcová & al. (2001); ²Krahulec & al. (2004); ³Krahulec & al. (2008); ⁴Krahulcová & al. (2009a); ⁵Krahulcová & al. (2012); ⁶Krahulcová & al. (2013); ⁷Krahulec & al. (2014); ⁸Krahulec & al. (2014); ⁹Rosenbaumová & Krahulec (2015).

The polyhaploid ($n + 0$) seed progeny generated at various rates by facultatively apomictic polyploids was detected in populations in the field in Bulgaria (Fig. 3A, 3B) and Central Europe (Krahulec & al. 2011). However, reliable detection of the polyhaploid origin is possible only in those progeny embryos and seedlings that can be compared with their certainly identifiable maternal parent (e.g. Krahulec & al. 2008). When examining the mature potentially polyhaploid plants already established in the field, this requirement is unfeasible. Nevertheless, mature triploid plants that were likely of polyhaploid origin were rarely found in two populations in the Czech Republic (Krahulec & al. 2008, 2014). This finding admits the importance of polyhaploids in microevolutionary processes that were simulated in experiments (Krahulec & al. 2011). Because no triploids that might potentially be of polyhaploid origin ($n + 0$) were detected among mature plants in populations in Bulgaria (Table 1), the polyhaploid progeny likely exhibits a minimal chance to establish and survive under field conditions there. Consequently, the haploid parthenogenesis that along with a sexual mating generates progeny not replicating the apomictic parent appears to be unsuccessful in populations in Bulgaria.

Evidence and impact of hybridization in other apomictic genera – examples in Asteraceae and Rosaceae

Hybridization has played an important role in evolutionary history of *Hieracium* s. str. that is closely related to *Pilosella*. Both genera, formerly classified as subgenera of the genus *Hieracium* s.l., share a traditional classification that is based on morphology and distinguishes the basic and intermediate (hybridogeneous) species. Surprisingly, using a combination of molecular markers, many of the species of *Hieracium* s. str. (diploid to tetraploid) that had traditionally been classified as basic ones, proved to be of hybrid origin (Krahulec & al. 2013). This suggests a stronger effect of hybridization on evolution of the genus that had been assumed previously. On the other hand, unlike to *Pilosella*, the recent *Hieracium* hybrids potentially originating in the species-mixed populations are very rare: using the combination of nuclear DNA markers and allozymes, one recent hybridization between two diploid species was still documented (Mráz & al. 2011).

Another agamic complex with a complicated reticulate structure, *Taraxacum* F. H. Wigg. (dandelions, *Asteraceae*), also comprises sexual diploids and apomictic polyploids similarly to hawkweeds (both *Hieracium* s. str. and *Pilosella*). However, unlike in *Pilosella*, apomixis is considered to be almost obligate in *Taraxacum*, with the exception of an occasionally fertilized unreduced female gamete (Mártonfióvá & al. 2010). Therefore, a rare manifestation of residual sexuality in apomictic dandelions limits a potential impact on interspecific hybridization. The asexual clones of *Taraxacum*

that had probably originated primarily from crosses between sexuals and apomicts have further been diversified by means of accumulation of mutations (Majeský & al. 2012). In fact, a perfect correlation was found between genotype fingerprints and morphotypes in polyploid obligatory apomictic accessions of *T. officinale* agg. (Majeský & al. 2012), and the clusters of apomictic taxa in *T. sect. Erythrosperma* (H. Lindb.) Dahlst. may also be well characterized by both molecular markers and morphology (Majeský & al. 2015).

