Production of polyhaploids by facultatively apomictic *Pilosella* can result in the formation of new genotypes via genome doubling

Autonomní vznik nových genotypů u apomiktických zástupců rodu *Pilosella* jako důsledek tvorby polyhaploidů a zdvojení jejich genomu

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D e d i c a t e d t o t h e m e m o r y o f L e o š K l i m e š

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Haploid parthenogenesis in facultatively apomictic *Pilosella* generated polyhaploid progeny (with half the maternal chromosome set) both in natural populations and garden experiments. Production of polyhaploids varied considerably among different species, hybridogenous species and hybrids. In the field (14 localities), the highest frequency of polyhaploids exceeded 80% of the total seed progeny produced by some recent hybrids. A similar diversity in the production of polyhaploids was also recorded in garden experiments. A two-step process by which new genotypes of both *P. aurantiaca* (tetraploid) and *P. rubra* (hexaploid) were formed under garden conditions during a polyploid–polyhaploid–polyploid cycle is described. In the first step, the maternal plants generated dihaploid and trihaploid F_1 progeny, respectively. Although a substantive part of this polyhaploid progeny was either non-viable or sterile, the apomictic polyhaploids occasionally doubled their genome. Consequently, the F_2 progeny resulting from the second step had a double ploidy level, identical to that of the original maternal parent. The complete process was autonomous, without contribution of pollen from parent genotype. This cycle necessarily implicates increasing homozygosity in F_2 progeny compared to the original maternal polyploid plant. The probabilities of particular steps of this process occurring in *Pilosella* and the variation in polyhaploids are estimated and described, and the ability of polyhaploid plants to survive under field conditions discussed. Probability of the complete cycle (haploid parthenogenesis followed by doubling of the genome), which occurred under garden conditions in *P. rubra*, is estimated to be in the order of hundredths of percent. Despite this low probability, it can result in the production of new homozygous genotypes in populations of apomicts, especially in those occurring in disturbed habitats with little competition.

K e y w o r d s: facultative apomixis, genome doubling, isozymes, *Pilosella*, polyhaploids, polyploid–polyhaploid–polyploid cycle, polyploidy

Introduction

Two types of reproduction, sexual and asexual, are usually distinguished. Although strange, the border between these two categories is not sharp, as there are several intermediate processes. Typically, sexual reproduction is characterized by meiosis during the course of sporogenesis in plants (oogenesis in animals), followed by fusion of reduced gametes. Nevertheless, some reproductive pathways actually occurring in both plants and

animals include only one of these steps. Haploid parthenogenesis bypasses the fusion of gametes but retains meiosis and recombination of the maternal genome and generates progeny that have half of the chromosome set of the original maternal parent (e.g. Asker & Jerling 1992). In spite of this inter-position of haploid parthenogenesis between sexuality (meiosis and recombination) and apomixis (parthenogenesis), some authors include it into broadly classified apomixis (e.g. Maheshwari 1950, Johri & Srivastava 2001). Haploid parthenogenesis occurs more frequently in polyploid than in diploid maternal plants, giving rise to (poly)haploid progeny (e.g. Kimber & Riley 1963, Barcaccia et al. 2006). Previous discussions on the potential evolutionary role of polyhaploids concentrate on the reversibility of polyploidization (Raven & Thompson 1964).

Another reproductive pathway in which meiosis does not occur and results in the production of unreduced gametes is relatively frequent in angiosperms. Unlike haploid parthenogenesis, the fusion of an unreduced gamete (male or female) with either a reduced (2n + n) or another unreduced gamete (2n + 2n), leads to polyploidization (the symbols follow Harlan & De Wet 1975). In terms of the supposed genetic control of apomixis operating in *Pilosella*, the two components of autonomous apomixis, i.e. apomeiosis and parthenogenesis, are decoupled, which results in the production of either polyhaploid (n + 0) or 2n + n progeny (Catanach et al. 2006). This implies that a particular reproductive pathway can completely dominate the reproduction of an individual. However, this is unknown in *Pilosella* in the field and did not occur in experimental garden crosses (Krahulcová et al. 2011), where there may be several reproductive pathways in each individual maternal plant.

Automixis occupies a special position among reproductive pathways. It includes the fusion of two maternal cells resulting from a meiotic process (megasporogenesis in angiosperms, oogenesis in animals). For example, automictic thelytoky, a type of parthenogenesis in which diploid females develop from auto-fertilized eggs and males are absent or rare (King & Stansfield 2002) occurs in some insect groups (Hartmann et al. 2005). In contrast, automixis is recorded much more rarely in plants, as e.g. in the blackberry *Rubus caesius* (Gerlach 1965, Antonius & Nybom 1995). In such automictic plants, the fusion of reduced (meiotic) cells occurs exclusively within the maternal parent. Consequently, automixis corresponds genetically to autogamy in an inbred homozygous line (Gerlach 1965). All of the distinct reproductive processes occurring in higher plants are reviewed by Asker & Jerling (1992).

The source of genetic variation in apomictic plants is still a subject of discussion (Hörandl & Paun 2007). Whereas minor variation may be caused by mutations, major variation is usually considered to be evidence of an independent origin of the different types (populations) (Gornall 1999). Due to residual sexuality operating in facultative apomicts, the occassional hybridization represents another substantive source of genetic variation (Hörandl & Paun 2007).

In previous crossing experiments involving the hexaploid facultativelly apomictic *P. rubra* an indispensable rate of polyhaploid production was recorded (Krahulcová et al. 2004, Krahulec et al. 2006). Some of the trihaploids were viable, setting at least some seed. Therefore, the reproductive behaviour of these trihaploid plants under garden conditions was examined. Ploidy screening of their progeny surprisingly detected two plants with a redoubled (hexaploid) ploidy level (R. Rosenbaumová et al., unpublished results). A similar phenomenon we also detected in another facultatively apomictic species, the tetraploid *P. aurantiaca*. Polyhaploids were also recorded among the progeny of polyploid

hybrids generated in experimental crosses, for example in the octoploid (2n = 8x = 72) hybrid *P. rubra* × *P. officinarum* (Krahulcová et al. 2011) and aneuploid hybrid *P. glomerata* × *P. officinarum*, with 2n = 42 (Krahulcová & Krahulec 2000). Consecutive investigations into the frequency of polyhaploid progeny, which are generated in natural populations, resulted in the first record of triploid *Pilosella* plants surviving in the field, which almost certainly had the polyhaploid (trihaploid) origin (Krahulec et al. 2008). The frequency of origin, fitness and reproductive behaviour of polyhaploids both in natural and garden populations, including their autonomous genome redoubling, which is rarely recorded, are summarized in this paper. The potential of polyhaploids in shaping the population variability in *Pilosella* is discussed.

Materials and methods

Origin of plants, their ploidy level and reproductive system

Polyhaploids generated by haploid parthenogenesis were recorded within the progeny of a whole set of apomictic *Pilosella* species, including hybrids, the seed of which was collected at 14 localities (Table 1). Most of the polyhaploid progeny recorded in the field were detected at the embryonic stage, either in samples of seed doublets using the conventional method of Flow Cytometric Seed Screen (FCSS; Matzk et al. 2000, Krahulcová et al. 2009) or in pooled samples of ten seeds using the modified FCSS method (Krahulcová & Suda 2006). To detect polyhaploids among the seedlings, the DNA ploidy level (Suda et al. 2006) of the seedlings was compared with that of the maternal plants, using flow cytometry. In this way, the polyhaploids that originated at Praha-Vysočany among seedlings cultivated from seeds in a greenhouse, were pre-screened using conventional flow cytometry (DAPI staining; Krahulcová et al. 2004) and subsequently, chromosomes were counted in each of these selected seedlings because of their potential for aneuploidy (Krahulcová et al. 2009). In some cases, root-tip squash preparations stained with lacto-propionic orcein were used (Krahulcová & Krahulec 1999) to compare the chromosome number in the progeny with that in the maternal plant. The frequency of polyhaploid progeny was also studied in garden experiments, involving crosses/emasculation/open pollination of the maternal facultatively apomictic plants. Approximately half of this data is published elsewhere. In Table 2 is a summary of the data on polyhaploids that have so far originated in experiments. The detailed description of experimental procedures used can be found in the references cited in Table 2.

