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The Detection, Rate and Manifestation of Residual Sexuality in Apomictic Populations of *Pilosella* (Asteraceae, Lactuceae)

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Abstract The effect of maternal, facultatively apomictic plants on population diversity was evaluated in seven hybridizing polyploid *Pilosella* populations, where apomictic (*P. bauhini* or *P. aurantiaca*) and sexual (*P. officinarum*) biotypes coexist. The ploidy level, reproductive system, morphology, clonal structure and chloroplast DNA haplotypes were used to characterize these plants and their hybrids. The reproductive origins of the progeny were assessed through either a flow cytometric seed screen and/or a comparison between the ploidy level of progeny embryos/seedlings and the maternal ploidy level. The cultivated progeny derived from residual sexuality in maternal apomicts were also identified based on their morphology and reproductive behaviour. The progeny different from their maternal parents (0.6–92.3 % of progeny embryos and 0–100 % of progeny seedlings) originated either sexually or via haploid parthenogenesis. Comparing the facultatively apomictic and sexual mothers, the progeny arrays generated in the field showed that apomictic mothers produce progeny that is more variable in ploidy level. This effect was demonstrated at both the embryonic and seedling stages of progeny development. Residual sexuality in apomicts was also effective in experimental crosses, generating progeny similar to spontaneous hybrids in the field. The $2n + n$ hybrids produced from an apomictic and a sexual parent displayed similar reproductive behaviour, producing polyhaploid, sexual and apomictic progeny in variable ratios. Repeated hybridizations between parental species and/or multi-step crosses can result in hybrid swarms rich in cytotypes and morphotypes. The variation recorded in these populations suggests prevailing introgressive hybridization towards the sexual species *P. officinarum*.

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Introduction

Because of residual sexuality in facultatively apomictic angiosperms, the dominant production of asexual seeds is commonly combined with occasional sexual mating on the female parent. Generally, asexuality with occasional sex is advantageous for the fixation and spread of adapted genotypes along with an increase of progeny variation through recombination and segregation (D'Souza and Michiels 2010). The promotion of genetic diversity and the simultaneous fixation of heterosis enhance the colonization ability of facultatively apomictic plants, a number of them successful invaders (Asker and Jerling 1992; Richards 2003). Fertilization of both unreduced and reduced egg cells might result in hybridization (e.g., Krahulcová et al. 2004), even between two apomictic taxa (e.g., Houliston and Chapman 2001; Krahulec et al. 2004). Crosses between either two apomicts or an apomictic plant and its sexual relative produce new biotypes, which are often apomictic (e.g., Krahulec et al. 2004). Some of these new products might surpass their parents in ploidy, reflecting the participation of unreduced gametes in a cross. Along with true sexual mating, haploid parthenogenesis (development of a reduced egg cell without fertilization) represents another pathway for the generation of progeny distinct from the polyploid apomictic maternal parent. While many of the resulting polyhaploids are weak plants, some progeny are vigorous and fertile (Asker and Jerling 1992; Richards 1997; Krahulec et al. 2011b). In addition to polyhaploids, the typical dominating apomictic progeny and the sexual progeny from either reduced or unreduced gametes are generated on polyploid, facultatively apomictic maternal plants (Asker and Jerling 1992). Thus, residual sexuality is manifested through both sexual and polyhaploid progeny, also referred to as "aberrant" or "off-type" progeny (Bicknell and Koltunow 2004).

Most studies concerning residual sexuality have been focused on apomictic plants of potential agricultural importance. Sexual reproduction rates have been determined in a number of taxa (Savidan et al. 2001; Hörandl and Paun 2007), ranging from a few percent to almost half of the entire resulting progeny when different species were compared. Similarly, considerable variation in the degree of apomixis has been revealed among different genotypes within species (Albertini et al. 2001; Matzk et al. 2005). The actual expression of apomictic reproduction can also be modified by the environment, the respective pollen donor or both (Nogler 1984; Asker and Jerling 1992; Koltunow 1993; Richards 1997). The expression of apomixis/sexuality, particularly in hybrids from an apomictic and a sexual parent, might vary among individual hybrid genotypes, reflecting interactions between parental genomes (e.g., Curtis and Grossniklaus 2007; Sharbel et al. 2009; Krahulcová et al. 2011). Rates of residual sexuality remain poorly understood among wild apomicts; moreover, the ability of particular biotypes to reproduce apomictically/sexually might vary in time and space in field populations as a result of ongoing hybridizations and backcrosses with sexual relatives (van Dijk 2003).

Pilosella (formerly classified as a subgenus of *Hieracium*) exhibits autonomous aposporous apomixis. Independence of fertilization (Bicknell and Koltunow 2004) simplifies not only experimental studies in the genus but also investigations into the processes that shape the structure of populations in the field. A perennial habit, vegetative propagation, efficient fertility in most hybrids and short generation time are additional advantages of using this polyploid agamic complex as an experimental model system (Bicknell and Koltunow 2004; Koltunow et al. 2011a). It is supposed that two independent, dominant genetic loci control apomeiosis and parthenogenesis (Catanach et al. 2006) and that their incomplete penetrance in ovules results in facultativeness of apomixis in *Pilosella* (Koltunow et al. 2011b). Facultative apomicts hybridize as both seed and pollen parents (Krahulcová et al. 2000; Rotreklová 2008). Both recent hybrids and stabilized hybridogenous taxa occur in nature (Fehrer et al. 2007; Krahulec et al. 2008; Krahulcová et al. 2009, 2011).

During previous field research, we recorded numerous populations in which facultatively apomictic and sexual species of *Pilosella* coexisted and hybridized (e.g., Krahulec et al. 2004, 2008; Křišťálová et al. 2010). When the parental taxa differed sufficiently in morphology, the hybrid swarms were recorded. These swarms were typically characterized by substantial morphological variation among coexisting biotypes. A search for sources of this variation in two hybrid swarms in the Czech Republic showed high clonal and/or cytotypic population diversity (Krahulcová et al. 2009). An analysis of the relationships between maternal plants and progeny in these two populations demonstrated the efficiency of polyploid maternal facultative apomicts in increasing population diversity, at least with respect to the ploidy level (Krahulcová et al. 2009). This phenomenon, however, was not confirmed in other hybridizing population in Germany as a result of the inconclusive identification of the maternal parent of the hybrids (Krahulcová et al. 2012).

We proposed that the coexistence of hybridizing facultatively apomictic and sexual parents (both polyploid) at other localities results in similar population structures based on similar relationships between maternal plants and progeny arrays. An analysis and comparison of these relationships should reveal the processes that shape the structure of these hybridizing populations. Accordingly, we chose four additional *Pilosella* populations in central Europe, where on each occasion, two hybridizing parental species exhibited distinctly different morphologies: the facultatively apomictic *P. aurantiaca* or *P. bauhini* and the sexual *P. officinarum*. The aim of this study was to evaluate the impact of residual sexuality in apomictic maternal parents on the variation among hybrid biotypes coexisting in mixed (apomictic and sexual) populations, particularly variation in ploidy level. The following component subjects were studied:

(1) The structure of mature plants coexisting at particular localities was compared with the structure of their progeny arrays generated in the field, with respect to ploidy level, morphotype (maternal or hybrid), reproductive mode and genotype.
(2) The facultatively apomictic maternal plants were (a) open-pollinated in the field and (b) hand-pollinated under garden conditions, and the resulting "off-type" progeny were detected and quantified. The hand-pollinations simulated putative spontaneous crosses in the field.
(3) The progeny variation in mixed (apomictic and sexual) populations was evaluated, comparing the progeny generated via apomictic *versus* sexual mothers.

