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ORIGINAL ARTICLE

The intriguing complexity of parthenogenesis inheritance in *Pilosella rubra* (Asteraceae, Lactuceae)

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Abstract Neither the genetic basis nor the inheritance of apomixis is fully understood in plants. The present study is focused on the inheritance of parthenogenesis, one of the basic elements of apomixis, in *Pilosella* (Asteraceae). A complex pattern of inheritance was recorded in the segregating F_1 progeny recovered from reciprocal crosses between the facultatively apomictic hexaploid *P. rubra* and the sexual tetraploid *P. officinarum*. Although both female and male reduced gametes of *P. rubra* transmitted parthenogenesis at the same rate in the reciprocal crosses, the resulting segregating F_1 progeny inherited parthenogenesis at different rates. The actual transmission rates of parthenogenesis were significantly correlated with the mode of origin of the respective F_1 progeny class. The inheritance of parthenogenesis was significantly reduced in F_1 $n + n$ hybrid progeny from the cross where parthenogenesis was transmitted by female gametes. In F_1 $n + 0$ polyhaploid progeny from the same cross, however, the transmission rate of parthenogenesis was high; all fertile polyhaploids were parthenogenetic. It appeared that reduced female gametes transmitting parthenogenesis preferentially developed parthenogenetically and only rarely were fertilized in *P. rubra*. The fact that the determinant for parthenogenesis acts gametophytically in *Pilosella* and the precocious embryogenesis in parthenogenesis-transmitting megagametophytes was suggested as the most probable explanations for this observation. Furthermore, we observed the different expression of complete apomixis in the non-segregating F_1 $2n + n$ hybrids as compared to their apomictic maternal parent *P. rubra*. We suggest that this difference is a result of unspecified interactions between the parental genomes.

Keywords Apomixis · Inheritance of parthenogenesis · Haploid parthenogenesis · *Pilosella rubra* · *Pilosella officinarum*

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Introduction

Apomixis is asexual reproduction through seeds in flowering plants (Asker and Jerling 1992; Nogler 1984a). Apomixis omits both meiosis and fusion of the gametes and thus produces progeny that are genetically identical to the maternal plant (Koltunow 1993). Plants reproducing exclusively through apomixis (obligate apomicts) are quite rare in nature (Asker and Jerling 1992). Most apomictic plants are facultative apomicts and retain some level of residual sexuality. Inheritance studies suggest that apomixis is a heritable trait that is controlled by one or only a few dominant loci. One apomixis locus has been reported in *Brachiaria*, *Cenchrus*, *Panicum*, *Paspalum*, *Pennisetum*, *Tripsacum*, and *Ranunculus*; several loci have been reported in *Erigeron*, *Hypericum*, *Poa*, and *Taraxacum* (reviewed in Bicknell and Koltunow 2004; Grossniklaus et al. 2001; Ozias-Akins and van Dijk 2007).

The genus *Pilosella*, formerly classified as *Hieracium* subgenus *Pilosella*, within the Asteraceae family, represents an established model system for studying apomixis (Bicknell 1994; Bicknell and Koltunow 2004; Catanach et al. 2006; Koltunow et al. 1995, 2011a, b). Apomixis in

the genus *Pilosella* occurs by gametophytic apomixis, which is characterized by three distinct developmental features: (1) mitotic embryo sac formation without prior meiosis (apomeiosis), (2) fertilization-independent (i.e. parthenogenetic) embryo development, and (3) autonomous (fertilization-independent) endosperm formation. The genus *Pilosella* comprises both apomictic and sexual species. Apomixis in *Pilosella* species is aposporous and facultative (Koltunow et al. 1998, 2011a, b). Apomictic *Pilosella* species thus have the capacity to reproduce sexually in addition to prevailing apomictic reproduction (Bicknell et al. 2003; Bicknell and Koltunow 2004; Fehrer et al. 2007; Ozias-Akins and van Dijk 2007).

Formerly, it was supposed that all the three elements of apomixis in *Pilosella* (apomeiosis, parthenogenesis, and autonomous endosperm formation) are conferred by a single dominant locus (Bicknell et al. 2000; Gadella 1991). More recent investigations in *Pilosella praealta*, however, show that apomixis is controlled by two dominant independent loci, one controlling apomeiosis and the other controlling parthenogenesis and autonomous endosperm formation (Catanach et al. 2006; Koltunow et al. 2011b). The loci are termed *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*), respectively. The transmission of both loci appeared to be subjected to distortion, which acted against transmission of the dominant allele for *LOA* but favoured transmission of the dominant allele for *LOP* (Catanach et al. 2006).

Despite all the inheritance studies cited above, the rules of apomixis inheritance still remain not fully understood in *Pilosella* species. In the present study, we focussed on inheritance of parthenogenesis, one of the basic elements of apomixis. Our study was based on an analysis of the mode of reproduction in ninety-eight F_1 progeny potentially segregating for parthenogenesis. The segregating F_1 progeny were recovered from reciprocal crosses between a facultatively apomictic *Pilosella rubra* ($2n = 6x = 54$) and a sexual *Pilosella officinarum* ($2n = 4x = 36$) (Table 1; Fig. 1). These crosses were performed within the scope of previous studies regarding the reproductive behaviour of *P. rubra* (Krahulcová et al. 2004; Krahulec et al. 2006). The segregating F_1 progeny originated from the reduced gametes of apomictic *P. rubra* but differed in the genome contribution of sexual *P. officinarum* (no contribution, reduced gametes, and unreduced gametes) and in the direction of the cross (Table 1; Fig. 1). Such diversity in the segregating F_1 progeny provided us with an opportunity to investigate whether and how the inheritance of parthenogenesis is correlated with the mode of progeny origin.