A various congruence of molecular markers and morphological characters delimiting the taxonomic groups at different levels was found in the family *Rosaceae*. Whereas the standard barcoding markers resolved the taxonomic groups in *Crataegus* L. poorly due to supposed hybridization between different lineages of the genus (Zarrei & al. 2015), a genetic evidence of parentage in 11 hybrid species of *Sorbus* L. in Central Europe was given, combining the chloroplast DNA and AFLP markers (Uhrinová & al. 2017). These *Sorbus* hybrids among sexual parents are polyploid and supposedly apomictic and may or may not be discriminated by morphology (Uhrinová & al. 2017). The combination of nuclear and chloroplast genetic markers was species-specific, distinguishing all of the few ancestral diploid species of *Rubus* subg. *Rubus* (Sochor & al. 2015). Furthermore, hybridogeneous origins and parentage were identified in several polyploid and apomictic units (series) of this subgenus that conventionally join the species sharing a similar morphology: these analysed polyploid *Rubus* taxa above species level are believed to be stabilized hybrids of ancient origin (Sochor & al. 2015). Furthermore, a genetic evidence of hybrid origin was accomplished in both stabilized apomictic species and local recent hybrids comprising one of the series of *R. subg. Rubus* (Šarhanová & al. 2017).

The pattern of reproductive variation and the processes that putatively shape population structures in *Pilosella* and *Rubus* subg. *Rubus* share some similar features. The level of apomixis/sexuality in the tetraploid *R. ser. Glandulosi* proved to be also different in two Central European regions: the same taxa were strictly sexual and partly apomictic, respectively (Šarhanová & al. 2012). Similarly to *Pilosella* in Central Europe, the embryos of $2n + n$ origin increase the ploidy level in the seed-progenies of *R. subg. Rubus* in the field (Šarhanová & al. 2012). These embryos that had originated via fertilization of an unreduced female gamete of both triploid and tetraploid mother plants were detected in three series of *Rubus* with a high degree of residual sexuality. In this way, the origin of the pentaploid and hexaploid species of *R. subg. Rubus* is suggested (Šarhanová & al. 2012). On the other hand, this type of rare sexual mating is probably the only opportunity for the manifestation of residual sexuality in apomictic mothers in *Rubus*, because no sexual $n + n$ progeny embryos were detected in apomictic taxa (Šarhanová & al. 2012). The hybridization in

R. subg. *Rubus* is thus almost exclusively unidirectional (Šarhanová & al. 2017), similarly to hybridization in apomictic taxa of *Taraxacum* (Mártonfiová & al. 2010).

Conclusions

The diploid fully sexual species of *Pilosella*, which are common in Bulgaria, hybridize only exceptionally as maternal parents with facultatively apomictic polyploids co-occurring in mixed populations in Bulgaria. One of the reasons limiting this hybridization is likely an inter-ploidy barrier. Comparing the residual sexuality in most of the facultatively apomictic *Pilosella* plants in Bulgaria and Central Europe, the common rate of the off-type progeny does not exceed 10% in any region (Fig. 3A, 3B here and fig. 3 in Krahulcová & al. 2014, respectively). The crucial difference between the two regions involves the occurrence of the high-polyploid $2n + n$ hybrids from an apomictic and a sexual parent that are occasionally and repeatedly formed in the mixed sexual-apomictic populations in Central Europe (Czech Republic and Germany), whereas they are exceptionally rare in Bulgaria. These recent $2n + n$ hybrids have a high degree of residual sexuality due to a low penetrance of apospory (Hand & al. 2015), thereby stimulating the on-going hybridizations in the mixed populations (Krahulcová & al. 2014). The tetraploid cytotype of *P. officinarum* that is common in Central Europe but absent in Bulgaria is the most frequent sexual pollen parent involved in the origin of such $2n + n$ hybrids. Sporadic facultatively apomictic plants of unknown origin that formed the off-type progeny embryos at a considerably high rate (namely up to one-third of all progeny) were rarely recorded in Bulgaria. However, the real impact of such exceptionally occurring plants on shaping the diversity of *Pilosella* populations in Bulgaria remains unclear. The parentage of many polyploid and morphologically defined interspecific hybrids in Central Europe is interpretable due to specific patterns of parental chloroplast-DNA haplotypes and combinations of ploidy levels. By contrast, the absence of these clues does not allow us to clearly retrace the origin of polyploid apomictic hybrid morphotypes that are most common in the mixed sexual-apomictic populations in Bulgaria. The presence of hybridogeneous species with high penetrance of apomixis leads to more complicated population structure in Central Europe in comparison with populations in Bulgaria.

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