Results of previous crossing experiments using the hexaploid *P. rubra* (Krahulcová et al. 2004) provided a set of 31 polyhaploid (trihaploid) F_1 progeny plants (not all of the crossing experiments that gave rise to trihaploid *P. rubra* are presented in Table 2). Before these trihaploids could be used in subsequent experiments, 14 of them either died or were sterile, i.e. they did not produce any germinable seeds after open pollination in the experimental garden. The remaining 17 seed-fertile trihaploids were cultivated under garden conditions and emasculated by cutting off the upper half of young capitula, which were then each enclosed in a nylon bag (Gadella 1987, Krahulcová & Krahulec 1999). In this way, any F_2 progeny must have been produced parthenogenetically (Fig. 1). Seeds obtained from all of the 17 emasculated polyhaploids were sown in sterilized garden soil in a greenhouse. In order to detect the hexaploid seedlings (those with double the ploidy of

Table 1. – Frequency of polyhaploid progeny detected in *Pilosella* populations in the field. Seeds or seedlings* cultivated from the seeds were screened for polyhaploids. The seeds collected at the different localities were analysed using Flow Cytometric Seed Screen (Matzk et al. 2000). Either seed doublets (Krahulcová et al. 2009) or pooled samples of 10 seeds (modified FCSS, Krahulcová & Suda 2006) were processed. The chromosome numbers of all the seedlings* cultivated were compared with that of the maternal parent. Only those progenies, in which some polyhaploids were detected, are presented. Published data relate to particular localities: [a]Krahulcová et al. (2011); [b]Krahulcová et al. (2009). Country: A – Austria, BG – Bulgaria, CZ – Czech Republic, D – Germany.

Taxon	Ploidy level (x = 9)	Country	Area	Locality	No. of individuals analysed	Frequency of polyhaploids (%)
Basic species						
P. aurantiaca	4x	CZ	Šumava Mts	Slučí Tah[a]	339	2.4
P. aurantiaca	4x	CZ	Šumava Mts	Zhůří near Železná Ruda[a]	247	0.4
P. aurantiaca	4x	CZ	Šumava Mts	Hadí vrch	204	2.0
P. aurantiaca	4x	D	North Rhine – Westphalia	Hagen[a]	266	0.4
P. aurantiaca	4x	A	Allgäuer Alps	Hirschegg	205	0.5
P. officinarum	6x	CZ	South Moravia	Rašovice near Slavkov	140	5.7
P. officinarum	6x	BG	Forebalkan	Mt Vrachanska near Vratsa	325	0.9
P. piloselloides subsp. *bauhini* and subsp. *magyarica*	6x	BG	Vitosha region	Kovachevitsa village	257	4.3
P. piloselloides subsp. *bauhini*	5x	CZ	South Moravia	Brno, Kamenný vrch[b]	134	1.5
P. piloselloides subsp. *magyarica*	6x	BG	Forebalkan	Mt Vrachanska near Vratsa	60	3.3
Stabilized hybridogenous species						
P. blyttiana	4x	CZ	Krkonoše Mts	Pomezní Boudy	240	0.8
P. floribunda	4x	CZ	Šumava Mts	Zhůří near Kašperské Hory	70	11.4
P. rubra	6x	CZ	Krkonoše Mts	Pomezní Boudy[a]	552	3.6
P. scandinavica	4x	CZ	Šumava Mts	Churáňov	140	1.4
Recent hybrids						
P. fuscoatra (*P. caespitosa* × *P. aurantiaca*)	4x	CZ	Krkonoše Mts	Pomezní Boudy	257	7.0
P. aurantiaca × *P. officinarum*	6x	CZ	Šumava Mts	Zhůří near Železná Ruda[a]	13	84.6
P. aurantiaca × *P. officinarum*	6x	CZ	Šumava Mts	Slučí Tah[a]	60	70.0
P. aurantiaca × *P. officinarum*	6x	D	North Rhine – Westphalia	Hagen[a]	210	43.8
P. officinarum × *P. densiflora*	5x	CZ	South Moravia	Rašovice near Slavkov	363	0.6
P. officinarum × *P. densiflora*	8x	CZ	South Moravia	Rašovice near Slavkov	190	43.2
P. piloselloides subsp. *bauhini* × *P. officinarum*	5x	CZ	North-East Bohemia	Lomnice n. Popelkou	268	1.9
P. piloselloides subsp. *bauhini* × *P. officinarum*	7x	CZ	Central-Bohemian Basin	Praha-Vysočany[b]	20*	25.0
P. hoppeana subsp. *testimonialis* / *P. officinarum* × *P. piloselloides*	5x	BG	Vitosha region	Kovachevitsa village	30	3.3
P. hoppeana subsp. *testimonialis* / *P. officinarum* × *P. piloselloides*	6x	BG	Vitosha region	Kovachevitsa village	440	3.4
P. hoppeana subsp. *testimonialis* / *P. officinarum* × *P. piloselloides*	6x	BG	Forebalkan	Mt Vrachanska near Vratsa	70	1.4

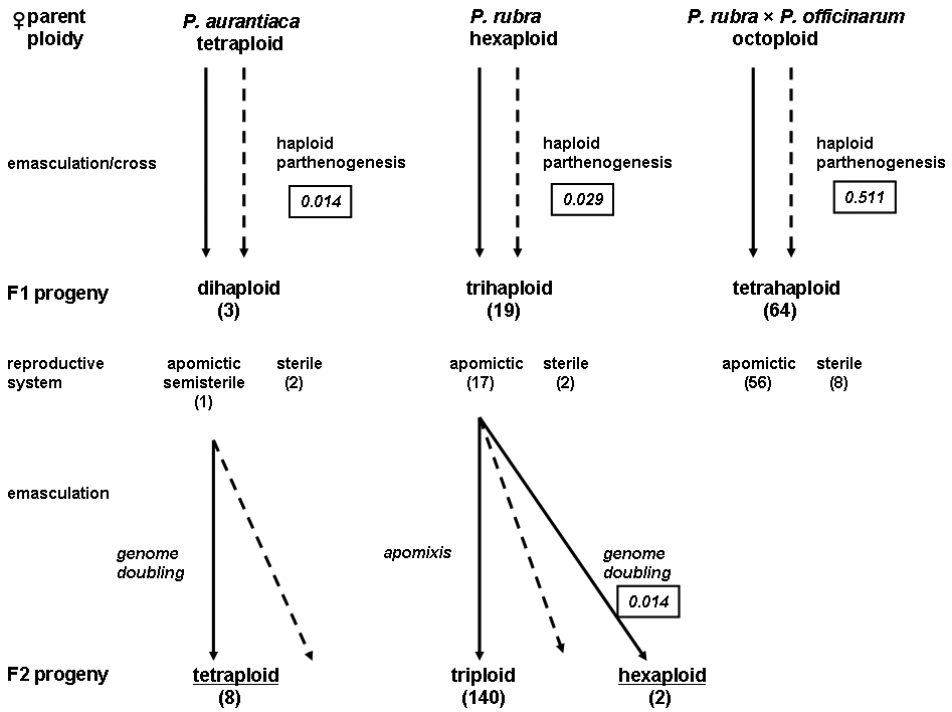


Fig. 1. – Polyhaploids generated parthenogenetically by three experimental maternal (symbol ♀) biotypes of *Pilosella*, which were either emasculated or crossed. Subsequent autonomous genome doubling, which proceeded in some emasculated apomictic polyhaploids, gave rise to a ploidy level (underlined) identical to that of the maternal parent. The respective reproductive processes are printed in italics and their frequency in particular biotypes framed. The data (Table 2) are based on plants cultivated from seeds. The number of experimental plants is given is parentheses. Dashed lines indicate non-viable embryos and/or those that perished or developed into sterile (not evaluated) plants.

their trihaploid maternal parents) their ploidy level was determined using flow cytometry (for the protocol see Krahulcová et al. 2004). This procedure was also used on the three dihaploid seedlings of *P. aurantiaca* that developed in the experiments (Table 2). Similarly, the reproductive system of the tetrahaploid seedling progeny, which were obtained from the emasculated octoploid hybrids *P. rubra* × *P. officinarum*, was determined by open pollination/emasculation of the F_1 tetrahaploid plants (Fig. 1, Table 2).