Some of the component results, which are summarized here, were formerly published elsewhere (Krahulec et al. 2008; Krahulcová et al. 2009, 2011, 2012), referring to a total of five hybridizing populations. The structure of mature plants in the field and the progeny structure of selected facultatively apomictic mothers were described in these papers. Through generalized conclusions based on the larger number of populations, we performed the following tasks:

(1) examined two additional hybrid swarms (localities 4 and 6 in Fig. **1**) with respect to both the population structure of mature plants (apomicts and sexual plants) and the variation of their progenies;
(2) completed the data concerning progeny generated through both facultatively apomictic and sexual maternal plants at three localities (localities 1, 2 and 3 in Fig. **1**); and
(3) performed experimental crosses, simulating the origin of selected hybridogenous types in the field.

We summarize and discuss common traits for all seven hybridizing populations investigated, including previously published component results (Krahulec et al. 2008; Krahulcová et al. 2009, 2011, 2012). A comparison of progeny and population structures in the wild provided an estimate of how similarly derived new biotypes are established in the field.

Material and Methods

Terminology

In this text, the term apomixis or apomictic actually means facultative apomixis or facultatively apomictic because apomixis in *Pilosella* is a facultative trait (e.g., Koltunow et al. 2011a,b). The term residual sexuality commonly refers to occasional sexual mating of facultatively apomictic maternal plants (Asker and Jerling 1992). The progeny of sexual mating via the conjugation of either reduced or unreduced gametes are named $n + n$, $n + 2n$, $2n + n$ and $2n + 2n$, and the first symbol represents the female gamete (Harlan and de Wet 1975). Two additional reproductive pathways, namely apomixis (in a narrow meaning) and haploid parthenogenesis, generate progeny without fertilization of either unreduced ($2n + 0$) or reduced ($n + 0$) egg cells, respectively. Haploid parthenogenesis combines meiosis (element of sexuality) with the avoidance of fertilization (element of apomixis). Although this reproductive pathway is occasionally included in the broadly defined term apomixis (for citations and discussions, see Krahulcová et al. 2004; Fehrer et al. 2007), variation is generated through the production of progeny distinct from the maternal parent (Krahulec et al. 2011b). In this study, the manifestation of maternal residual sexuality is considered as all categories of progeny that differ from the maternal parent ("off-type" progeny). Thus, we include the products of both sexual mating and haploid parthenogenesis in the progeny generated through residual sexuality of the apomictic seed parent. The reproductive system in $2n + n$ hybrids from apomictic and sexual parents is referred to as "variable" because the hybrids form apomictic, polyhaploid and sexual progeny at variable rates (Krahulec et al. 2008; Krahulcová et al. 2009, 2011, 2012).

Plant Material

The progeny of particular biotypes coexisting in the field were studied in altogether seven hybridizing populations. The tetraploid sexual cytotype of the species *P. officinarum* Vaill. (syn.: *Hieracium pilosella* L.) coexisted with its facultatively apomictic counterpart, namely the tetraploid *P. aurantiaca* (L.) F.W. Schultz & Sch. Bip. (syn.: *Hieracium aurantiacum* L.) or the pentaploid *P. bauhini* (Schult.) Arv.-Touv. (syn.: *Hieracium bauhini* Schult.). The morphological characters (height and branching of the stem, density of the stellate hairs underleaf, size of the inflorescences and colour of the capitula in the case of *P. aurantiaca versus P. officinarum*) markedly distinguished the parental taxa (Chrtek 2004), thus facilitating the easy identification of their hybrids in the field. All morphotypes that showed the combined characters of two parental species were classified as hybrids without any detailed taxonomic evaluation. The names of the parental taxa follow the convention of Bräutigam (2011).

The location and numbering of the studied populations is provided in Fig. **1**; for a more detailed description, see Appendix **1**. An examination of the population structure of mature plants in the field was previously performed at two localities in the Šumava Mts (localities 2 and 3 in Fig. **1**, both described in Krahulec et al. 2008) and a locality in Germany, North-Rhine Westphalia (loc. 1 in Fig. **1**, described in Krahulcová et al. 2012). The structure of mature plants and their progeny arrays in two populations were published previously (localities 5 and 7 in Fig. **1**, Krahulcová et al. 2009). The two remaining populations in the Czech Republic (localities 4 and 6 in Fig. **1**) were newly studied. The number of plants sampled at particular localities (see Appendix **1**) differed according to the perceptible variation among coexisting morphotypes and the area occupied by the hybridizing population. We tried to obtain a representative sample of plants from each population, covering the morphological variation observed at the locality. A complete list of the parental species and hybrids sampled is provided in Table **1**. Voucher specimens of both mature plants from the field and their cultivated progeny were deposited in the herbarium of the Institute of Botany, Průhonice, Czech Republic (PRA).

A separate study aimed at expression of apomixis (Krahulcová et al. 2011; localities 1, 2 and 3 in Fig. **1** here) evaluates the progeny of two maternal facultatively apomictic species *P. aurantiaca* (tetraploid) and *P. rubra* (hexaploid) when crossed in the field with the sexual tetraploid *P. officinarum*. Here, we present the complete progeny structures derived from all maternal plants (involving both the facultatively apomictic and newly the sexual plants) in these three hybridizing populations. In addition, we present complete data characterizing the progenies of both apomictic and sexual parents from two hybridizing populations (localities 4 and 6 in Fig. **1**).

Plant and Seed Sampling, Cultivation and Detection of Ploidy Level

The individual plants sampled in the field were transplanted to the experimental garden in Průhonice for cultivation. The DNA ploidy level of these mature plants was determined using flow cytometry (Krahulcová et al. 2004). Each individual sample of the fresh leaf was simultaneously analyzed with the leaf tissue of an internal standard with a known chromosome number. Only those peaks in the histograms where the coefficient of variation (CV) did not exceed 3 % were considered. In several

Table 1 Population structure of the mature plants from the seven localities (for their numbering and location, see Fig. 1 and Appendix **1**), where the apomictic and sexual species of *Pilosella* coexist and hybridize. The plants in each population were characterized with respect to morphotype (either parental species or their hybrids), ploidy level, reproductive system (RS) and clonal structure (nr. of genotypes, not studied at Locality 7). For a detailed explanation of the plants' characteristics, see Material and Methods. The hybrids and their maternal apomictic parents, which explicitly shared a characteristic chloroplast DNA haplotype within the locality, are given in **bold**. Abbreviations and explanatory notes: apo − apomictic; sex − sexual; var − variable; ster − sterile; apo• − parthenogenesis was detected using emasculation experiments, without testing theprogeny origins: consequently, two pathways, namely, the true apomixis and haploid parthenogenesis, might be involved; ns − not studied

