

## Reproduction mode in the allopolyploid facultatively apomictic hawkweed *Hieracium rubrum* (Asteraceae, *H.* subgen. *Pilosella*)

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The versatility of the breeding system in the hybridogenous hexaploid, *Hieracium rubrum*, was demonstrated in emasculatation and crossing experiments. The flow-cytometric ploidy analysis of 1095 seedlings amongst its' progeny enabled the determination and quantification of the reproductive pathway (apospory, haploid parthenogenesis, cross-fertilization of both reduced and unreduced female gametes) responsible for each progeny class. The progeny profiles were stable between two successive years. The percentage of hybrids arisen from crosses with tetraploid sexual *H. pilosella* was 7.9%. The rate of trihaploids generated by *H. rubrum* when crossed to *H. pilosella* was 3.95%, similar to that of pentaploid ( $n+n$ ) and octoploid ( $2n+n$ ) hybrids (3.95% and 3.39%). Unreduced pollen from *H. pilosella* contributed to hybridization much less frequently (0.56% of all progeny), than unreduced eggs of *H. rubrum*. The increased frequency of trihaploids formed by crossed compared to emasculated plants suggests an interaction between the presence of pollen and the autonomous development of meiotic embryo sacs. Although the environmental conditions (garden compared to unheated glasshouse) influenced the progeny following the emasculatation, no corresponding response to environment was detected amongst the autonomously derived progeny following pollination with *H. pilosella*. However, there was an influence of pollen parent on progeny which varied under the different environmental treatments. The importance of residual sexuality in reproduction of apomicts is evident. The non-maternal progeny was generated with highly variable frequency especially in the glasshouse, with some capitula reaching up to 50%. Having an autonomous endosperm development, *Hieracium* subgen. *Pilosella* is almost unique among other aposporous genera, in which such variable reproduction mode is connected with pseudogamy.

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*Hieracium* subgen. *Pilosella* (Hill) S.F. Gray has an extremely diverse breeding system. Besides clonal growth, both sexual reproduction and aposporous apomixis are present (KRAHULCOVÁ et al. 2000; BICKNELL et al. 2003). Although the sexuals are predominantly allogamous (GADELLA 1984), autogamy is stimulated under the influence of foreign pollen (KRAHULCOVÁ et al. 1999). Facultatively apomictic polyploids, acting as seed parents, can generate hybrids as well as an autonomously derived progeny via both somatic and haploid parthenogenesis (SKALIŇSKA 1971). Moreover, the apomicts are capable as pollen donors (GADELLA 1987; KRAHULCOVÁ and KRAHULEC 2000). The crosses involving the fusion of unreduced gametes,  $2n+n$  or  $2n+2n$ , respectively, give rise to “addition hybrids” (SKALIŇSKA 1976; GADELLA 1988; CHAPMAN and BICKNELL 2000; BICKNELL et al. 2003), i.e. B<sub>III</sub> hybrids in the sense of RUTISHAUSER (1967). In nature, the fertilization of unreduced egg cells by reduced sperm cells seems to be more common than vice versa, especially with facultative apomicts as seed parents (unpubl.).

The initial formation of a meiotic (reduced) embryo sac in an ovule, usually suppressed by the rapid development of ameiotic (unreduced) female gametophyte, is characteristic of apospory (KOLTUNOW 1993; KOLTUNOW et al. 1998), although it is possible for both sexual and aposporous embryo sacs to coexist within one ovule. *Hieracium* subgen. *Pilosella* species produce endosperm autonomously, which is rather exceptional among aposporous apomicts, as most are pseudogamous, requiring pollination for endosperm production (RICHARDS 1997). The dominant genetic control of aposporous apomixis has been demonstrated in several plant genera (KOLTUNOW et al. 2000), including *Hieracium* subgen. *Pilosella* (GADELLA 1987; BICKNELL et al. 2000). The introduction of molecular developmental markers into sexual and apomictic plants of *Hieracium* subgen. *Pilosella* showed that sexual and apomictic pathways are closely interrelated, sharing regulatory programs (TUCKER et al. 2003). In general, the degree of apomixis, i.e. the expression of residual sexuality, can be influenced by the dosage of an aposporous gene, as in the polyploid *Ranunculus auricomus* (NOGLER