Isozyme analysis

The extraction and protocol follow Krahulec et al. (2004). Isozyme phenotypes of the system AAT1 and AAT2 (aspartate-aminotransferase) were used to compare the maternal hexaploid *P. rubra* with its 25 trihaploid progeny (Fig. 2). This enzyme system was chosen because it was the most variable of four enzyme systems (AAT, PGM, LAP, EST) used previously in a trial analysis in which the maternal hexaploid was compared with only a few trihaploid individuals. In the case of the isozyme phenotypes of the hexaploid–trihaploid–hexaploid cycle (Fig. 2C) the four enzyme systems used were: AAT PGM, LAP and EST.

Table 2. – Frequency of polyhaploid progeny produced by *Pilosella* in garden experiments. All maternal plants (symbol ♀) are facultatively apomictic. Polyhaploids were screened among either seeds or seedlings cultivated from the seeds, respectively. Seed samples were analysed using Flow Cytometric Seed Screen (Matzk et al. 2000), either as seed doublets (Krahulcová et al. 2009), or as pooled samples of 10 seeds (modified FCSS, Krahulcová & Suda 2006). Screening of the seedlings was carried out using conventional flow cytometry (DAPI staining, Krahulcová et al. 2004). In some cases, the polyhaploid origin of the respective progenies was inferred, comparing the chromosome number in the cutivated seedlings/germinating seeds with that of the maternal parent. The individual maternal genotypes collected in the field were distinguished within particular taxa and ploidy level, using the combination of four isozyme phenotypes. Abbreviations used: EM – emasculation; OP – open pollination. Published data related to respective experiments: [a]Krahulcová et al. (2004); [b]Krahulec et al. (2006); [c]Krahulec et al. (2008); [d]Krahulcová et al. (2009); [e]Krahulcová et al. (2011); [f]Krahulcová & Krahulec (2000); [g]Fehrer et al. (2007).

Parental taxa used in experiments	Experimental treatment/♀ genotype	Ploidy level of ♀ parent (x = 9)	Progeny analysed	No. of individuals analysed	Frequency of polyhaploids (%)
♀Basic species or stabilized hybrids					
♀*P. aurantiaca*	EM	4x	[c]seeds	100	3.0
♀*P. aurantiaca*	EM	4x	seedlings	202	1.0
♀*P. aurantiaca* × ♂*P. officinarum*	cross	4x	[f]seedlings	20	5.0
♀*P. iserana*	EM	4x	seeds	60	1.7
♀*P. officinarum*	EM	6x	seeds	70	2.9
♀*P. piloselloides* subsp. *bauhini*	EM/genotype A	5x	seeds	100	2.0
♀*P. piloselloides* subsp. *bauhini*	EM/genotype B	5x	seeds	100	1.0
♀*P. piloselloides* subsp. *bauhini* × ♂*P. officinarum*	cross	6x	[g]seedlings	849	1.2
♀*P. piloselloides* subsp. *magyarica* × ♂*P. lactucella*	cross/genotype A	6x	seeds	60	3.3
♀*P. piloselloides* subsp. *magyarica* × ♂*P. officinarum*	cross/genotype A	6x	seeds	200	4.5
♀*P. piloselloides* subsp. *magyarica* × ♂*P. onegensis*	cross/genotype A	6x	seeds	52	15.4
♀*P. piloselloides* subsp. *magyarica* × ♂*P. onegensis*	cross/genotype A	6x	seedlings	51	15.7
♀*P. piloselloides* subsp. *magyarica* × ♂*P. hoppeana* subsp. *testimonialis*	cross/genotype A	6x	seeds	48	14.6
♀*P. piloselloides* subsp. *magyarica* × ♂*P. hoppeana* subsp. *testimonialis*	cross/genotype A	6x	seedlings	13	15.4
♀*P. piloselloides* subsp. *magyarica* × ♂*P. pavichii*	cross/genotype B	6x	seeds	200	1.0
♀*P. rubra*	EM	6x	[a]seedlings	312	1.6
♀*P. rubra*	EM	6x	[b]seeds	1370	5.9
♀*P. rubra* × ♂*P. officinarum*	cross	6x	[a]seedlings	354	4.0
♀*P. rubra* × ♂*P. officinarum*	cross	6x	[b]seeds	1190	11.8
♀Recent hybrids originated in the field					
♀*P. piloselliflora* × ♂*P. officinarum*	cross	6x	[g]seedlings	134	3.0
♀(*P. aurantiaca* × *P. officinarum*) × ♂*P. officinarum*	cross/genotype A	6x	[c]seedlings	118	39.0
♀(*P. aurantiaca* × *P. officinarum*) × ♂*P. officinarum*	cross/genotype B	6x	[c]seedlings	60	66.7
♀*P. aurantiaca* × *P. officinarum*	EM/5 genotypes	6x	[c]seeds	206	26.0-93.8
♀(*P. floribunda* × *P. officinarum*) × ♂*P. officinarum*	cross/genotype A	6x	[c]seedlings	140	82.1
♀*P. floribunda* × *P. officinarum*	EM/genotype A	6x	seeds	27	74.1
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype A	8x	[d]seedlings	75	48.0
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype B	8x	[d]seeds	60	76.7
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	OP/genotype B	8x	[d]seedlings	25	8.0
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype A	7x	[d]seedlings	84	82.1
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype B	7x	seeds	97	46.4
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype C	7x	seeds	80	66.2
♀*P. piloselloides* subsp. *bauhini* × *P. officinarum*	EM/genotype D	7x	seeds	40	52.5
♀*P. officinarum* × *P. densiflora*	EM	8x	seedlings	175	9.1
♀Recent hybrids originated from garden crosses					
♀*P. aurantiaca* × *P. officinarum*	EM/13 genotypes	6x	[c]seeds	658	36.0-89.1
♀*P. aurantiaca* × *P. officinarum*	OP/7 genotypes	6x	[c]seeds	339	0.0-66.7

Parental taxa used in experiments	Experimental treatment/♀ genotype	Ploidy level of ♀ parent (x = 9)	Progeny analysed	No. of individuals analysed	Frequency of polyhaploids (%)
♀*P. rubra* × *P. officinarum*	EM/8 genotypes	8x	[c]seeds	470	53.3-84.0
♀*P. rubra* × *P. officinarum*	EM/2 genotypes	8x	seedlings	208	71.7-73.4
♀*P. rubra* × *P. officinarum*	OP/19 genotypes	8x	[c]seeds	1115	0-80
♀*P. rubra* × *P. officinarum*	EM/19 genotypes	8x	seedlings	137	51.1 on average
♀*P. glomerata* × *P. officinarum*	OP	2n = 41	[f]germinating seeds	19	10.5