Additionally, to demonstrate whether the sexual *P. officinarum* genome contribution directly affects the expression of apomixis in our experimental system, the reproductive behaviour of parental *P. rubra* was compared with that of 19 non-segregating octoploid $2n + n$ F_1 hybrids (Table 1; Fig. 1; hybrid terminology according to Harlan and de Wet 1975). This comparison is a follow-up to our previous study that examined differences in the expressivity of apomixis between the stabilized facultative apomicts and their non-segregating $2n + n$ hybrids with

Table 1 F_1 progeny originating from the reciprocal crosses between facultatively apomictic hexaploid *Pilosella rubra* ($2n = 6x = 54$) and sexual tetraploid *P. officinarum* ($2n = 4x = 36$)

Maternal genome contribution	Paternal genome contribution	F_1 progeny class	No. of F_1 germinated seedlings[a]	No. of mature F_1 plants used in this study[a]
Cross A: ♀ *P. rubra* ($2n = 6x = 54$) × ♂ *P. officinarum* ($2n = 4x = 36$)				
Reduced gametes ($n = 3x$)	–	*Trihaploids*	26	19
Reduced gametes ($n = 3x$)	Reduced gametes ($n = 2x$)	*Pentaploid hybrids*	33	31
Reduced gametes ($n = 3x$)	Unreduced gametes ($2n = 4x$)	*Heptaploid hybrids*	3	3
Unreduced gametes ($2n = 6x$)	Reduced gametes ($n = 2x$)	*Octoploid hybrids*	20	19
Unreduced gametes ($2n = 6x$)	–	Hexaploids[b]	459	–
Cross B: ♀ *P. officinarum* ($2n = 4x = 36$) × ♂ *P. rubra* ($2n = 6x = 54$)				
Reduced gametes ($n = 2x$)	–	Tetraploids[c]	12	–
Reduced gametes ($n = 2x$)	Reduced gametes ($n = 3x$)	*Pentaploid hybrids*	44	44
Unreduced gametes ($2n = 4x$)	Reduced gametes ($n = 3x$)	*Heptaploid hybrids*	1	1

Reciprocal crosses resulted in the formation of progeny at six ploidy levels (from triploid to octoploid), each representing a unique combination of meiosis/apomeiosis and fertilization/parthenogenesis; the segregating F_1 progeny and the non-segregating $2n + n$ F_1 octoploid hybrids used in this study are given in *italics*

[a] Part of germinated F_1 seedlings died before reaching maturity and thus they could not be scored for mode of reproduction

[b] Progeny from apomixis (including progeny from autogamy)

[c] Progeny from autogamy

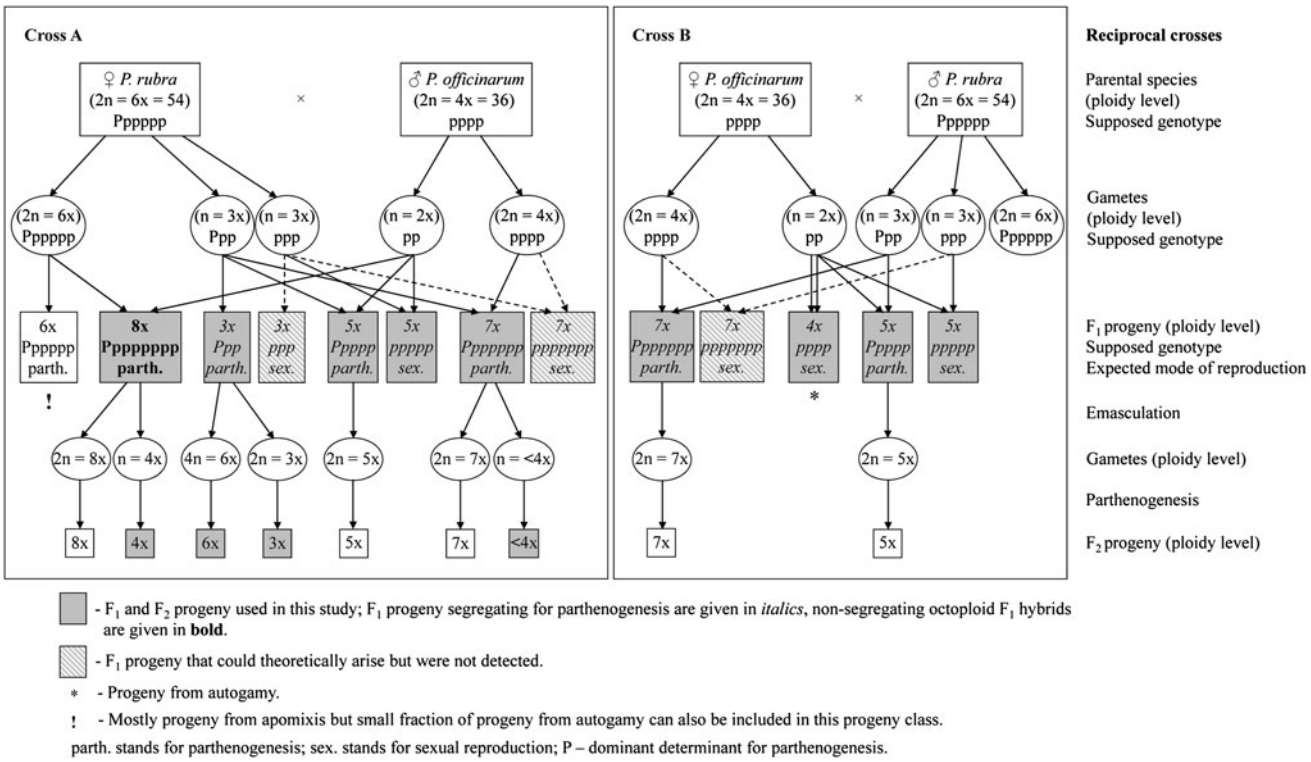

Fig. 1 Design of reciprocal crosses A and B between the facultatively apomictic *Pilosella rubra* ($2n = 6x = 54$) and the sexual *P. officinarum* ($2n = 4x = 36$), F_1 progeny from these crosses and F_2 progeny from the emasculated capitula of the segregating F_1 progeny and non-segregating F_1 octoploid hybrids. The supposed genotypes of parental species and their reduced and unreduced gametes and of the F_1 progeny from reciprocal crosses A and B with respect to parthenogenesis are included

sexual pollen parent (Krahulcová et al. 2011). By analysing the origins of progeny at the embryo stage, Krahulcová et al. (2011) have demonstrated that expressivity of apomixis in the $2n + n$ hybrids was variable and profoundly lower than that in their apomictic maternal parent. In spite of the presence of a complete maternal "apomictic" genome in the $2n + n$ hybrids, their resulting reproductive behaviour was most likely influenced by regulatory interactions between parental genomes (Krahulcová et al. 2011). This study is primarily focussed on expressivity of apomixis in progeny arisen from reciprocal crosses between the same parental species.