	Plants assigned to	*P. aurantiaca*	*P. bauhini*				*P. officinarum*			Hybrids								Total nr. of plants	Total nr. genotypes
Locality 1	Ploidy/RS (nr. of plants)	4*x*/apo (3)					4*x*/sex (3)			4*x*/sex (5)				6*x*/var (3)				14	
	Nr. of genotypes (nr. of plants analyzed)	1 (3)					3 (3)			5 (5)				2 (3)					11 out of 14 plants
Locality 2	Ploidy/RS (nr. of plants)	**4*x*/apo (5)**					4*x*/sex (4)			**3*x*/apo (2)**				**6*x*/var (2)**				13	
	Nr. of genotypes (nr. of plants analyzed)	1 (5)					ns			1 (2)				1 (2)					3 out of 9 plants
Locality 3	Ploidy/RS (nr. of plants)	**4*x*/apo (4)**					4*x*/sex (3)			4*x*/sex (1)				**6*x*/var (1)**				9	
	Nr. of genotypes (nr. of plants analyzed)	1 (4)					ns			1				1					3 out of 6 plants
Locality 4	Ploidy/RS (nr. of plants)		5*x*/apo (9)				4*x*/sex (2)			5*x*/apo (11)				7x/var (3), 7x/apo• (1)				26	
	Nr. of genotypes (nr. of plants analyzed)		1 (9)				2 (2)			1 (11)				4 (4)					8 out of 26 plants
Locality 5	Ploidy/RS (nr. of plants)		4*x*/apo (3)	**5*x*/apo (7)**	6*x*/ns (3)	**7*x*/var (1)**	4*x*/sex (17)	5*x*/sex (3)		**4*x*/sex (4)**	4*x*/apo (4)	5*x*/sex (1)	**5*x*/apo (5)**	6*x*/sex (2)	**7*x*/var (8)** **7*x*/apo• (6)**	8*x*/var (3)	**aneuploids**/ both **sex** and apo (12)	79	
	Nr. of genotypes (nr. of plants analyzed)		2 (3)	4 (7)	ns	1	14 (16)	2 (3)		3 (4)	3 (3)	1	2 (5)	1	9 (13)	1 (3)	11 (12)		54 out of 72 plants
Locality 6	Ploidy/RS (nr. of plants)		**5*x*/apo (6)**				4*x*/sex (3)			4*x*/sex (5)		4*x*/apo (3)		**7*x*/var (1)**				18	
	Nr. of genotypes (nr. of plants analyzed)		1 (6)				2 (2)			4 (5)		1 (3)		ns					8 out of 16 plants
Locality 7	Ploidy/RS (nr. of plants)		5*x*/apo (15)		6*x*/apo (4)		6*x*/sex (15)	6*x*/apo (7)	6*x*/ster (5)	4*x*/sex (1)		5*x*/ster (5)		6*x*/apo (1)	6*x*/ster (1)		8*x*/var (2) 8x/apo• (1)	57	

problematic cases, the chromosomes were counted using the root-tip meristems stained with lacto-propionic orcein (Krahulcová and Krahulec 1999).

The seeds, which were sampled separately from the individual plants in the field, were examined using a stereomicroscope to discard both aborted (empty) and damaged seeds. The amounts of viable (filled) seeds collected from particular maternal plants varied considerably, reflecting either the reduced fertility in some plants or the damage of developing seeds by insects. In the case of a sufficient amount of filled seeds per individual plant, the proportion of the seeds remaining after flow cytometric seed screening (see below) was sown, and the seedlings were cultivated to maturity. The DNA ploidy level in the cultivated progeny seedlings was determined using flow cytometry following the procedure described above. Aneuploidy, a potential cause of inter-ploidy peaks in the histograms obtained by analyses of cultivated plants/seedlings, was detected by chromosome counting. Because the parental species markedly differ in monoploid genome size (see Crossing Experiments below), the varying proportion of parental genomes may contribute to variation in DNA content in hybrid progeny, in addition to aneuploidy and differences in ploidy level. During the screening for embryo ploidy in seeds (see below), it was impossible to exclude or detect aneuploidy. In the case of the inter-ploidy peak in the FCSS histogram, the embryo ploidy level was therefore estimated as a decimal number after the symbol "~" (see Table **S1** in Electronic Supplementary Material). The plant/seed sampling, progeny cultivation and experimental design for processing plant material follow the procedure described by Krahulcová et al. (2009).

Reproductive System and Progeny Origins

The reproductive system (i.e., sexual *versus* apomictic) can be ascertained (1) by comparing the seed set of emasculated and open-pollinated capitula (Gadella 1984; Krahulcová and Krahulec 1999) and/or (2) using the flow cytometric seed screen (FCSS) method (Matzk et al. 2000). The latter method allows to distinguish the following mating systems: true apomixis ($2n + 0$ progeny), haploid parthenogenesis ($n + 0$ progeny), sexual mating via reduced ($n + n$) or unreduced ($2n + n$, $n + 2n$, $2n + 2n$) gametes. The $2n + n$ and $n + 2n$ progeny embryos could be discriminated by comparing the endosperm/embryo DNA content (C-value) ratios in different reproductive pathways (Matzk et al. 2000; Krahulcová and Rotreklová 2010), although both embryos might have identical ploidy levels. Similarly, the $2n + 0$ progeny of autonomous apomictic origin without pseudogamy (endosperm/embryo C-value ratio 4 : 2) can be distinguished from the $n + n$ progeny derived from autogamy (endosperm/embryo C-value ratio 3 : 2). The modification of the FCSS method for pooled samples of ten seeds (Krahulcová and Suda 2006) was used to screen the embryos according to their ploidy levels. Consequently, the progeny origins can be inferred from the ploidy level of the respective individual embryos compared with that of their maternal and putative pollen parents. Plants sampled in the field and subsequently cultivated in the experimental garden were at first screened for their reproductive system (sexual *versus* apomictic): the presence/absence of seeds was scored in at least three emasculated/open-pollinated capitula of each individual plant. The reproductive origins of the progeny were precisely determined in embryos (seeds) sampled from both open-pollinated maternal plants in the field and

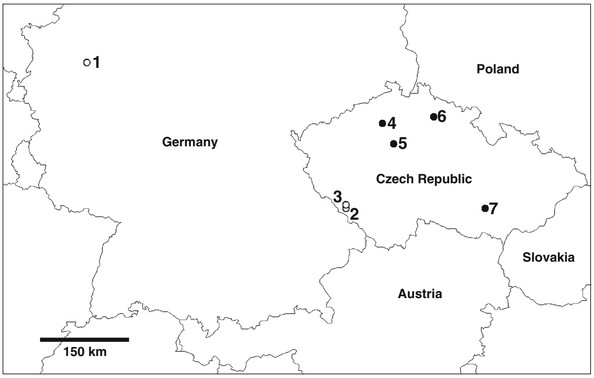

Fig. 1 Location of the seven hybridizing populations studied. For a detailed description of the localities, see Appendix **1**. The populations were derived from crosses between the following parental species: apomictic *Pilosella aurantiaca* × sexual *P. officinarum* (white circles) and apomictic *P. bauhini* × sexual *P. officinarum* (black circles)

emasculated (cut) capitula in the garden. Using both variants of the FCSS method (see above), the CVs that did not exceed 5 % were accepted. For details of the flow cytometric procedures used for the quantification of particular reproductive pathways in *Pilosella,* see Krahulcová et al. (2009, 2011).