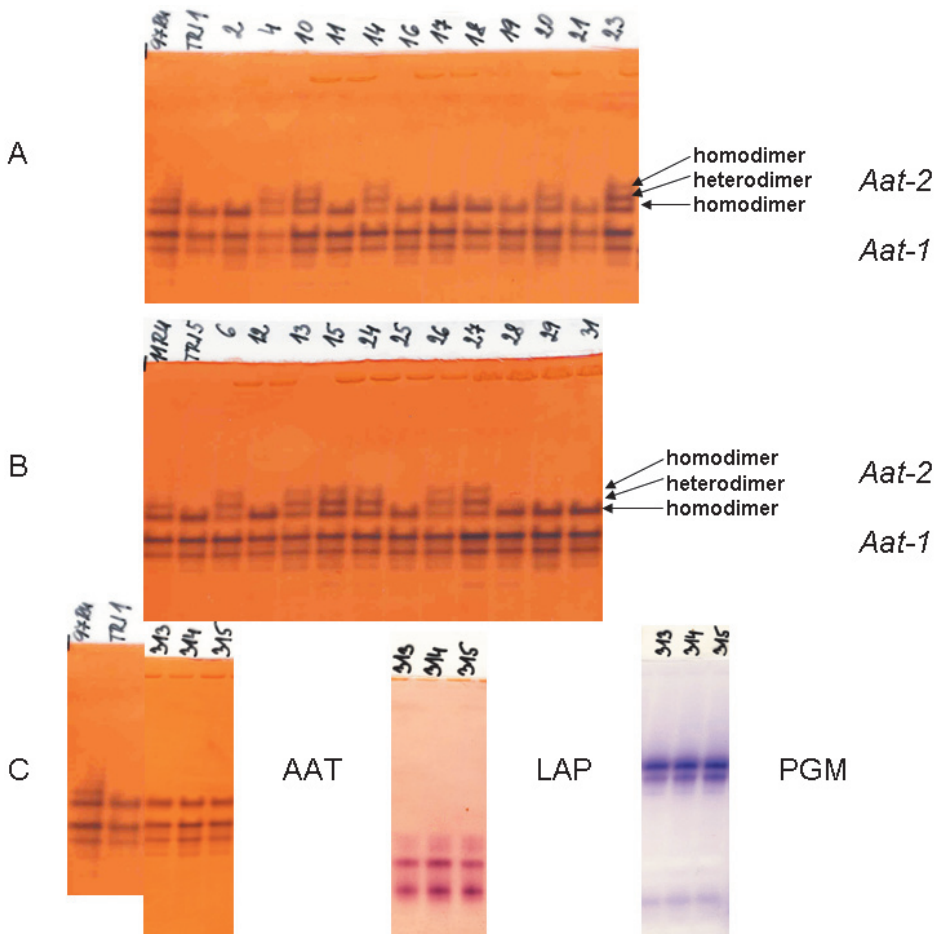


Fig. 2. – Zymograms of trihaploid plants of *Pilosella rubra* compared with that of the mother plant. A – mother plant 97RU and the trihaploids it produced; B – mother plant 11RU and the trihaploids it produced; C – mother plant 97RU, trihaploid (TRI1 and 313) and two autonomously produced progenies, triploid (315) and hexaploid (314). Note that 97RU and 11RU were determined as belonging to the same clone, different numbers represent plants collected at two localities. The uppermost band (arrowed in panels A, B) in the zymograms representing the system *Aat-2* is more intense in some of the trihaploids and not clearly detectable in their maternal plants.

Results

Rate of haploid parthenogenesis in Pilosella

Polyhaploid embryos were generated in the field at frequencies from tenths of a percent to a percent (Table 1). This holds for both basic and stabilized hybridogenous species. Nevertheless, a rather high variation was recorded among the populations/taxa studied. Whereas no polyhaploid embryos were detected in seeds sampled at number of localities (not shown in Table 1), one of the hybridogenous species (*P. floribunda*) and especially recent hybrids between apomictic and sexual parents with hexaploid and higher ploidy levels displayed haploid parthenogenesis at rates of the order of tens of percent (Table 1). This tendency of the high-polyploid hybrid (apomictic × sexual) genomes to be unstable was also confirmed by the experiments (see below and Table 2). Data in Table 1 relates to particular well defined species, most of them represented just by a single population. For this reason there are only a few records for particular taxa that can be compared between different localities/populations.

The frequencies of polyhaploid progeny recorded in the garden experiments are mostly presented in our previous experimental studies on *Pilosella*. These data are only briefly summarized here, in order to reveal the variation (Table 2). Due to selection acting during germination and early growth of the seedlings, the frequencies of polyhaploid embryos (detected in seeds) are higher than of polyhaploid seedlings, which developed from the same experimental seed set (Table 2, Krahulec et al. 2006). Extremely high percentages of polyhaploids (up to around 90%) were recorded in the seed progeny of some of the hexaploid hybrid plants, which had arisen as recently via 2n + n hybridisation between the tetraploid facultatively apomictic *P. aurantiaca* (seed parent) and tetraploid sexual *P. officinarum* (Table 2). Similarly, the frequency of polyhaploids produced by some either octoploid or heptaploid 2n + n hybrids between another apomictic maternal species and the sexual *P. officinarum* exceeded 70% (Table 2). Interestingly, haploid parthenogenesis can occur even in maternal plants that have odd chromosome numbers, such as pentaploids, heptaploids and aneuploids (Table 2).

Frequency of production of polyhaploid seedlings by the experimental maternal plants *P. aurantiaca* (tetraploid), *P. rubra* (hexaploid) and the hybrid *P. rubra* × *P. officinarum* (octoploid) can be calculated using the data in the Table 2 as follows: *P. aurantiaca* (both crossed and emasculated) 0.014 (three dihaploids out of 222 seedlings), *P. rubra* (both crossed and emasculated) 0.029 (19 trihaploids out of 666 seedlings) and the hybrid *P. rubra* × *P. officinarum* (emasculated only) 0.511 (70 tetrahaploids out of 137 seedlings). The frequency of polyhaploids produced in these experiments increased with increasing ploidy of the maternal parent (Fig. 1).

Variation within polyhaploids

Thirty one trihaploid plants that originated from the hexaploid maternal *P. rubra* (see Materials and methods) were assembled. Because *P. rubra* itself is a hybridogeneous species (result of 2n + n hybridisation *P. aurantiaca* × *P. officinarum*; Suda et al. 2007), the morphological characters of individual trihaploid plants differed due to segregation (haploid parthenogenesis is a meiotic process – Fig. 3). The characters that segregated were, for example, the size and shape of leaves and especially the colour of ligules ranging from

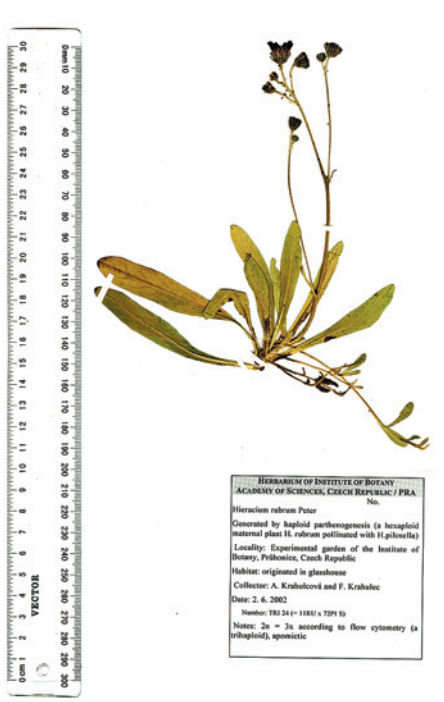
yellow to orange. The trihaploid plants were more slender than the maternal *P. rubra*, with small capitula and a thin stem. Some of the plants in the original set of 31 trihaploids died in the first or second year of cultivation (see Materials and methods). The dihaploid *P. aurantiaca* grew even less well. The three cultivated dihaploid plants (Table 2) were small, rather weak and produced few flowers. Under garden conditions they did not survive for more than one or two seasons. On the other hand, some of the tetrahaploids, produced by the octoploid 2n + n hybrids between *P. rubra* (6x) and *P. officinarum* (4x) (Krahulcová et al. 2011, R. Rosenbaumová et al., unpublished results) were also weaker and more slender than their octoploid mothers, but about half of the progeny grew well.