Materials and methods

Plant material

The present study primarily focussed on the F_1 progeny potentially segregating for parthenogenesis that were recovered from reciprocal crosses between the hexaploid facultatively apomictic *Pilosella rubra* (Peter) Soják ($2n = 6x = 54$) and the tetraploid sexual *Pilosella officinarum* F.W.Schultz and Sch.Bip. ($2n = 4x = 36$) (Table 1; Fig. 1). The cross *P. rubra* × *P. officinarum* was performed as part of previous studies (Krahulcová et al. 2004; Krahulec et al. 2006). The reciprocal cross was performed newly. For purposes of this study, the cross *P. rubra* × *P. officinarum* was labelled as cross A and the reciprocal cross was labelled as cross B; the breeding scheme is detailed in Fig. 1. *Pilosella rubra* is assumed to be a spontaneous allopolyploid $2n + n$ hybrid between the tetraploid ($2n = 4x = 36$) apomictic *P. aurantiaca* (maternal parent) and the tetraploid ($2n = 4x = 36$) sexual *P. officinarum* (Krahulcová et al. 2004; Suda et al. 2007; hybrid terminology according to Harlan and de Wet 1975). The crosses between *P. rubra* and *P. officinarum* thus essentially represented backcrosses (Krahulcová et al. 2004, 2011). Parental accessions of *P. rubra* and *P. officinarum* were originally collected in the Krkonoše Mountains (the Sudetes Mountains), Czech Republic, where *P. rubra* behaves as a stabilized hybridogenous species (Fehrer et al. 2007); collected accessions were maintained as vegetatively propagated lines, each derived from a single plant (Krahulcová et al. 2004).

Parthenogenesis is controlled by a single dominant locus termed *LOSS OF PARTHENOGENESIS* (*LOP*, here abbreviated as "P") in the genus *Pilosella* (Catanach et al. 2006; Koltunow et al. 2011b). As none of our results indicate otherwise, we suppose that *P. rubra* is simplex for

the dominant P-allele in accordance with the concept of parthenogenesis inheritance proposed by Catanach et al. (2006) and Koltunow et al. (2011b). Thus, the supposed genotypes of the hexaploid apomictic parent *P. rubra* and the tetraploid sexual parent *P. officinarum* with respect to parthenogenesis are Pppppp and pppp, respectively. Supposed genotypes of parental gametes and of the F_1 progeny from reciprocal crosses A and B are summarized in Fig. 1.

Both crosses A and B resulted together in the formation of six different types of progeny distinct in ploidy level, each representing a unique combination of meiosis/apomeiosis and fertilization/parthenogenesis (Table 1; Krahulcová et al. 2004). All the segregating F_1 progeny originated from the reduced gametes (n) of the apomictic parent *P. rubra* but differed in the amount of genome contribution from the sexual parent *P. officinarum*: no contribution (0), reduced gametes (n), and unreduced gametes ($2n$) (Table 1; Krahulcová et al. 2004). Thus, this F_1 progeny occurred at three ploidy levels: triploid ($n + 0$), pentaploid ($n + n$), and heptaploid ($n + 2n$ in the cross A, $2n + n$ in the cross B), respectively (Table 1; Fig. 1; Krahulcová et al. 2004). We cultivated the surviving F_1 progeny seedlings up to maturity and classified the matured plants into five progeny classes according to their origin and ploidy: (1) trihaploid progeny originated from cross A via parthenogenetic development of the reduced female gametes of *P. rubra* (19 plants), (2) pentaploid hybrids originated from cross A (31 plants), (3) pentaploid hybrids originated from cross B (44 plants), (4) heptaploid hybrids originated from cross A (three plants), and (5) heptaploid hybrids originated from cross B (one plant) (98 plants together; Tables 1, 2). In addition to the segregating F_1 progeny, the non-segregating F_1 progeny (19 octoploid $2n + n$ hybrids originated from cross A) were analysed for their reproductive behaviour (Tables 1, 2).

For more information on these crossing experiments and a detailed description of *P. rubra* and *P. officinarum*, see our previous papers (Krahulcová et al. 2004, 2011).

Mode of reproduction

Ninety-eight F_1 progeny potentially segregating for parthenogenesis and 19 non-segregating octoploid F_1 hybrids were scored for mode of reproduction (parthenogenesis, sexuality, and sterility; Table 2). The potential for parthenogenesis was defined as the ability of the plant to set viable seeds after emasculation: this was done by decapitation of the upper part of an immature capitulum (Ostenfeld 1906; Koltunow et al. 1995). The F_1 plants that formed some seeds after emasculation were scored as parthenogenetic. The F_1 plants that did not form any seeds after emasculation but formed them in the open-pollinated capitula were scored as sexual. Finally, the F_1 plants that did not form seeds either after emasculation or after open pollination were scored as sterile. At least three emasculated and three open-pollinated capitula were scored from each analysed F_1 plant.

Table 2 Mode of reproduction of analysed F_1 progeny from reciprocal crosses between facultatively apomictic *Pilosella rubra* and sexual *P. officinarum*

F_1 progeny class (no. of plants)	Mode of reproduction in F_1 progeny, no. of plants		
	Parthenogenetic	Sexual	Sterile
Cross A: ♀ *P. rubra* ($2n = 6x = 54$) × ♂ *P. officinarum* ($2n = 4x = 36$)			
Trihaploids (19)	17	0	2
Pentaploid hybrids (31)	2	27	2
Heptaploid hybrids (3)	3	0	0
Octoploid hybrids (19)	19	0	0
Cross B: ♀ *P. officinarum* ($2n = 4x = 36$) × ♂ *P. rubra* ($2n = 6x = 54$)			
Pentaploid hybrids (44)	22	20	2
Heptaploid hybrids (1)	1	0	0

F_1 progeny potentially segregating for apomixis (trihaploids, and pentaploid and heptaploid hybrids) are given in *italics*

Parthenogenesis can be preceded either by apomeiosis or meiosis in apomictic members of the genus *Pilosella* (Bicknell et al. 2000), which results in apomixis or haploid parthenogenesis, respectively. Additional analyses were undertaken to determine which of these two reproductive pathways had occurred in our emasculated F_1 plants. Apomixis produces progeny with the maternal ploidy level whereas haploid parthenogenesis produces progeny with half of the maternal ploidy level. Ploidy level screening therefore can be used to distinguish between the two reproductive pathways. The well-developed achenes obtained from the emasculated capitula of the F_1 plants were germinated in pots filled with sterilized garden soil in the greenhouse. The ploidy level of recovered F_2 seedlings was determined using flow cytometry (see "Ploidy level estimation" below). The origin of F_2 seedlings (apomixis versus haploid parthenogenesis) was inferred from the relationship between their ploidy level and the ploidy level of their maternal parent—the respective emasculated F_1 plant (Fig. 1; Table 3). Those F_2 seedlings that according to our assessment originated either from haploid parthenogenesis or from rare chromosome doubling of the maternal plant (see the paragraph on "Trihaploids" in the "Results") were grown to maturity. Both the emasculation experiments and open pollinations were also used to investigate the ability of these F_2 plants to reproduce parthenogenetically (Table 3). Again, at least three emasculated and three open-pollinated capitula were scored from each analysed F_2 plant.