The origins of the progeny at locality 5 were analyzed exclusively at the seedling stage, whereas only seeds were analyzed in the case of the progeny from locality 7 (for details, see Krahulcová et al. 2009). Seeds from the maternal plants with variable reproductive system (Fig. **2**) were unavailable from three populations (localities 4, 6 and 7).

Clonal and Haplotype Structure of the Populations

Since the apomictic reproductive system implies the spread of the maternal clone through seeds, the clonal structure (genotype identity) of the plants in the field was studied particularly in the facultatively apomictic biotypes coexisting at individual localities. In addition, with respect to the clonal growth in *Pilosella* (vegetative spread of individual genotypes through stolons), four plants representing the sexual parental species *P. officinarum* were sampled from distant sites at localities 4 and 6 and analyzed for genotype identity (Table **1**). The minimum number of individual genotypes comprising one morphotype/cytotype at each locality was inferred, irrespective of the reproductive system, from a unique pattern of isozyme phenotypes derived from a combination of the four enzymes tested (AAT, LAP, 6-PGDH/PGM and EST). These systems previously showed a competent variability to provide sufficient resolution efficiency in *Pilosella* (Krahulec et al. 2004; Krahulcová et al. 2009).

The maternal lineages among the coexisting plants were studied through an analysis of chloroplast DNA (hereafter referred to as haplotypes; for details of the procedure, see Fehrer et al. 2005). The chloroplast *trn*T−*trn*L intergenic spacer mediated the distinction of several individual sub-haplotypes within the two primary haplotype groups (called I and II) in *Pilosella* (Fehrer et al. 2005). The matroclinal inheritance of the chloroplast DNA (cpDNA) was verified in *Pilosella* (Fehrer et al. 2005). If the parental taxa analyzed in the individual populations differed in their haplotypes, the analysis of the haplotypes in the hybrids indicated the direction of the crosses from which the hybrids might have originated.

Crossing Experiments

Experimental crosses were performed in the greenhouse using the method of Krahulcová et al. (2004) to generate interspecific hybrids between *P. aurantiaca*/*P. bauhini* and *P. officinarum*, which were actually identified in the field. Spontaneous hybrids with a facultatively apomictic maternal parent (either *P. aurantiaca* or *P. bauhini*), as shown in previously analyzed haplotype lineages (Krahulec et al. 2008 and the results published here), were selected. Namely, the following hybrids were considered: the heptaploid hybrid *P. bauhini* × *P. officinarum* from locality 6 (Table **1**) and the hexaploid hybrids *P. aurantiaca* × *P. officinarum* from localities 2 and 3 (Table **1** here and Krahulec et al. 2008). The apomictic accessions used in the crosses had the same genotypes as those observed at the respective localities. Accordingly, residual sexuality in both apomictic maternal parents of these interspecific hybrids, the tetraploid *P. aurantiaca* and the pentaploid *P. bauhini*, was detected in experimental crosses with the tetraploid sexual *P. officinarum*. Two different accessions of the tetraploid sexual *P. officinarum* were used in these crosses (Table **2**).

The seeds, sampled from cross-pollinated maternal facultatively apomictic plants, were sown in garden soil, and the progeny was cultivated in a greenhouse. The sexual pollinating species *P. officinarum* differs from its apomictic maternal counterpart (both *P. aurantiaca* and *P. bauhini*) in monoploid genome size (Suda et al. 2007a). This difference, exceeding 13 % in both cases, is adequate for the identification of homoploid $n + n$ hybrids using flow cytometry, when the hybrids are analyzed simultaneously with their maternal parent (Suda et al. 2007b). Hence, the progeny seedlings were screened according to DNA ploidy level using DAPI staining (for details of the procedure, see Krahulcová et al. 2009). Seedlings showing a separate peak in the flow cytometric histograms when analyzed simultaneously with their apomictic maternal parent were assigned to aberrant (non-maternal-like) progeny, not replicating the maternal apomictic parent. Other progeny, which exhibited only a single peak in simultaneous analyses with their apomictic maternal parent, were assigned to apomictic, mother-replicating progeny. Only the peaks with a coefficient of variation that did not exceed 3 % were accepted. The prospective homoploid $n + n$ hybrids from experimental crosses were pre-screened using this method at the seedling stage. Subsequently, their actual hybrid origin was assessed at the mature plant stage, comparing their morphological characters with those of their maternal parent. As a consequence of the demands on adequate space and time, no reciprocal crosses were performed for both parental combinations. Nevertheless, one inflorescence of *P. officinarum* from locality 6 was tentatively pollinated with the pollen of *P. bauhini* from the same locality.

The reproductive pathways generating the progeny produced in the crossing experiments were inferred from the DNA ploidy level and the morphological characters in the cultivated progeny, compared with those in their facultatively apomictic maternal plant. Because crossing experiments yielded an insufficient amount of seeds, we preferred cultivation of all viable progeny over FCSS screening, a method that destroys seeds. The flow cytometric screening of the progeny seedlings according to their DNA ploidy level was performed using the same method as that used to screen mature plants from the field (see above).

Results

Population Structure

Two populations were newly studied to assess the population structure of mature plants (localities 4 and 6 in the Table 1 and in Fig. 2). The apomictic pentaploid *P. bauhini* parent was represented by a single genotype (clone) within each population, while the two tetraploid *P. officinarum* plants, sampled at each of the two localities, belonged to different clones (Table 1).

Most of the hybrids were either tetraploids (both sexual and apomictic) or apomictic pentaploids (Table 1, Fig. 2). These ploidy levels in hybrids, which maintain the ploidy of either putative parent, suggest $n + n$ hybridization. Whereas the apomictic $n + n$ hybrids were represented through a single clone at each of the two localities, the sexual $n + n$ hybrids at locality 6 comprised four genetically different clones. Heptaploid hybrids were recorded in both populations (Table 1, Fig. 2). Their reproductive system was variable, as these hybrids primarily formed progeny via haploid parthenogenesis and/or $n + n$ mating, and apomixis was the minority reproductive pathway (Fig. 3). Whereas only a single heptaploid hybrid was recorded at locality 6, four genotypically different heptaploid hybrid accessions were successively sampled at locality 4 (Table 1, Fig. 2).

Both parental species at locality 4 had identical haplotypes, namely, the subtype II/7 (classification follows Fehrer et al. 2005). Thus, the maternal parent of hybrids at this locality could not be identified. The parental species at locality 6 showed different haplotype subtypes, namely, the subtype II/7 (shared by tetraploid *P. officinarum* and the tetraploid sexual hybrids) and the subtype II/11 (shared by pentaploid *P. bauhini* and the heptaploid hybrid). Consequently, a pattern of variation in the haplotypes at this locality (Table 1) facilitated the identification of the sexual *P. officinarum* as the maternal parent of the sexual tetraploid hybrids and the apomictic *P. bauhini* as the maternal parent of the heptaploid hybrid (Table 1). As inferred from the ploidy level in both parents and the markedly prevailing maternal (*P. bauhini*) morphological characters in the heptaploid hybrids at localities 4 and 6, these hybrids were likely derived from $2n + n$, suggesting that an unreduced egg cell of the maternal *P. bauhini* was fertilized via a reduced male gamete of *P. officinarum*. An additional haplotype, the subtype I/1, was identified among the plants at locality 6, namely, in the apomictic tetraploid hybrid (Table 1). The occurrence of this haplotype, however, cannot be explained through the simple hybridization between *P. bauhini* and *P. officinarum* at this locality. This haplotype was likely introgressed through a past hybridization with another species present in close proximity.