When the isozyme phenotypes of the enzyme system AAT2 in 25 trihaploid plants were compared with that of their hexaploid maternal parent *P. rubra*, each trihaploid plant was found to differ from its maternal parent in the pattern of its isozyme bands. Moreover, variation in isozyme phenotypes was detected within the trihaploid progeny array, which originated from a single maternal plant (Fig. 2A, 2B). In the mother plant, only one homodimer and heterodimer of AAT2 was detected, whereas the trihaploids had two homodimers and a heterodimer or only one homodimer.

Reproductive system and genome doubling in polyhaploids

With respect to seed set, the dihaploids of *P. aurantiaca* appeared to be less fertile than the trihaploids of *P. rubra*; however, the number/percentage of filled seed was not quantified. One of the three dihaploid *P. aurantiaca* cultivated (Table 2) produced some seed after emasculation, but the seed set and germination of these seeds were very low (only eight seedlings survived). Nevertheless, this plant could be classified as apomictic and semi-sterile. The remaining two dihaploid plants of *P. aurantiaca* were emasculated/pollinated by the diploid *P. lactucella*: they appeared to be sterile, producing only undeveloped (aborted) seeds which did not germinate. Consequently, these two dihaploids were classified as sterile.

In spite of reduced seed fertility, both dihaploid and trihaploid plants cultivated in the experimental garden, produced at least some progeny with a doubled chromosome number, i.e. the progeny had the same ploidy level as the original maternal polyploid plant (Figs 4 and 5). A doubled DNA ploidy level was recorded for all eight seedling progeny produced by a single fertile emasculated dihaploid *P. aurantiaca*. The species *P. rubra* provided more accurate estimates of the frequency of genome doubling because more trihaploids survived. Namely, 19 trihaploid plants (out of an original set of 31) survived in the garden until the requisite experiments started. Among them, two were sterile and 17 were apomictic with a low rate of seed germination (R. Rosenbaumová et al., unpublished results). Of the 140 seedlings (F_2 generation) produced by the 17 emasculated apomictic trihaploids, two of the F_2 progeny had doubled (hexaploid) DNA contents (R. Rosenbaumová et al., unpublished results). Thus, the frequency of genome doubling among the progeny of apomictic trihaploids was 0.014. If the frequency of trihaploid formation in *P. rubra* (0.029; see above and Fig. 1) and the genome doubling recorded in the apomictic trihaploids are taken into consideration, the resulting frequency of both successive steps is very low, approximately 0.04%. However, the actual frequency of the complete process of the formation of trihaploids followed by genome doubling is even lower, because neither the non-viable nor the sterile progeny were taken into account (Fig. 1).


HERBARIUM OF INSTITUTE OF BOTANY
ACADEMY OF SCIENCES, CZECH REPUBLIC / PRA
No.
Hieracium rubrum Peter
Generated by haploid parthenogenesis (a hexaploid maternal plant H. rubrum pollinated with H.pilosella)
Locality: Experimental garden of the Institute of Botany, Průhonice, Czech Republic
Habitat: originated in glasshouse
Collector: A. Krahulcová and F. Krahulec
Date: 2. 6. 2002
Number: TRI 24 (= 118IU x 72PI 5)
Notes: 2n = 3x according to flow cytometry (a trihaploid), aposmictic


HERBARIUM OF INSTITUTE OF BOTANY
ACADEMY OF SCIENCES, CZECH REPUBLIC / PRA
No.
Hieracium rubrum Peter
Locality: Experimental garden of the Institute of Botany, Průhonice, Czech Republic
Habitat: originated in glasshouse
Collector: A. Krahulcová and F. Krahulec
Date: 25. 8. 2002


HERBARIUM OF INSTITUTE OF BOTANY
ACADEMY OF SCIENCES, CZECH REPUBLIC / PRA
No.
Hieracium rubrum Peter
Generated by haploid parthenogenesis (a hexaploid maternal plant H. rubrum pollinated with H.pilosella)
Locality: Experimental garden of the Institute of Botany, Průhonice, Czech Republic
Habitat: originated in open field
Collector: R. Rosenbaumová Date: 27. 6. 2003
Number: TRI 1 (= 97IU x 39 IIIu)
Notes: 2n = 3x according to flow cytometry (a trihaploid)


HERBARIUM OF INSTITUTE OF BOTANY
ACADEMY OF SCIENCES, CZECH REPUBLIC / PRA
No.
Hieracium rubrum Peter (seed parent)
Locality: cultivated in the experimental garden Průhonice
Origin: F1 progeny, the seeds obtained of open-pollinated seed parent in the garden
Collector: F. Krahulec and A. Krahulcová
Date: 25. 5. 1999
Number: 11 IIU, v. sp. 1
Notes: 2n = 27 (trihaploid), the seed parent (2n = 54) aposmictic

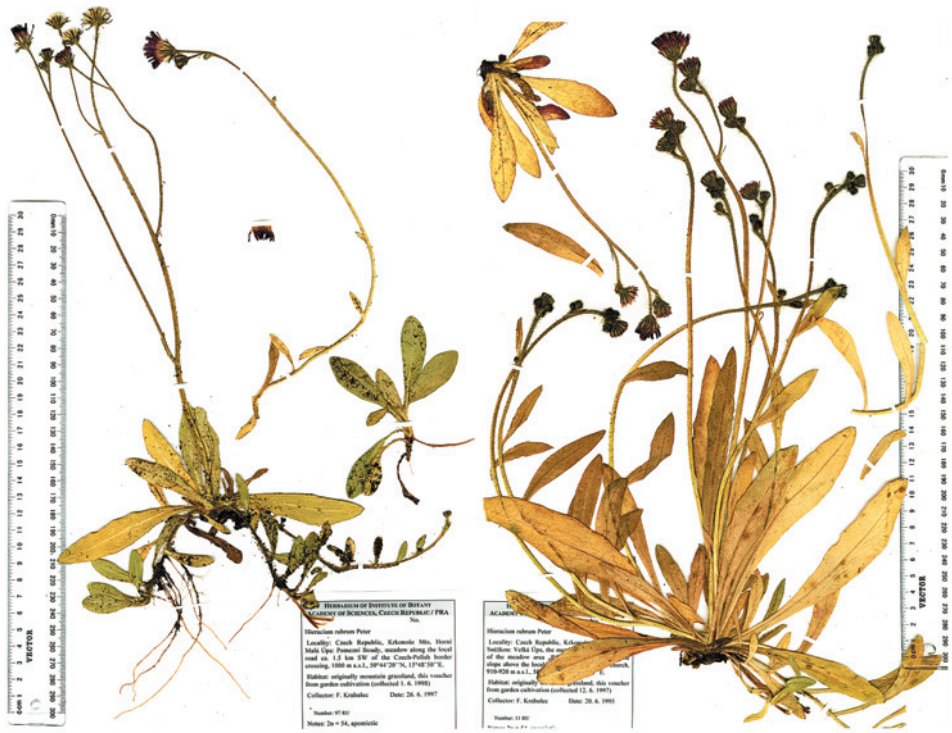


Fig. 3. – Variation of trihaploids that originated from hexaploid *Pilosella rubra* (left page) and two plants of *P. rubra*.