Table 3 Mode of reproduction in F_2 progeny obtained from emasculated capitula of F_1 progeny presented in Table 2

Maternal F_1 progeny (no. of plants)	Genome contribution of the maternal F_1 progeny	F_2 progeny (no. of plants)	Mode of reproduction in F_2 progeny, no. of plants		
			Parthenogenetic	Sexual	Sterile
F_2 progeny obtained from emasculated capitula of F_1 progeny potentially segregating for parthenogenesis					
F_1 Trihaploids (17)	Unreduced gamete ($2n = 3x$)	Triploids[a] (138)	n.d.	n.d.	n.d.
	Doubled unreduced gamete ($4n = 6x$)	Hexaploids[b] (2)	2	0	0
F_1 Pentaploid hybrids (24)	Unreduced gamete ($2n = 5x$)	Pentaploids[a] (561)	n.d.	n.d.	n.d.
F_1 Heptaploid hybrids (4)	Reduced gamete ($n < 4x$)	Polyhaploids[c,d] (1)	1	0	0
	Unreduced gamete ($n = 7x$)	Heptaploids[a] (14)	n.d.	n.d.	n.d.
F_2 progeny obtained from emasculated capitula of non-segregating octoploid F_1 hybrids					
F_1 Octoploid hybrids (19)	Reduced gamete ($n = 4x$)	Tetrahaploids[d] (70)[e]	56	0	0
	Unreduced gamete ($n = 8x$)	Octoploids[a] (67)	n.d.	n.d.	n.d.

n.d. not determined

[a] Progeny from apomixis

[b] Progeny from chromosome doubling

[c] Aneuploid, nearly tetraploid plants

[d] Progeny from haploid parthenogenesis

[e] Six plants died before they could be scored for mode of reproduction and eight plants were nearly sterile, thus we were not able to asses their mode of reproduction properly

Ploidy level estimation

We used flow cytometry to determine the ploidy level of the F_2 progeny, as stated above. A Partec PA II flow cytometer (Partec GmbH., Münster, Germany) was used with the modified two-step procedure originally described by Otto (1990). For each seedling analysed, approximately 0.5 cm^2 of a fresh young leaf was chopped together with the leaf tissue of an internal standard (the seedling's maternal parent) with a sharp razor blade in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5 % Tween 20). The suspension was filtered through a 42-µm nylon mesh. After a 20-min incubation (room temperature with occasional shaking), 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) with 4 µg/ml of the AT-selective fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) and 2 µg/ml β-mercaptoethanol was added. The staining lasted 1–2 min. The fluorescence of at least 5,000 nuclei was recorded for each sample. Only measurements with coefficients of variance (CV) less than 5 % were taken into account; measurements with CV exceeding 5 % were discarded, and the samples were re-measured.

Statistical analyses

The frequencies of parthenogenetically and sexually reproducing plants were determined for each analysed group of segregating F_1 progeny (Table 4). Sterile and dead plants were separately considered either as having arisen independently of parthenogenesis/sexuality or as representing either dysfunctional parthenogenetic plants or dysfunctional sexual plants. In the former case, they were omitted from the analysis entirely, whereas in the latter cases they were either assigned to the parthenogenetic or to the sexual progeny class, respectively. The chi-squared test of independence was used to test whether there was a relationship between the transmission rate of parthenogenesis and the reproductive pathway by which the respective segregating F_1 progeny class has originated. Attention was paid to the evaluation of the effect of both the direction of cross and the genome contribution of *P. officinarum* (no contribution/reduced gametes/unreduced gametes) (Table 4). The null hypothesis was that the transmission rate of parthenogenesis is independent of the way by which the segregating F_1 progeny originated. Unfortunately, the number of heptaploid F_1 hybrids was very low, most likely due to the rarity of unreduced gametes in sexual *P. officinarum*, and therefore, they could not be included into some of the conducted chi-squared tests of independence (see paragraph "Statistical analyses" in the "Results"). The significance level for the rejection of the null hypothesis was 5 %.

Results

Mode of reproduction in F_1 progeny potentially segregating for parthenogenesis

Trihaploids

Out of 19 F_1 trihaploids, 17 plants were assessed as parthenogenetic and two plants were sterile (Table 2). Both

Table 4 Statistical analyses—chi-square tests of independence

Progeny included in chi-square test	Mode of reproduction, no. of plants		Chi-square test of independence		
	Parthenogenetic	Sexual	χ^2	*d.f.*	*p*
Do both female and male reduced gametes of *P. rubra* transmit parthenogenesis at the same rate?					
Whole segregating F_1 progeny from cross A	22	27	0.38[a]	1	0.5376[a]
Whole segregating F_1 progeny from cross B	23	20	0.68[b]	1	0.4096[b]
Do those F_1 progeny differing only in the direction of cross but not in the genome contribution of the sexual parent *P. officinarum* inherit parthenogenesis at the same rate?					
Pentaploid F_1 hybrids from cross A	2	27	13.89[a]	1	0.0002[a]
Pentaploid F_1 hybrids from cross B	22	20	15.86[b]	1	<0.0001[b]
Do those F_1 progeny differing only in the genome contribution of the sexual parent *P. officinarum* but not in the direction of cross inherit parthenogenesis at the same rate?					
Trihaploid F_1 progeny from cross A	17	0	34.58[a]	1	<0.0001[a]
Pentaploid F_1 hybrids from cross A	2	27	38.32[b]	1	<0.0001[b]

[a] Yates chi-square, corrected for continuity

[b] Pearson chi-square, uncorrected for continuity

parthenogenetic and sterile F_1 trihaploids were smaller and less vigorous than their maternal hexaploid parent *P. rubra*. Parthenogenetic F_1 trihaploids showed low seed-set production and produced achenes with very low germinability. More than 3,000 well-developed achenes obtained from the emasculated capitula of all 17 parthenogenetic F_1 trihaploids were sown, but only 140 F_2 seedlings (4.7 %) were recovered. Among them, two hexaploids (1.4 %) were recovered, probably resulting from a chromosome-doubling event during megagametophyte/embryo development; each hexaploid was from another F_1 trihaploid plant (Table 3; Fig. 1). Both recovered F_2 hexaploids reproduced parthenogenetically (Table 3). The remaining 138 recovered F_2 seedlings were apomictically derived triploids (Table 3; Fig. 1); no plant originating from haploid parthenogenesis was detected among them.