Progeny Generated in the Field

The origins of the newly investigated progeny from both apomictic and sexual maternal parents were examined at five localities (localities 1, 2, 3, 4 and 6 in Fig. 3). The

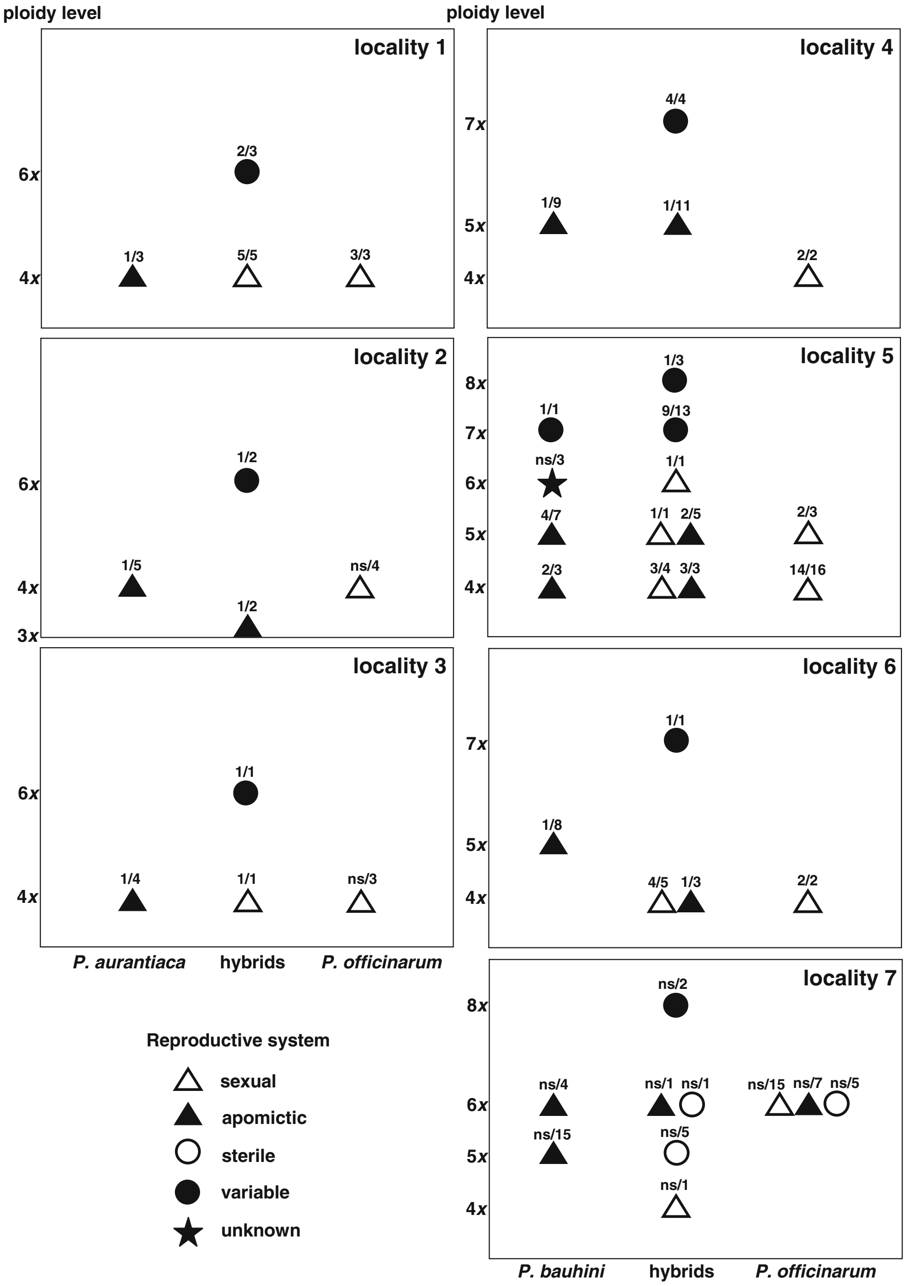

Fig. 2 Charts of the population structure detected at individual localities, where the facultatively apomictic *Pilosella* species (*P. aurantiaca* and *P. bauhini*) hybridized with the sexual *P. officinarum* species. Either parental or hybrid morphotypes were distinguished in each plant; the pictogram represents the reproductive system. The numbers above the pictograms refer to the number of genotypes/number of plants analyzed for the respective cytotype (ns − not studied). At locality 5, a total of 12 aneuploid plants (not shown, ploidy range from hypertetraploid to hyperhexaploid, reproductive system predominantly sexual), were recorded in addition to euploid cytotypes (Krahulcová et al. 2009). For the source data, see Table 1; for the identification of genotypes, see Material and Methods

variation in the reproductive pathways in apomictic mothers indicates that less than 10 % of their progeny were formed via an alternative reproductive pathway to apomixis. Even fewer progeny of sexual mothers were produced through pathways other than sexual $n + n$ mating, namely via the conjugation of unreduced gametes. The ability to produce high-polyploid progeny via unreduced gametes was more manifested in apomictic than in sexual mothers (Fig. 3). In fact, most of this progeny originated via $2n + n$ hybridization, as inferred from the relationship between the ploidy of the embryo and the endosperm using the conventional FCSS method for progeny screening. Moreover, the $2n + n$ origin of the high-polyploid seedlings was inferred when comparing the maternal and progeny ploidy levels and the markedly predominating maternal morphological characters in these cultivated hybrids. The only $2n + 2n$ octoploid embryo was observed among the *P. aurantiaca* seed progeny sampled at locality 2.

Crossing Experiments

Both apomictic maternal parents, the tetraploid *P. aurantiaca* and pentaploid *P. bauhini*, generated apomictic ($2n + 0$) progeny at a high frequency when pollinated by the tetraploid sexual *P. officinarum* (Table 2). In addition, two categories of hybrids, namely $n + n$ and $2n + n$, were rarely formed. Whereas the frequency of the $n + n$ sexual progeny was identical in both maternal species, the $2n + n$ hybrids were detected approximately four times more frequently among the progeny of *P. aurantiaca* than among the progeny of *P. bauhini* (Table 2). Both $2n + n$ hybrid cytotypes sampled in the field at localities 2, 3 and 6 (Fig. 2, Table 1) were also successfully produced in experimental crosses (Table 2). The same direction of these experimental crosses was used, as shown in the origin of the respective $2n + n$ hybrids from the field, i.e., the apomictic maternal plant was pollinated by a sexual parent. Most of the $2n + n$ hybrids from the experimental crosses exhibited poor seed set (not quantified) after open-pollination in the experimental garden. Thus, the experimental $2n + n$ hybrids resembled mature plants sampled in the field in this respect.

No polyhaploid seedlings were observed among the progeny of experimental crosses. An aneuploid $n + n$ hybrid ($2n = 40$) was generated from the pollination of tetraploid sexual *P. officinarum* (maternal accession 1417PI, $2n = 36$) with the pentaploid *P. bauhini* (accession 1422BA, $2n = 45$). Another aneuploid hybrid ($2n = 41$) was detected from a reciprocal cross between these parental species (Table 2). However, no aneuploids were observed among the mature hybrid plants sampled at locality 6 (Table 1), where both experimental parental accessions (1417PI, 1422BA) originated.