Based on these four systems (AAT, PGM, LAP and EST), the three isozyme phenotypes referred to the trihaploid maternal plant and its progeny, which were generated (i) by chromosome doubling and (ii) by apomixis, respectively, are exactly identical (the AAT phenotypes are shown in the Fig. 2C).

Altogether 64 tetrahaploids, which were produced by the octoploid hybrids *P. rubra* × *P. officinarum* (Table 2), were scored for reproductive system. Eight plants were nearly sterile and the remaining 56 plants were apomictic, i.e. they set seed after emasculation (R. Rosenbaumová et al, unpublished results). However, the possible occurrence of octoploid individuals with a double DNA content was not evaluated within this parthenogenetically produced progeny of tetrahaploids. The polyhaploid production of *P. aurantiaca*, *P. rubra* and the octoploid hybrids *P. rubra* × *P. officinarum* and the reproductive behaviour of the cultivated polyhaploids and reproductive pathways recorded in the fertile polyhaploids are summarized in Fig. 1.

Survival of polyhaploids in the field

Generally it is believed that polyhaploids cannot grow in the field. To test this experimentally, a set of plants comprised of (i) different *Hieracium* species, (ii) their experimental hybrids that had diverse cytotypes including aneuploids and (iii) experimental

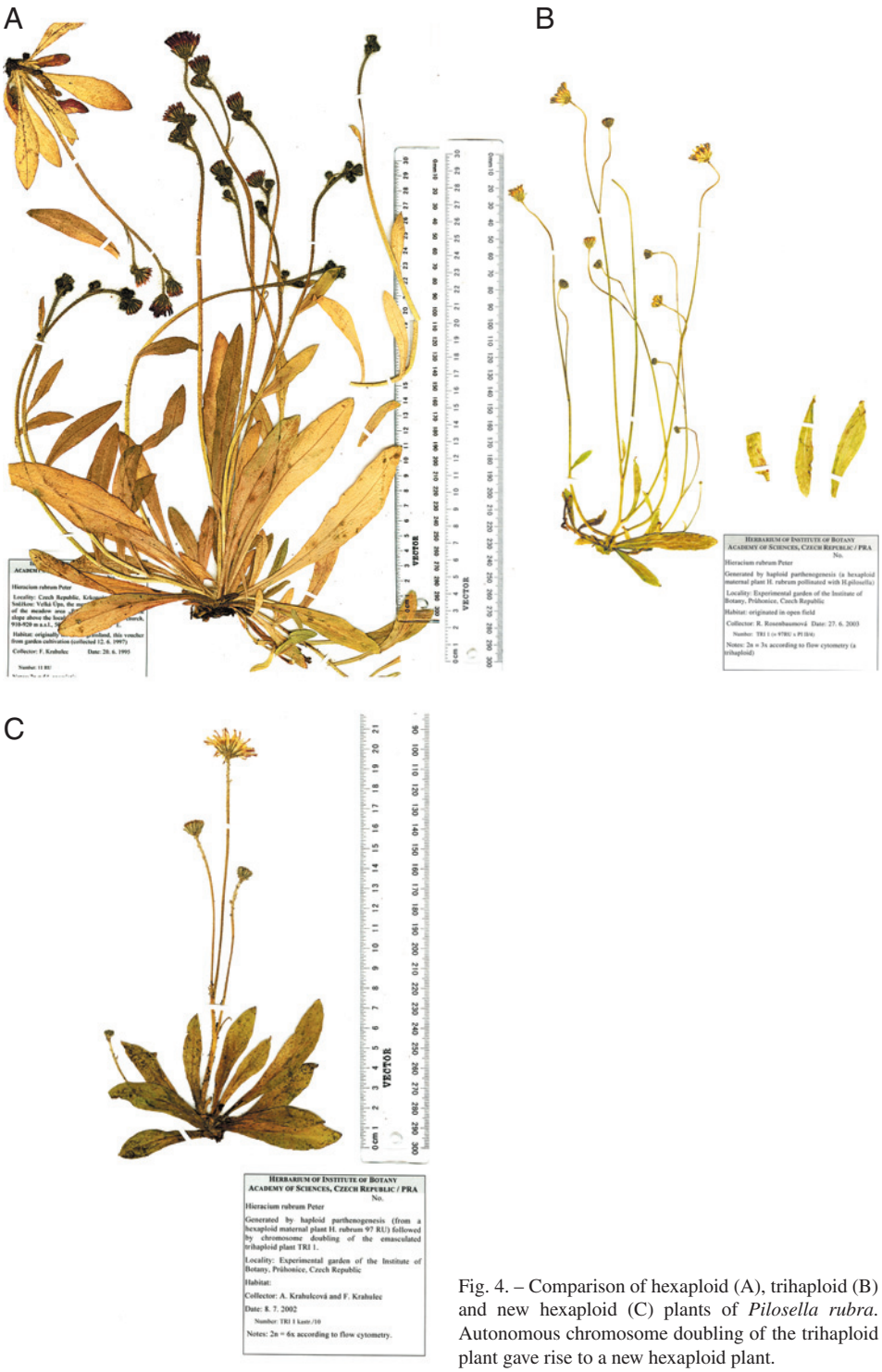


Fig. 4. – Comparison of hexaploid (A), trihaploid (B) and new hexaploid (C) plants of *Pilosella rubra*. Autonomous chromosome doubling of the trihaploid plant gave rise to a new hexaploid plant.

Fig. 5. – Comparison of tetraploid (A), dihaploid (B) and a new tetraploid (C) plants of *Pilosella aurantiaca*. Autonomous chromosome doubling of the dihaploid plant gave rise to a new tetraploid plant.

polyhaploids, were planted in dense mountain grassland situated at an altitude of 880 m a.s.l. (F. Krahulec et al., unpublished data that are not presented in this paper). The seven trihaploid plants of *P. rubra* planted had different genotypes, which were previously identified using isozyme phenotypes (see Materials and methods). As these seven trihaploids grew well in the lowland experimental garden, they were chosen for the field experiment, where they were exposed to a harsh and long winter and inter-specific competition. Of them, two grew poorly (one died after flowering in the first season) and five grew relatively well. Six of the trihaploids flowered in early autumn, immediately after they were planted out in summer and one also the following year. It is evident that at least some of the trihaploids can grow and even flower in the field and survive there over winter. Although unequivocal identification of polyhaploids in the field is disputable (see the Discussion below), two triploid plants were recorded at one locality in the Šumava Mts, which were very probably of polyhaploid origin (Krahulec et al. 2008).

Tetrahaploid plants (Table 2), which were more slender than their maternal parent, were detected among the progeny of the octoploid accession corresponding to hybrid *P. piloselloides* subsp. *bauhini* × *P. officinarum.* Similar tetraploid plants were also recorded in the field at the locality where the octoploid accession was collected. However, it was not possible to prove they are of polyhaploid origin. On the other hand, the fitness of some of the tetrahaploids may be rather high. For example, at least half of the tetrahaploid progeny produced by another octoploid maternal parent, an artificial hybrid between *P. rubra* and *P. officinarum* (Table 2), grew well under garden conditions and did not differ in morphology from the maternal plants (see above paragraph Variation within polyhaploids). Hence, such tetrahaploids might have a good chance of surviving in the field.

Discussion

How can natural polyhaploids be detected?