Pentaploid hybrids

Out of 31 F_1 pentaploid hybrids from cross A, two were assessed as parthenogenetic, 27 were sexual, and two were sterile (Table 2). The transmission rate of parthenogenesis observed in this cross, namely two plants out of 31 (0.065), was very low. In contrast, out of 44 F_1 pentaploid hybrids from cross B, 22 were assessed as parthenogenetic, 20 were sexual, and two were sterile (Table 2). The transmission rate of parthenogenesis in this cross was 22/44 (0.500). All F_2 progeny derived from the emasculated capitula of the 24 parthenogenetic F_1 pentaploid hybrids from both crosses A and B were apomictically derived pentaploids (Table 3; Fig. 1). No plant that originated from haploid parthenogenesis was detected among them (Table 3). In total, 561 F_2 progeny were analysed.

Heptaploid hybrids

The three F_1 heptaploid hybrids recovered from cross A and the only F_1 heptaploid hybrid from reciprocal cross B were all assessed as parthenogenetic (Table 2). This heptaploid F_1 progeny did not contain any sexual or sterile plant. The achenes obtained from the emasculated capitula of the F_1 heptaploid hybrids displayed a rather low germination rate: only 15 F_2 seedlings (4.5 %) were recovered from 331 well-developed achenes. One of the recovered F_2 seedlings resulted from haploid parthenogenesis and was scored as parthenogenetic (Table 3; Fig. 1). The remaining 14 F_2 seedlings were apomictically derived heptaploids (Table 3; Fig. 1).

Mode of reproduction in non-segregating octoploid F_1 hybrids

All 19 octoploid F_1 hybrids from cross A were assessed as parthenogenetic (Table 2). The F_2 progeny derived from the emasculated capitula of octoploid F_1 hybrids consisted of apomictically derived octoploids (51 plants) and tetrahaploids (70 tetraploid plants generated by haploid parthenogenesis) (Table 3; Fig. 1). Out of 70 F_2 tetrahaploids, six plants died before they could be scored for mode of reproduction, 56 plants were assessed as parthenogenetic, and the remaining eight plants were nearly sterile, so we were unable to exactly score their reproductive behaviour (Table 3); no fertile sexual plant was detected among these F_2 tetrahaploids.

Statistical analyses

We first estimated whether the transmission of parthenogenesis is independent of the direction of the cross. We

conducted two chi-squared tests of independence to determine the following: (1) whether both female and male reduced gametes of the apomictic parent *P. rubra* transmitted parthenogenesis at the same rate and (2) whether those segregating F_1 progeny differing only in the direction of cross but not in the genome contribution of the sexual parent *P. officinarum* inherited parthenogenesis at the same rate.

(1) We compared the frequencies of parthenogenetically and sexually reproducing plants between the whole segregating F_1 progeny from cross A and the whole segregating F_1 progeny from the reciprocal cross B (Table 4). The test of independence ($\chi^2 = 0.38$, *d.f.* $= 1$, $p = 0.5376$) showed that there is no correlation between the direction of the cross and the transmission rate of parthenogenesis in whole segregating F_1 progeny. Both female and male reduced gametes of *P. rubra* transmitted parthenogenesis at the same rate. Assignment of the sterile and dead plants (Tables 1, 2) to either the parthenogenetically or the sexually reproducing F_1 progeny group did not change the conclusion. Results of these tests of independence were $\chi^2 = 0.01$, *d.f.* $= 1$, $p = 0.9203$ and $\chi^2 = 2.01$, *d.f.* $= 1$, $p = 0.1563$, respectively.

(2) We compared the frequencies of parthenogenetically and sexually reproducing plants between F_1 pentaploid hybrids from cross A and F_1 pentaploid hybrids from the reciprocal cross B (Table 4). The test of independence ($\chi^2 = 13.89$, *d.f.* $= 1$, $p = 0.0002$) showed that there is a significant correlation between the transmission rate of parthenogenesis among F_1 pentaploid hybrids and the direction of the cross. F_1 pentaploid hybrids from cross A inherited parthenogenesis at a significantly lower rate than F_1 pentaploid hybrids from reciprocal cross B. Again, assignment of the sterile and dead plants (Tables 1, 2) to either the parthenogenetically or the sexually reproducing F_1 progeny group did not change the conclusion. Results of these tests of independence were $\chi^2 = 9.01$, *d.f.* $= 1$, $p = 0.0027$ and $\chi^2 = 14.98$, *d.f.* $= 1$, $p = 0.0001$, respectively. Unfortunately, it was impossible to conduct a similar test on the F_1 heptaploid hybrids from crosses A and B because their total number was insufficient to make a statistically valid conclusion.

Further, we estimated whether the transmission of parthenogenesis is independent of the genome contribution of the sexual parent *P. officinarum*. We tested whether the individual classes of the segregating F_1 progeny that differed in the genome contribution of the sexual *P. officinarum* but not in the direction of the cross inherited parthenogenesis at the same rate. Because the number of F_1 heptaploid hybrid plants was insufficient and they were omitted from the statistical analyses, we conducted the chi-squared test only with F_1 trihaploids and F_1 pentaploid hybrids from cross A (Table 4). The test of independence ($\chi^2 = 34.58$, *d.f.* $= 1$, $p < 0.0001$) revealed a significant correlation between the transmission rate of parthenogenesis and the genome contribution of the sexual *P. officinarum*. F_1 trihaploids, which originated parthenogenetically without any contribution of *P. officinarum*, inherited parthenogenesis at a significantly higher rate than the F_1 pentaploid hybrids, which originated from the reduced gametes of both parents, *P. officinarum* and *P. rubra*. Assignment of the sterile and dead plants (Tables 1, 2) to either the parthenogenetically or the sexually reproducing F_1 progeny group did not change the conclusion. Results of these tests of independence were $\chi^2 = 35.99$, *d.f.* $= 1$, $p < 0.0001$ and $\chi^2 = 20.08$, *d.f.* $= 1$, $p < 0.0001$, respectively.