Discussion

Common Attributes in the Hybridizing Populations

Although two different pairs of parental species were involved, most of the populations under study shared certain common features. The intrapopulational clonal homogeneity of an apomictic parent was detected in five of the seven populations studied (Appendix 1, Fig. 1). In addition, the three populations (localities 1, 2 and 3 in Fig. 2) shared the same genotype (isozyme phenotype) of the tetraploid apomictic parental species *P. aurantiaca* (Krahulec et al. 2008; Krahulcová et al. 2011). Consequently, both apomictic species (*P. aurantiaca* and *P. bauhini*) effectively spread throughout their respective localities via apomictic (mother replicating) seeds and vegetative growth. Nevertheless, using the same method for the identification of individual clones, we observed remarkable diversity in coexisting clones within other populations of *P. bauhini* in central Europe (Krahulcová et al. 2009; Krahulec et al., unpubl. data). The intrapopulational clonal diversity detected in the parental species *P. officinarum* (Table **1**) is consistent with sexual reproduction in the biotypes studied accompanied by vegetative growth (see the clonal structure at locality 5 in Fig. **2**).

The $n + n$ hybrids (the ploidy did not exceed the parental ploidy) derived from the conjugation of reduced gametes were recorded in six of the seven populations studied (Table **1**, Fig. **2**). These hybrids had diverse reproductive modes, sexual and apomictic, or were sterile. Sexual hybrids exhibiting diverse genotypes were identified at localities 1, 5 and 6. Such genotypic heterogeneity suggests independent origins from repeated hybridizations between parental species and/or multi-step hybridizations with either parental species. In the case of differing parental cpDNA haplotypes, the maternal parent of the $n + n$ hybrids was detected (populations at localities 2, 3, 5 and 6 in the Table **1**). Although most of the $n + n$ hybrids originated via fertilization of the sexual mother plant by an apomictic pollen donor, the haplotype structure at locality 5 showed reciprocal $n + n$ hybridization between apomictic and sexual parents (Table **1** here, Krahulcová et al. 2009).

High-polyploid hybrids (the ploidy exceeded the parental ploidy) were detected in all seven populations (Table **1**). A comparison of the ploidy in these hexaploid, heptaploid and octoploid F_1 hybrids with the ploidy of their parents in the respective populations, suggested hybridization via unreduced gametes from either parent, namely, either $2n + n$ or $n + 2n$ hybridization. A comparison of the haplotypes in the parental species and their high-polyploid hybrids at four localities (localities 2, 3, 5 and 6 in Table **1**) demonstrated the involvement of apomictic mothers in these interspecific hybridizations. Furthermore, the genome sizes of two parental species, *P. aurantiaca* and *P. officinarum* (both tetraploid), and their hexaploid hybrids at localities 1, 2 and 3 were consistent with a $2n + n$ origin of these hybrids (Krahulcová et al. 2011). Similarly, the morphological characters of the apomictic parent markedly prevailed over those of the sexual parent in all high-polyploid hybrids from the field, further suggesting the $2n + n$ origin of these hybrids (Krahulec et al. 2008; Krahulcová et al. 2009, 2011, and this paper). However, plants from locality 7 were not evaluated with respect to haplotype structure, genome size or morphology, and the origin of the octoploid hybrids at this locality remains ambiguous (Krahulcová et al. 2009). Consequently, fertilization of the unreduced female gamete from an apomictic parent by reduced pollen from a sexual parent generated high-polyploid hybrids in six of the seven investigated populations.

The two apomictic species, *P. aurantiaca* and *P. bauhini*, formed $2n + n$ hybrids at different frequencies when crossed with the sexual *P. officinarum* (Table **2**). However, the occurrence of $2n + n$ hybrids among the progeny of *P. bauhini* was too rare (a single seedling only) to obtain a meaningful statistical evaluation. The two interspecific crosses were performed in two different years. Potential climatic differences between years could result in different ratios of the apomictic/sexual progeny derived from crosses (e.g., Naumova et al. 1999; Houliston et al. 2006), but cultivation in a greenhouse might reduce this effect. The octoploid and decaploid seedlings in experimental crosses (Table **2**) probably originated autonomously from the maternal plant through chromosome doubling in the proembryo. The origin of rare progeny with doubled chromosome numbers compared with the apomictic maternal parent has been described in *Pilosella* (Krahulcová and Krahulec 2000; Krahulec et al. 2011b).

*Role of 2*n *+* n *Hybrids in the Mixed Apomictic/Sexual Populations*

The high-polyploid hybrids from the unreduced female gamete of an apomictic parent could originate repeatedly, at least in three of the investigated populations (localities 1, 4 and 5 in the Table **1**, Fig. **2**), as inferred from the genotype diversity of the respective $2n + n$ hybrids. A reduced set of viable seed in these hybrids and the recent origin of hybrid swarms (Krahulcová et al. 2009, 2011, 2012, and Appendix **1** in this paper) suggests that rather than a long-term genetic diversification via multi-step hybridizations or mutations, an independent repeated origin of hybrids from interspecific $2n + n$ hybridizations occurs. The variable reproductive system in these $2n + n$ hybrids results in rather low and deficient production of apomictic ($2n + 0$) progeny replicating the maternal genome. For this reason, genomic instability in subsequent generations is expected in these high-polyploid hybrids in the field. Nevertheless, the $2n + n$ hybrids represent important components of hybridizing populations, producing variable progeny and thus stimulating the origin of entirely new hybridogenous types.

Gene Flow between Apomictic and Sexual Biotypes

With respect to morphological characters and reproductive behaviour, the characteristic complex structure observed in advanced hybrid swarms comprised apomictic (either *P. aurantiaca* or *P. bauhini*) and sexual parents (*P. officinarum*) and their diverse hybrids (Table **1**, Fig. **2**). The high-polyploid hybrids of the $2n + n$ origin were closer to their apomictic parent than the other hybrids coexisting at the locality. Hybrids that are close to the sexual parent are typically comprised of several morphotypes, often without sharp borders between individual hybrid plants. Such hybrids can arise from primary interparental (interspecific) hybridizations, from backcrosses between the primary hybrids and their sexual parental species and secondary hybridizations between previously established hybrid lines. Repeated backcrosses smooth the border between the sexual parental species and the hybrids: the pattern of variation in morphology, reproductive behaviour and haplotype structure (at locality 1, Krahulcová et al. 2012) suggests introgressive hybridization from an apomictic parental species towards sexual parental species (Krahulcová et al. 2009, 2012; Krahulec et al. 2011a). The tendency of the sexual tetraploid *P. officinarum* to absorb genetic material from other cross-compatible species via interspecific hybridizations is also evident through a comparison of the genome size variation (Cx-values) in *P. officinarum* and related species and their hybrids (Suda et al. 2007a).