Polyhaploids are easily indentified if the maternal plant is known because polyhaploids have half the maternal chromosome set (Fig. 6). With respect to morphology, dihaploid and trihaploid plants of *Pilosella* are usually weak with small and narrow leaves. They often have many leaves and many stolons, which can be of aberrant appearance. Such "strange" plants are sometimes found in the field, where their maternal parents are unknown. On the other hand, the morphology of at least some of the experimentally produced trihaploid plants of *P. rubra* and also some of the tetrahaploid plants originating from the octoploid 2n + n hybrids between *P. rubra* and *P. officinarum*, give no indication of their origin. This makes it very difficult to distinguish polyhaploids from standard hybrids in the field. As far as is known, no test exists for revealing whether a plant in a natural population is polyhaploid (for discussion see also Krahulec et al. 2008). The tetraploid hybrid individuals were recorded, for example, in the hybridizing population of *P. piloselloides* subsp. *bauhini* (pentaploid) and *P. officinarum* (tetraploid). Hybrids were identified based on morphology (Krahulcová et al. 2009). These hybrids had the same appearance as the tetrahaploids cultivated from the seed generated by the octoploid hybrid, which came from the same hybrid swarm (Table 2). However, an alternative origin of the assumed "natural tetrahaploids" cannot be excluded, namely a recent hybridization between both putative parents, which co-occured at this locality.

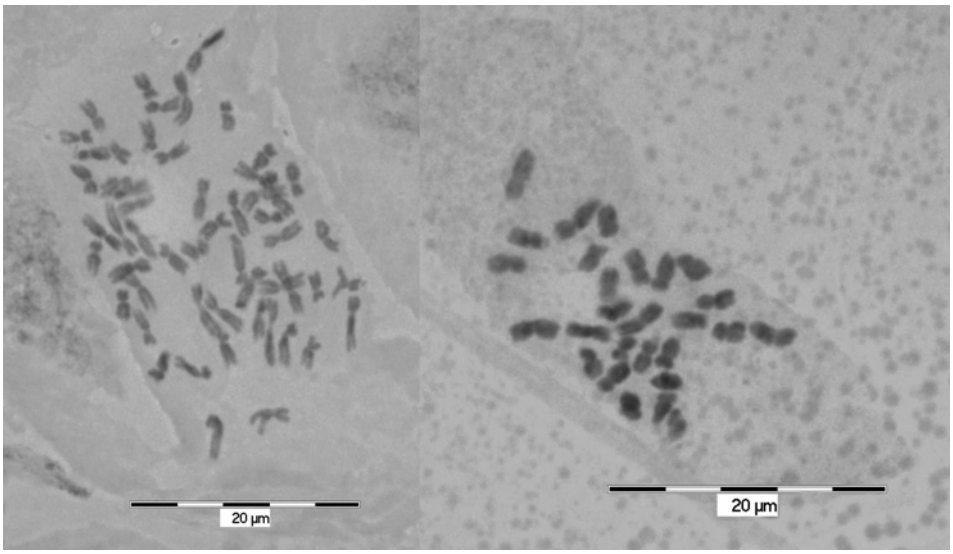


Fig. 6. – Microphotograps of somatic metaphases in hexaploid (2n = 6x = 54; left) and trihaploid plants (2n = 3x = 27; right) of *Pilosella rubra*.

Indeed, weak plants in the field that resemble polyhaploids may be of another origin, such as hybridisation or somatic mutation. Despite possible differences in ploidy level/chromosome number between the products of either hybridisation or somatic mutation and their maternal plants, such progenies can never have half of the chromosome set of their maternal parent. When the fertility of the pollen (measured in terms of its stainability) of experimental polyhaploids was checked it was found to be very variable (R. Rosenbaumová, unpublished results). In particular, pollen stainability ranged from 0.40–63% for the trihaploids produced by the hexaploid *P. rubra*. The highest stainability was of the same magnitude as that recorded for some hybridogenous species of *Pilosella* sampled in the field (Rotreklová 2008; A. Krahulcová et al., unpublished results). Consequently, a low incidence of stained pollen grains (suggesting low pollen fertility) might serve as an indication of polyhaploid origin, whereas a medium incidence of stained pollen grains (estimated as medium pollen fertility) does not exclude another origin of the plant.

Variation in the isozyme phenotypes of trihaploids

Isozyme phenotypes (AAT2) of the trihaploid progeny produced by the hexaploid *P. rubra* strongly differed from those of the maternal plant: whereas the maternal plant had two clearly detectable bands, the trihaploid progeny had one or three bands (Fig. 2A, B). This is surprising, but due to AAT2 being a dimeric enzyme forming two homodimers and a heterodimer. Because the maternal *P. rubra* is hexaploid, it probably has an uneven number of alleles determining the formation of two enzyme components. For that reason only one homodimer and heterodimer was clearly detectable in the hexaploid plant and the other homodimer was not formed in detectable or visible amounts. In the trihaploid prog-

eny the proportion of both alleles was more balanced, or one of them was absent. For that reason the trihaploids had either one homodimer or both homodimers and a heterodimer. Comparison of the phenotypes of all the trihaploids analysed revealed a stable and strongly stained band (arrowed in Fig. 2A, B) corresponding to a particular homodimer. This corresponds with a ratio of alleles of 5:1 in hexaploid and 2:1 or 3:0 in trihaploids. Consequently, in this way it is possible to explain why the polyhaploid progeny have more bands than the mother plant. Again, the identical AAT isozyme phenotypes found in the trihaploid and in its descendants, which originated via chromosome doubling (Fig. 2C), can be explained by the same proportions of alleles determining the formation of the homodimer and the heterodimer, respectively (see above). Although the genome of the trihaploid was doubled, the proportion of the alleles in the resulting hexaploid remained unchanged. In the literature, however, there are no similar cases recorded. Even when enzymes are dimeric, only two clearly detectable bands are referred to.

Probability of the spontaneous rise of polyhaploids in the field

There are great differences in the rates at which polyhaploids appear in natural populations of *Pilosella*. Most of the data (Table 1) refers to the frequency of polyhaploid embryos in seeds, i.e. the embryonic stage of development of progeny. The development of mature polyhaploid plants depends on, in addition to the frequency of polyhaploid seed production, the conditions at the locality, namely whether the conditions are suitable for seed germination, early growth and establishment of the seedlings (Krahulec et al. 2006). Such conditions usually occur at localities where the ground is disturbed and vegetation canopy open. In an attempt to maximize the number of polyhaploid plants surviving in the garden experiments the conditions for both seed germination and seedling growth were optimized. Data based on these experiments (summarized in Fig. 1) indicate that the percentage survival of polyhaploid seedlings ranged from percents (in dihaploids and trihaploids) to tens of percent (in tetrahaploids). Thus, at least in this experiment, the production of polyhaploid progeny increased with increasing ploidy level of the maternal parent (Fig. 1). This finding might be consistent with optimal and stable ploidy levels in *Pilosella* that are most frequent in natural populations. Namely, tetraploids and pentaploids are the most common cytotypes in the field (Fehrer et al. 2007).

Polyploid–polyhaploid–polyploid cycle

Cyclic changes in ploidy level in consecutive generations of progeny are described in a few genera of angiosperms. However, such changes in ploidy level are combined with changes in the mode of reproduction. An example of this is the *Bothriochloa-Dichanthium* complex in the *Poaceae* (De Wet 1968, Asker 1979). Here, the tetraploid facultative apomicts predominate in natural populations in addition to rare sexual diploids. The genome doubling via fusion of two unreduced gametes sometimes occurs in diploids, giving rise to sexual autotetraploids. This step facilitates subsequent gene exchange at the tetraploid level. The apomictic tetraploids, which arise from such inter-tetraploid crosses between sexuals and apomicts (the latter hybridize as pollen parents), give rise to new sexual diploids via haploid parthenogenesis. Finally, these sexual dihaploids can again undergo genome doubling. A similar cycle, in another agamic complex in the *Poaceae*, was detected in crossing experiments involving *Panicum maximum* (Savidan & Pernés 1982). The authors assume that such

diploid–tetraploid–dihaploid cycles facilitate gene exchange between diploid sexual and tetraploid apomictic populations of *Panicum maximum* in the field (Savidan & Pernés 1982). Another type of the diploid–tetraploid–diploid cycle occurs in *Potentilla argentea*, where the diploids are facultatively apomictic (Asker 1979). Their chromosome doubling (via unreduced gametes) generates sexual tetraploids. Subsequent backcrosses with diploids result again in apomictic diploids, through the bridge comprised of sexual triploids and aneuploids. This type of cycle does not involve haploid parthenogenesis.