Discussion

Parthenogenetic development of the embryo is one of the basic elements of apomixis in plants. In the present study, we revealed some interesting features concerning the inheritance of parthenogenesis in the facultatively apomictic plant *Pilosella rubra*. It appeared that both female and male reduced gametes of *P. rubra* transmitted parthenogenesis at the same rate in reciprocal crosses with the sexual *P. officinarum* (Table 4). However, each class of the segregating F_1 progeny inherited parthenogenesis at a different transmission rate. The actual transmission rate of parthenogenesis appears to be significantly correlated with the mode of origin of the respective class of segregating F_1 progeny, including both the direction of cross and the genome contribution of sexual parent *P. officinarum* (Table 4).

Inheritance of parthenogenesis in the F_1 trihaploid progeny from cross A

The F_1 trihaploid $n + 0$ progeny from cross A either reproduced parthenogenetically or were sterile, but none were sexual (Table 2). The same effect applied to the reproductive mode of the other polyhaploid ($n + 0$) progeny analysed in this study (namely, 70 F_2 tetrahaploids from the F_1 octoploid hybrids, and one F_2 polyhaploid with nearly tetraploid DNA ploidy level from the F_1 heptaploid hybrid; Table 3). In trihaploids, the absence of sexual plants can be explained by irregular or non-functional meiosis that is frequently observed at this ploidy level. Nevertheless, this explanation cannot be used in the case of tetrahaploids because their meiosis is expected to be regular. The observed lack of sexual polyhaploids is in agreement with previous studies, as no sexual polyhaploid plant was published to date in the genus *Pilosella*. Most of the published polyhaploid plants were assessed as apomictic, in addition to a minor fraction of (semi)sterile

plants (Bicknell 1997; Koltunow et al. 2000; Krahulcová and Krahulec 2000; Krahulec et al. 2011).

Assuming that the genotype of the hexaploid apomictic parent *P. rubra* is Pppppp with respect to parthenogenesis, reduced gametes with both the Ppp and the ppp genotype could theoretically participate in cross A (Fig. 1). Both parthenogenetic and sexual F_1 trihaploids should thus be expected. However, only parthenogenetic (or sterile) trihaploids were observed (Table 2). According to Catanach et al. (2006) and Koltunow et al. (2011b), the determinant for parthenogenesis (*LOP*) acts gametophytically in *Pilosella* and also confers the ability for autonomous endosperm formation. This implies that only those reduced embryo sacs that inherit parthenogenesis (the genotype Ppp) can undergo fertilization-independent seed development and give rise to viable polyhaploid plants, which is in congruence with our study.

Interestingly, we detected two hexaploid plants during determination of ploidy level of F_2 progeny from emasculated capitula of parthenogenetic F_1 trihaploids (Table 3; Fig. 1). These hexaploids, which arose from different F_1 trihaploid plants, probably resulted from a chromosome-doubling event during megagametophyte/embryo development. Thus, we supposed that their genotype was PPpppp (Ppp + Ppp) with respect to parthenogenesis, showing a double dose of P-allele. The two recovered F_2 hexaploids reproduced parthenogenetically (Table 3). In nature, the role of haploid parthenogenesis and chromosome doubling remains unknown in the genus *Pilosella*. The field investigations showed that haploid parthenogenesis occurs in natural populations but detection of polyhaploid plants is rather complicated (Krahulec et al. 2008, 2011). The chromosome doubling is known only from experimental conditions (Krahulec et al. 2011).

Low viability of the F_1 trihaploid progeny from cross A

The F_1 trihaploid progeny from cross A showed a rather high mortality rate. From the 26 germinated F_1 trihaploids, only 19 (73.1 %) survived to maturity and only 17 (65.4 %) were able to set seed (Tables 1, 2). Even higher mortality among the F_1 trihaploid progeny from cross A was observed during seed germination and early stages of seedling growth (Krahulec et al. 2006). Krahulec et al. (2006) used some of the mature seeds from the cross labelled as cross A in this study and quantified the proportions of individual F_1 progeny classes using flow cytometry. The ascertained proportions of individual F_1 progeny classes at the embryo stage were compared with the proportions of individual F_1 progeny classes among the young germinated and established seedlings. At the embryo stage, the trihaploids constituted 11.8 % of all F_1 progeny from cross A. At the seedling stage, however, trihaploids represented only 4.0 % of the progeny, showing a profound and statistically significant difference (Krahulec et al. 2006). No significant difference was detected in any other F_1 progeny class from cross A (Krahulec et al. 2006).

In addition to high mortality, other characteristics such as low plant vigour and low seed set imply that the F_1 trihaploid progeny has some handicap against other F_1 progeny classes (Krahulec et al. 2006, 2011, and the present study). As apomixis represents a form of asexual reproduction, genomes of apomictic plants tend to accumulate deleterious mutations in a mechanism known as Muller's ratchet (Muller 1964). Poor viability of trihaploids thus may be a consequence of high mutational load carried by the genome of the maternal parent *Pilosella rubra*. The lethal effect of accumulated mutations is masked in the maternal polyploid genome, but it could be exposed after meiosis and reduced viability of the trihaploid progeny. The lethal effect, however, seems to be effectively masked by fertilization, since no measurable selection was observed against the F_1 pentaploid and heptaploid hybrids from cross A (Krahulec et al. 2006).

The selection against deleterious mutations could act more effectively on the male gamete population compared to the female gamete population due to different quantity of meiotic products concerned. Consequently, fitness of the reduced female gametes transmitting parthenogenesis may be more affected by deleterious mutations than fitness of the reduced male gametes in *P. rubra*. Theoretically, this effect may also contribute to observed differential inheritance of parthenogenesis transmitted by the reduced female versus male gametes. Nevertheless, to compare the viability of both types of gametes is impossible in this experimental system.

Inheritance of parthenogenesis in the F_1 pentaploid hybrid progeny from cross A and B

The F_1 pentaploid hybrids from crosses A and B represented $n + n$ hybrids (Table 1; Fig. 1). Regarding parthenogenesis, the hexaploid apomictic parent *P. rubra* could theoretically produce reduced gametes with the genotypes Ppp and ppp whereas the tetraploid sexual parent *P. officinarum* could only produce reduced gametes with the genotype pp (Fig. 1). Hence, pentaploid hybrids carrying both the Ppppp and pppppp genotype were expected in both crosses A and B (Fig. 1). In agreement with this expectation, both parthenogenetic and sexual pentaploids were observed (Table 2).