The potential of facultative apomicts to cross with their sexual relatives is attributed more to the male function of apomictic taxa in hybridizations than to the female function through residual sexuality (Hörandl and Paun 2007; Mogie et al. 2007). Indeed, many apomicts have at least partially fertile, meiotically reduced pollen that mediates the fertilization of sympatric sexual relatives and, consequently, the generation of new hybrid (backcrossed) genotypes (Whitton et al. 2008). However, factors, such as geographic separation, ecological differentiation, cytotype distribution, or gender, might differently act as reproductive barriers in different agamic complexes. For example, the importance of hybridizations between sexual plants and apomicts in enriching genotype diversity is negligible in natural populations of *Ranunculus cassubicus* complex, *Antennaria rosea* complex (reviewed in Hörandl and Paun 2007) or *Taraxacum* sect. *Erythrosperma* (Mártonfiová et al. 2010); however, these hybridizations produce important genotypic variation in populations of *Taraxacum* sect. *Ruderalia* (Hörandl and Paun 2007; Mártonfiová 2011). In the latter case, both the male and female functions of the facultative apomicts contribute to gene flow, which is directed towards higher ploidy levels to enhance the gene pool of apomicts through the genes of sexual plants in the populations of *Taraxacum* sect. *Ruderalia* (Mártonfiová 2011). By contrast, we demonstrated that an opposite direction of the gene flow between apomicts and sexual plants is present in the hybridizing apomictic/sexual populations of *Pilosella*, at least in those populations where the tetraploid *P. officinarum* represents the sexual parent. This assumed effect is also supported through prevailing sexuality in the hybrids recovered from reciprocal crosses between the facultatively apomictic *P. rubra* and sexual *P. officinarum* (Rosenbaumová et al. 2012).

Impact of Residual Sexuality of Apomicts on the Population Structure

The range of ploidy variation in maternal parents and their progeny provides a different picture in apomictic and sexual mothers (Fig. **4**). An evaluation of the data available from six populations (localities 1, 2, 3, 4, 6 and 7 in Fig. **1**, for the source data, see Table **S1** in Electronic Supplementary Material), revealed that the range of ploidy variation was much higher in apomictic mothers towards their progeny than the corresponding variation in the progeny of sexual mothers. This difference was more conspicuous when evaluating the progeny embryos in mature seeds (Fig. **4**, upper row) than in the progeny of cultivated seedlings (Fig. **4**, bottom row), which probably reflects a selection mechanism at germination and the early seedling stage (Krahulec et al. 2006). Namely, in the case of progeny embryos, the maternal parents ranged from $4x - 6x$ in both apomictic

Table 2 The origins of the progeny produced from the experimental crosses. The ploidy level/chromosome number in the parents and their progeny seedlings is shown in parentheses. For determination of the progeny classes, see Material and Methods. Nr. − number of progeny screened using flow cytometry; • − perished seedlings, originated most likely from the autonomous genome doubling of the proembryo in the maternal apomictic parent.

♀ parent	Progeny class									
	$2n + 0$		$n + 0$		$n + n$		$2n + n$		Total	
	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
	♂ parent *P. officinarum*, accession 74PI ($4x$)									
P. aurantiaca, four accessions ($4x$)	500 ($4x$) + 1• ($8x$)	94.7	0	0	9 ($4x$)	1.7	19 ($6x$)	3.6	529	100
	♂ parent *P. officinarum*, accession 1417PI ($4x$)									
P. bauhini, accession 1422BA ($5x$)	115 ($5x$) + 1• ($10x$)	97.5	0	0	1 ($2n = 41$) + 1 ($5x$)	1.7	1 ($7x$)	0.8	119	100

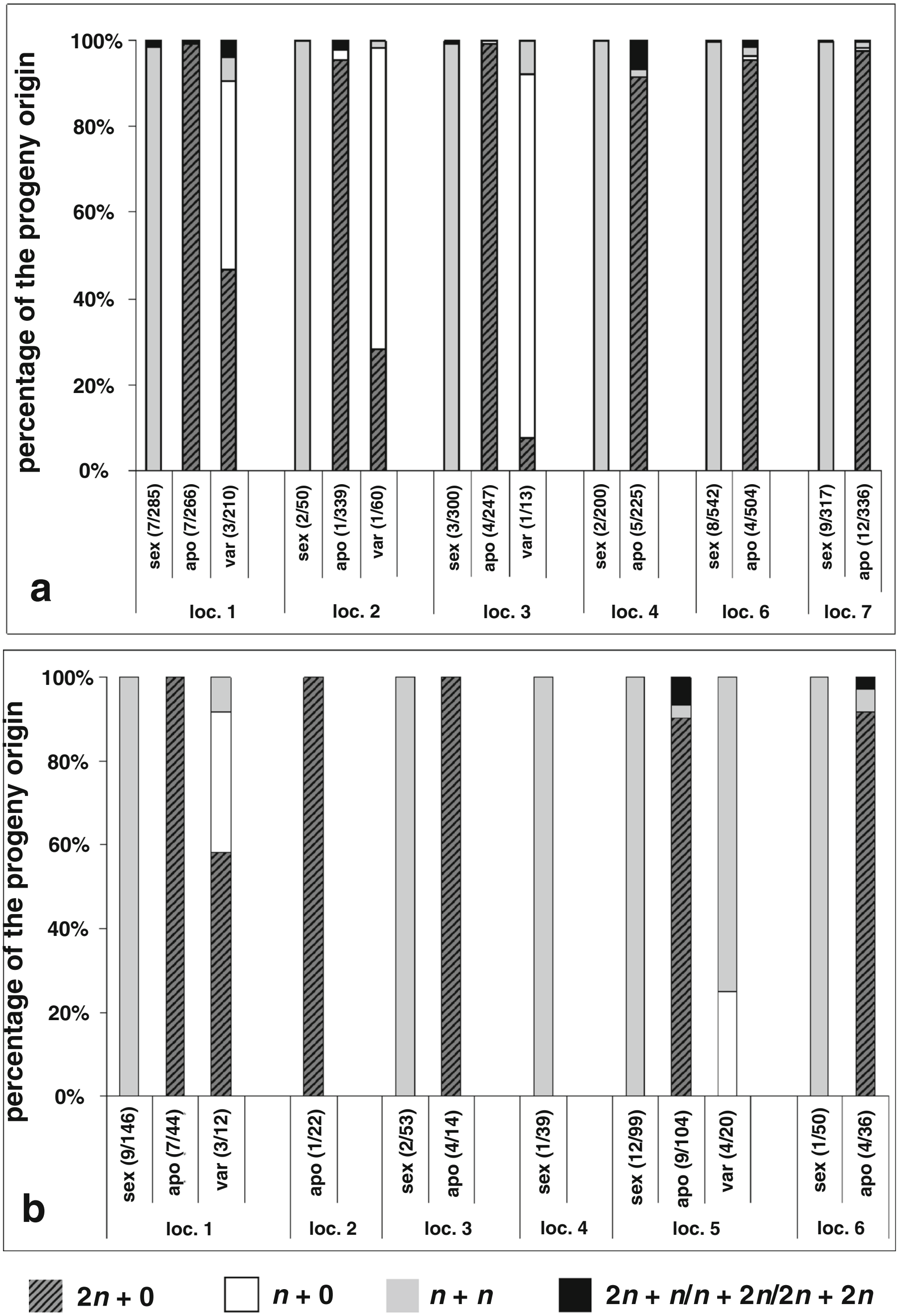

Fig. 3 Frequency of the reproductive pathways for progeny generated in the field, depending on reproductive system (RS) of the respective maternal plants. The number of maternal/progeny individuals analyzed is shown in parentheses behind the symbol of the maternal RS. The ploidy of the progeny was detected at the embryonic stage (**a**, analysis of mature seeds using the FCSS method) and/or the seedling stage (**b**, analysis of seedlings). Maternal RS: sex − sexual; apo − apomictic; var − variable. The locality (loc.) numbering corresponds to the numbers assigned in Fig. 1 and Table 1