Unlike the cycle presented here for *Pilosella*, both of the cycles reported in *Bothriochloa-Dichanthium* complex and in *Potentilla argentea* are evidently not autonomous, because the sexual biotypes require fertilization. In both the cyclic processes mentioned above, the genome doubling is ensured by fertilization of unreduced gametes. In contrast, in *Pilosella*, the complete process of genome "haploidisation" and subsequent doubling is independent of gametes from a second parent. Thus, the genome doubling in apomictic polyhaploids of *Pilosella* might occur at either the prezygotic stage of development via fusion of two unreduced female gametes (possibly evoking automixis), or the postzygotic stage via somatic chromosome doubling in the proembryo. The latter possibility might also account for the origin of rare octoploids that arise among the progeny of open pollinated tetraploid facultatively apomictic *P. aurantiaca* (Krahulcová & Krahulec 2000). It is relevant to mention a few additional rare cases of autonomous genome doubling that occurred in progeny of apomictic maternal plants, which was recorded during the experiments on *Pilosella*: for example, another in *P. aurantiaca* and the pentaploid apomictic *P. officinarum*. In these cases, however, the genome doubling in progeny was not preceded by haploid parthenogenesis. The potential for haploid parthenogenesis and genome doubling, respectively, may be associated with genetic determinants of apomixis in *Pilosella* (R. Rosenbaumová et al., unpublished results).

The probability of the complete two-step process occurring in *Pilosella* (Fig. 1) is a product of both probabilitities, i.e., of a polyhaploid occurring and of the consequent doubling of chromosome set. Using the experimental data for the hexaploid–trihaploid–hexaploid cycle in *P. rubra* (Fig. 1), the probability is relatively low, in the order 10^{-4}. *Pilosella rubra* forms clones with many stems, each of which has several capitula, producing several hundreds of seeds, so that the whole population can certainly produce at least hundreds of thousands of seeds per year. What is unknown, are the probabilities of seed germination and seedling establishment (see above). However, under conditions of low competition, as on road margins, slopes with landslides etc., the probability of establishment of seedlings that arise from both steps could be greater than in closed grassland. Hence, many polyploid species and hybrids of *Pilosella* are common in disturbed habitats with little competition from surrounding vegetation.

Consequences of the existence of plants that mainly produce polyhaploids

Plants that produce mainly polyhaploids make studies on breeding systems and testing for apomixis difficult. *Pilosella* plants that produce seeds after emasculation (decapitation of the capitula before anthesis) are considered to be apomictic. However, if haploid parthenogenesis is excluded from the definition of apomixis, these plants cannot be considered as apomictic. Hence, the routinely used procedure of decapitation tests for parthenogenesis not apomixis,which replicates the maternal genome.

The second consequence that has already been mentioned is that the determination of chromosome number based on the chromosome numbers of the progeny is problematic, even of facultatively apomictic plants. As in some cases (Tables 1, 2) most of the progeny seeds may have half of the chromosome set of the mother plant, care needs to be exercized when estimating chromosome numbers based on chromosome counts in germinating seeds or seedlings.

General discussion

Although haploid parthenogenesis is mostly considered to be an important phenomenon in the production of pure lines in different crops, data on its importance in the field are rare. This study indicates that this process might contribute to the generation of variation within apomictic species. There is a need to study its importance in the field. The most limiting factor in such research is likely to be the absence of an unequivocal test of the polyhaploid origin of suspected plants.

Theoretically, the production of homozygous progeny with a doubled half of the original maternal chromosome set could occur in one step. Such a process, without involvement of a polyhaploid plant, would involve the doubling of chromosomes during the early development of an embryo generated by haploid parthenogenesis. However, such progeny would be difficult to distinguish from those that arise from selfing. Although the genus *Pilosella* is usually considered to be allogamic and self-incompatible (Gadella 1984, 1987) selfing is possible when pollinated by foreign pollen (mentor effect; Krahulcová et al. 1999).

A possible pathway that results in the generation of the variation in apomicts without sex, namely without the fusion of gametes, is suggested. As far as is known, the process described for *Pilosella*, comprising haploid parthenogenesis in a polyploid parent followed by autonomous chromosome doubling, has not previously been considered as a possible source of variation in wild apomictic plants. This process corresponds to that used in crop breeding to obtain pure homozygous lines used for F_1 production. As one half of the genome of the original polyploid mother is lost during meiosis in the course of megasporogenesis, the new F_2 progeny (generated by F_1 polyhaploids via genome doubling) must differ from the original maternal parent, namely they have a higher level of homozygosity. The cycle of diploid–tetraploid–dihaploid is reported in some other groups of angiosperms (see one of the previous subheads). What is described here is the cycle polyploid–polyhaploid–polyploid. In this process a new, more homozygous strain of polyploid is formed without the fusion of gametes. Unlike previously described cycles in the *Bothriochloa–Dichanthium* complex, *Panicum maximum* and in *Potentilla*, the process in *Pilosella* is completely autonomous and independent of a fertilizing partner. This possibility should be considered in theoretical discussions on the origin of variation within apomictic complexes, and, in general within asexual plants.

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Souhrn

V rodu *Pilosella* produkují fakultativně apomiktické druhy procesem haploidní partenogeneze potomstvo s polovičním počtem chromozomů, a to jak v přírodě, tak v experimentálních podmínkách. V množství tohoto potomstva jsou velké rozdíly mezi jednotlivými základními a hybridogenními druhy i recentními hybridy. U rostlin sbíraných v terénu (14 lokalit) přesáhla nejvyšší frekvence polyhaploidních embryí 80% z celkové produkce nažek; šlo o recentní hybridy. Podobná úroveň produkce polyhaploidního potomstva byla zjištěna i v experimentech. V této práci popisujeme dvoustupňový proces, ve kterém *P. aurantiaca* (tetraploidní) a *P. rubra* (hexaploidní) produkovaly v zahradě zcela nové genotypy během cyklu polyploid–polyhaploid–polyploid. Tento proces zahrnuje pouze jednu část sexuálního procesu, meiozu; nezahrnuje fúzi gamet. Nové genotypy jsou tedy produkovány bez účasti otcovského pylu. Během prvního kroku mateřská rostlina produkovala dihaploidy (u *P. aurantiaca*) nebo trihaploidy (*P. rubra*). Velká většina z tohoto potomstva měla sníženou vitalitu i fertilitu. Apomiktické polyhaploidy produkovaly vzácně rostliny s dvojnásobnou ploidií, která tak byla identická s původní mateřskou rostlinou. Celý tento proces je autonomní. Polyhaploidní genotyp byl ale jen výsekem genetického materiálu mateřské rostliny, a proto nově produkované rostliny se zdvojeným polyhaploidním genomem mají vyšší úroveň homozygozity ve srovnání s původní mateřskou rostlinou. Odhadli jsme pravděpodobnost tohoto dvoustupňového procesu jako součinu pravděpodobností jednotlivých kroků. V případě cyklu hexaploid–trihaploid–hexaploid byla řádově 10^{-4}. V přírodě má tento proces evidentně ještě nižší pravděpodobnost vlivem kompetice a stanovištních podmínek. Zjištění této pravděpodobnosti v přírodě je komplikováno skutečností, že není k dispozici metoda vedoucí k jednoznačnému stanovení, že nalezená rostlina je polyhaploid. Pravděpodobnost cyklu polyploid-polyhaploid-polyploid je ovšem zvýšena velkým množstvím opakování; to je dané jak množstvím apomiktických rostlin určitého genotypu, tak i množstvím jimi produkovaných nažek.

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