Nevertheless, the proportion of parthenogenetic pentaploids was not identical in both crosses A and B. The chi-squared test revealed significant association between the transmission of parthenogenesis and direction of the cross in the F_1 pentaploid $n + n$ hybrids (Table 4). Pentaploid

$n + n$ hybrids from cross A, where parthenogenesis was transmitted through female gametes, inherited parthenogenesis at a significantly lower rate than pentaploid $n + n$ hybrids from reciprocal cross B, where parthenogenesis was transmitted through male gametes. In cross B, the segregation rate of parthenogenesis was approximately 1:1. Additionally, the low transmission rate of parthenogenesis in the F_1 pentaploid $n + n$ hybrids from cross A seems to be significantly correlated with the high transmission rate of parthenogenesis in the F_1 trihaploid $n + 0$ progeny from cross A (Table 4). Formation of the trihaploids is fertilization independent and relies on the presence of all genetic factors required for successful parthenogenetic embryo development. Development of the pentaploid hybrids, however, requires fertilization; successful formation of the pentaploid hybrids thus depends on the success of the cross. We are aware that these two processes are not fully comparable and thus the results of statistical analyses must be interpreted with some caution.

All in all, it might seem that Ppp female gametes of *P. rubra* preferentially developed parthenogenetically instead of being fertilized and giving rise to parthenogenetically reproducing pentaploid hybrids. It appears that the determinant of parthenogenesis not only enabled reduced female gametes to develop parthenogenetically (see "Inheritance of parthenogenesis in the F_1 trihaploid progeny from cross A" above in the "Discussion") but also prevented their fertilization. Koltunow et al. (2011b) reported resistance to fertilization in unreduced as well as in reduced embryo sacs carrying the determinant for parthenogenesis (*LOP*) in *P. praealta* and proposed precocious initiation of embryo and endosperm development as the possible explanation. The same mechanism may also play a role in partial prevention of fertilization of reduced embryo sacs inheriting parthenogenesis in *P. rubra*. Nevertheless, the fertilization was not inhibited completely in *P. rubra* because two apomictic pentaploid hybrids and three apomictic heptaploid hybrids arose from the fertilization of reduced female gametes of *P. rubra* in cross A (Table 2). The same also applies to unreduced embryo sacs of *P. rubra* because, in addition to prevailing $2n + 0$ maternal progeny resulting from apomixis, 20 F_1 $2n + n$ octoploid hybrids arose in cross A from the fertilization of unreduced female gametes, that is, the gametes transmitting the determinant for parthenogenesis (Table 1).

A hint of the resistance to fertilization in reduced female gametes transmitting apomixis or its elements may be spotted also in $n + n$ maize-*Tripsacum* hybrids (Grimanelli et al. 1998) and *Pennisetum* hybrids (Roche et al. 2001). Furthermore, it seems that in *Tripsacum* the resistance to fertilization is correlated with the formation of prevalently apomictic polyhaploids (Grimanelli et al. 1998). Also in *Pennisetum*, only apomictic or sterile, but no sexual polyhaploids, was identified (Dujardin and Hanna 1986). Nevertheless, it is unlikely to be a feature of all apomictic plants because in *Ranunculus* some fertile sexual plants were detected among a group of prevalently apomictic polyhaploids (Nogler 1984b). In *Panicum* and *Bothriochloa-Dichanthium* complex, sterile and occasionally sexual, but no fertile, apomictic polyhaploids were observed (Savidan and Pernes 1982; de Wet 1968).

Reproductive behaviour in non-segregating octoploid hybrids

Depending on the environmental conditions, 92.6–100.0 % of seedling progeny recovered from the emasculated capitula of the hexaploid *P. rubra* originated from apomixis; the remaining progeny originated from haploid parthenogenesis (Krahulcová et al. 2004). Nineteen non-segregating $2n + n$ octoploid F_1 hybrids, which originated from the fusion of an unreduced female gamete of apomictic *P. rubra* with a reduced male gamete of sexual *P. officinarum* (Krahulcová et al. 2004, Table 1; Fig. 1), behaved quite differently. Although all 19 non-segregating $2n + n$ octoploid F_1 hybrids were scored as parthenogenetic (Table 2), the proportion of apomictically derived F_2 progeny ($2n + 0$) was highly variable, ranging from 0 to 100 % among individual octoploid $2n + n$ F_1 hybrids (Fig. 2). This proportion did not exceed 50 % in 14 (73.7 %) out of 19 octoploid $2n + n$ F_1 hybrids tested. These results suggest that the simple addition of the reduced genome of the sexual *P. officinarum* profoundly affected the expressivity of apomixis, decreasing it on behalf of haploid parthenogenesis in the majority of the analysed octoploid F_1 hybrids. The effect was more obvious when the origin of the progeny was studied directly at an embryonic level (see our previous study Krahulcová et al. 2011).

A similar difference in the expressivity of apomixis was also observed between the tetraploid facultatively apomictic *P. aurantiaca* and its recent hexaploid $2n + n$ hybrids with the sexual tetraploid *P. officinarum* (Krahulcová et al. 2011). It appears that the $2n + n$ hybridization between apomictic and sexual species is followed by a prevailing decline in the expression of apomixis, probably due to the change in genetic background and the interactions between the parental genomes. In natural populations of *Pilosella*, octoploid plants are rather rare (Krahulcová et al. 2000), possibly because they reproduce mainly through haploid parthenogenesis and produce progeny with reduced ploidy levels instead of replicating their own ploidy level (Krahulcová et al. 2009).