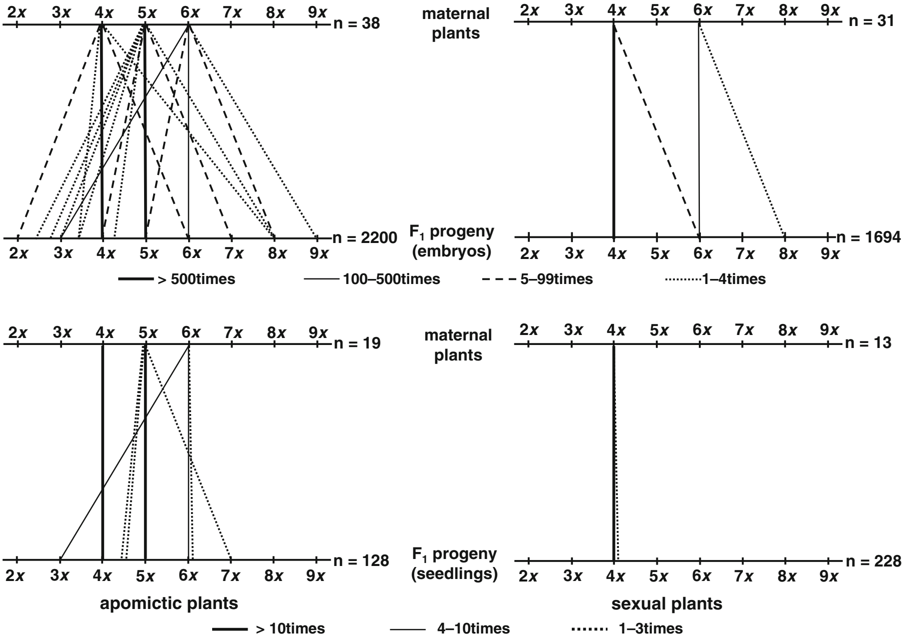

Fig. 4 Ploidy variation in the maternal plants and their progeny embryos (upper row, data summarized for localities 1, 2, 3, 4, 6 and 7) and cultivated seedlings (bottom row, data summarized for localities 1, 2, 3, 4 and 6). The numbers (n) of the maternal plants and their progeny are given beside the respective axis, illustrating the ploidy level. The diagram for locality 5 was previously shown in Fig. 2 in Krahulcová et al. (2009). The relationship between the maternal and progeny ploidy levels is illustrated with differently structured lines, according to the observed frequency of progeny types. The data are arranged irrespective of the taxonomic status of the maternal plants, summarizing the data for the parental species and the hybrids. For the source data, see Table **S1** in Electronic Supplementary Material

and sexual mothers, while the embryo progeny ranged from $2x - 9x$ in the progeny of apomictic mothers; however, a range of only $4x - 8x$ was observed in the progeny of sexual mothers (Fig. 4, upper row). In the case of progeny seedlings (Fig. 4, bottom row), only the sexual tetraploid mothers produced a sufficient amount of seeds for progeny cultivation, exclusively producing progeny seedlings with the same ploidy level, including one aneuploid seedling ($2n = 4x + 1 = 37$). By contrast, apomictic mothers (maternal ploidy range $4x - 6x$) showed an increased ploidy range in their progeny seedlings (seedling ploidy range $3x - 7x$). Consequently, the tendency to extend ploidy variation in the progeny is more apparent in apomictic maternal plants than in sexual plants, and this phenomenon has previously been shown in one population in Prague (population 5 in this paper; the details previously published by Krahulcová et al. 2009).

Residual sexuality of apomicts was also effective in experimental crosses, producing progeny similar to those detected in the field. We also demonstrated that some "off-type" progeny, namely the polyhaploids, were generated through some of the maternal apomicts at a high rate (Fig. 4). In fact, some of the polyhaploids might be sufficiently vigorous to survive in hybrid swarms, but these plants are only rarely detected in the field (Krahulec et al. 2008, 2011b), potentially reflecting the selection during germination/early seedling development (Krahulec et al. 2006) and the lack of a reliable test for the detection of these plants (Krahulec et al. 2008, 2011b). Thus, an estimation of the potential impact of polyhaploids on the structure of hybridizing populations is impossible so far.

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Appendix 1 Study sites and plants sampled

(1) Germany, North Rhine-Westphalia: a nutrient-poor fallow land in the south-western periphery of the town of Hagen, 260–280 m a.s.l., 51°20′38″ N, 07°28′39″ E. Parental hybridizing species: *P. aurantiaca, P. officinarum.* Collecting years: 2003 (plants), 2007 (seeds from individual maternal plants), 2008 (plants and seeds). For data on population structure, see Krahulcová et al. (2012).

(2) Czech Republic, the Šumava Mts: grassland bordering the parking place in the settlement of Slučí Tah near the village of Hartmanice, 876 m a.s.l., 49°09′10.9″ N, 13°21′00.2″ E. Parental hybridizing species: *P. aurantiaca, P. officinarum.* Collecting years: 2001 (plants), 2005 and 2007 (plants and seeds). For data on population structure, see Krahulec et al. (2008).

(3) Czech Republic, the Šumava Mts: mountain grasslands in the former settlement of Zhůří near the town of Železná Ruda, 940 m a.s.l., 49°10′39.8″ N, 13°22′21.4″ E. Parental hybridizing species: *P. aurantiaca, P. officinarum.* Collecting years: 2002 (plants) and 2007 (plants and seeds). For data on population structure, see Krahulec et al. (2008).

(4) Czech Republic, central Bohemia: the village of Přestavlky near the town of Roudnice nad Labem, grassy place (clearing in the wood) N of the military area ca. 0.7 km SW of the village, 210 m a.s.l., 50°24′10″ N, 14°11′25″ E. Parental hybridizing species: *P. bauhini, P. officinarum.* Collecting years: 2008 (plants), 2002, 2007 and 2009 (plants and seeds).

(5) Czech Republic, Praha-Vysočany: grassy slope above the railway line, 210 m a.s.l., 50°06′38″ N, 14°29′00″ E. Parental hybridizing species: *P. bauhini, P. officinarum.* The complete data were published in Krahulcová et al. (2009).

(6) Czech Republic, northeastern Bohemia: the village of Ploužnice close to the town of Lomnice nad Popelkou, on the grassy slope at the road turning, 520 m a.s.l., 50°30′41.7″ N, 15°23′09.2″ E. Parental hybridizing species: *P. bauhini, P. officinarum.* Collecting years: 2002 (plants) and 2007 (plants and seeds).

(7) Czech Republic, Brno: Kamenný kopec hill, 350 m a.s.l., 49°11′02″ N, 16°32′38″ E. Parental hybridizing species: *P. bauhini, P. officinarum.* The complete data were published in Krahulcová et al. (2009).