Possibility of multiple dose of P-allele in nature

The fact that in *Pilosella rubra* both the unreduced and the reduced female gametes carrying the P-allele can be

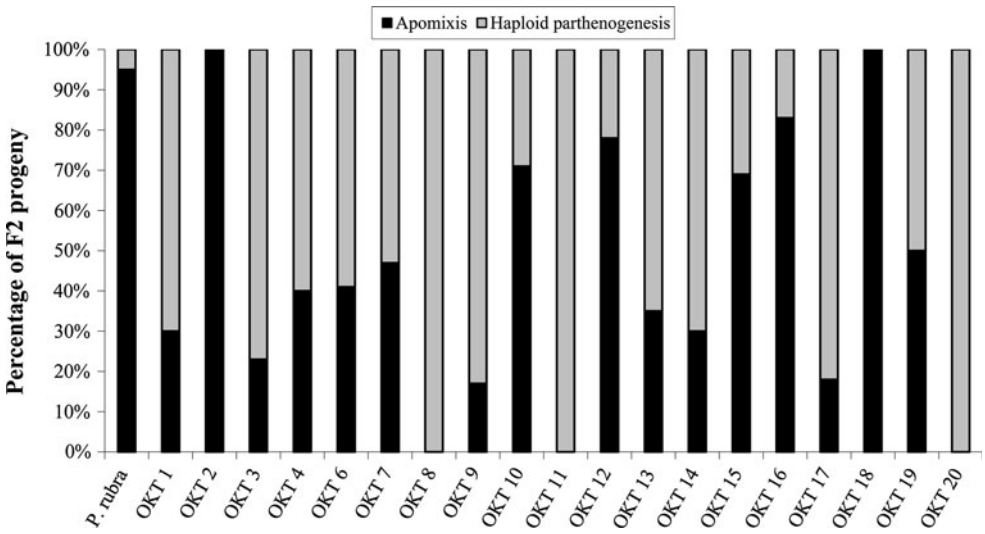

Fig. 2 The proportion of progeny originated through apomixis and haploid parthenogenesis in emasculated capitula of nineteen non-segregating F_1 $2n + n$ octoploid hybrids and their maternal parent apomictic *Pilosella rubra*. Octoploids are labelled OKT 1–OKT 20; OKT 5 was not included because it died at seedling stage

fertilized suggests that, in theory, progeny with double dose of the P-allele can arise from hybridization with an apomictic male parent. For example, from hybridization with tetraploid apomictic male parent (Pppp), the octoploid $2n + n$ hybrids (PPppppppp) and the pentaploid $n + n$ hybrids (PPppp) can arise. The presently accepted one dose model of parthenogenesis inheritance (Catanach et al. 2006; Koltunow et al. 2011b) does not allow for this possibility. From our field investigations, we know that numerous hybrids and hybridogenous species have an apomictic plant as the maternal parent and some of these reproduce apomictically (Krahulec et al. 2004, 2008). Nevertheless, we have no knowledge of their exact genetics. It will be interesting to study this question in greater depth.

Comparison with previous studies on apomixis inheritance in *Pilosella*

Our findings regarding the inheritance of parthenogenesis in *P. rubra* do not seem to be fully consistent with some findings in *P. praealta* (Catanach et al. 2006; Koltunow et al. 2011b). According to Catanach et al. (2006), apomixis is controlled by two dominant independent loci, *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*). Furthermore, the transmission of both loci appears to be distorted by selection acting against transmission of the dominant allele for *LOA* but favouring transmission of the dominant allele for *LOP* (Catanach et al. 2006). As a result, a high proportion (52 %) of progeny carrying the dominant allele of the *LOP* locus and the recessive allele of the *LOA* locus was detected among the F_1 $n + n$ hybrids that originated from the cross between the sexual tetraploid *P. officinarum* (maternal parent) and the apomictic aneuploid (nearly tetraploid) *P. praealta* (Catanach et al. 2006). These "*loa/LOP*" F_1 $n + n$ hybrid progeny could reproduce exclusively via haploid parthenogenesis after emasculation (bud decapitation), giving rise to polyhaploids, the progeny with a reduced ploidy level (Table 2 in Catanach et al. 2006). The cross between the sexual *P. officinarum* and the apomictic *P. praealta* corresponds to our cross B where the apomictic plant similarly served as a paternal parent. We, nevertheless, did not detect any F_1 $n + n$ hybrid that would reproduce exclusively via haploid parthenogenesis among the F_1 $n + n$ pentaploid hybrids from cross B. All the 22 F_1 pentaploid $n + n$ hybrids from cross B that were scored as parthenogenetic (Table 2) also seem to be able to perform apomeiosis, as they produced only the pentaploid F_2 progeny but no polyhaploids after emasculation (Table 3). The same holds for almost all of the remaining segregating F_1 progeny from both crosses A and B. The only exception was one F_1 heptaploid $n + 2n$ hybrid from cross A which formed one F_2 polyhaploid after emasculation (Table 3). These data suggest a rather close linkage between the determinant for parthenogenesis and the determinant for apomeiosis in *P. rubra*. We have to note that some of our F_1 progeny were found to be sterile and could represent sterile "*loa/LOP*" plants, although sterile plants represented only 6 % of segregating F_1 progeny.

The lack of haploid parthenogenesis in emasculated capitula of almost all segregating F_1 progeny from both crosses A and B is striking; the only exception is represented by F_2 polyhaploids originating from an emasculated capitulum of one heptaploid hybrid from cross A (Table 3). Here we propose two possible explanations of this phenomenon. First, an effective haploid parthenogenesis can be prevented by disturbances in meiosis in the respective segregating F_1 progeny—especially in trihaploids, regular meiosis is not very probable. Alternatively, it may be a consequence of a high mutational load carried by the genome of the maternal parent *Pilosella rubra*. The lethal

effect of the mutational load could be exposed in reduced gametes of the segregating F_1 progeny and could preclude their parthenogenetic development. In our previous long-term experimental and field studies in *Pilosella,* we have demonstrated that pentaploid apomicts, including hybrids, are able to form polyhaploid embryos (detected using flow cytometry), but always alongside other classes of progeny, namely apomictic and sexual progeny (Krahulec et al. 2011). To date, we did not detect any *Pilosella* hybrid producing exclusively polyhaploid progeny, irrespective of the ploidy level (Krahulcová and Krahulec 2000; Krahulcová et al. 2011; Krahulec et al. 2011).

The observed incongruence with previous studies may be caused by the fact that we did not use the same experimental system as Catanach et al. (2006) and Koltunow et al. (2011b). They used the aneuploid *P. praealta* ($2n = 4x - 1 = 35$) as the apomictic parent, whereas we used the hexaploid *P. rubra*. Okada et al. (2011) studied markers associated with the *LOA* locus in several *Pilosella* species and reported the absence of the *LOA*-linked markers in two accessions of apomictic *P. aurantiaca*. They suggested that apomeiosis may have evolved independently several times in the aposporous *Pilosella* species. This is interesting because the species *P. aurantiaca* represents the proposed maternal parent of the hybridogenous species *P. rubra*, which we have used in this study. The observed difference between this and previous studies thus may be a consequence of a difference in the genetic basis of apomeiosis between the model species.

In spite of efforts devoted to investigations of apomixis, it appears that our knowledge of the inheritance of apomixis in *Pilosella* is still incomplete and that many gaps must be filled to fully understand this process.

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