1984). It can also be influenced by the pollen donor or environmental factors, e.g. photoperiod and temperature (ASKER and JERLING 1992; KOLTUNOW 1993; RICHARDS 1997). The environmental control of the timing of development of the sexual and aposporous embryo sacs within an ovule is likely to be critical for the mechanism of environmental control. Conditions of high temperature, low humidity and intense insolation are suggested to increase the frequency of embryos derived from sexual embryo sacs in facultatively aposporous *Hieracium pilosella* (TURESSON 1972).

BICKNELL et al. (2003) quantified the proportions of particular progeny classes formed by two facultatively apomictic *Hieracium* species, *H. aurantiacum* and *H. piloselloides*, by screening seedling groups to determine their origins. The isolation of non-maternal individuals was based on the segregation and inheritance of introduced marker genes. The progeny class frequencies were estimated using a set of equations corresponding to particular pollination treatments. To study the reproduction in *H. rubrum*, we chose a different, less complicated method, which enabled an unambiguous detection of particular reproduction pathways operating in the polyploid facultatively apomictic mother plants. The reproductive origins, reflected in ploidy levels of the progeny, were sorted in this respect by flow cytometry. This approach was facilitated by an appropriate choice of ploidy for the pollen plants used in crosses. Another method proposed for detection of reproductive origins of progeny (MATZK et al. 2000), is based on flow-cytometric assessment of the embryo/endosperm ploidy ratio in seeds. To assess and quantify the progeny classes in *H. rubrum*, we performed the analysis of seeds as well. The comparison of the two methods, which can detect the possible influence of progeny selection at the stage of germination and establishment of seedlings, will be published separately.

The following studies aimed i) to detect the capacity for diverse reproduction modes in *H. rubrum* in intentional crosses with defined pollen parents; ii) to estimate the contributions of particular reproductive pathways, which are reflected in particular cytotypes within the progeny ( $n+n$  hybrids,  $2n+n$  hybrids,  $n+2n$  hybrids, trihaploids, hexaploids originating from apospory). To determine if there is a difference in the frequency of particular reproduction modes iii) between different maternal plants and pollen donors, iv) between two years of experiments and v) under different environmental conditions.

## MATERIAL AND METHODS

### *Plant material*

All plants used in experiments (Table 1) originated from the Krkonoše Mts (the Sudeten Mts), Czech Republic. Three accessions of *H. rubrum* Peter collected at different localities were studied. The isozyme analysis of 6 loci (PGDH-1, PGDH-2, EST, SOD, LAP, AAT) identified all three accessions as one isozyme phenotype. Moreover, no significant differences were found among the progeny profiles from the three accessions in emasculation and crossing experiments in the year 2000. Consequently, in most cases, the accessions of *H. rubrum* were evaluated as one clone, i.e. the results of experiments were summed together and considered to represent one entity. The sympatric occurrence of the putative parental species of *H. rubrum* (*H. aurantiacum* L. and *H. pilosella* L., both tetraploid), is not rare in the Krkonoše Mts. The analysis of cp-DNA haplotypes indicated that the former species was the seed parent of the *H. rubrum* accession investigated, while the latter was most likely the pollen parent (unpubl.). Two sexual species, the tetraploid *H. pilosella* and the diploid *H. lactucella* Wallr., were chosen as pollen parents in experimental crosses with *H. rubrum*, as they are potential crossing partners of this species in nature. The isozyme analysis assigned the pollen plants to one isozyme phenotype for *H. lactucella*, and to two for *H. pilosella* (Table 1).

The origin of *H. rubrum* is assumed to be via a spontaneous hybridization between *H. aurantiacum* and *H. pilosella* (or, especially in the Alps, *H. hoppeanum* Schultes and *H. aurantiacum*). The morphological characters of *H. rubrum* more resemble *H. aurantiacum* than either of the other putative parents (PETER 1881; NÄGELI and PETER 1885; BRÄUTIGAM and SCHUHWERK 2002). A third parental species, *H. caespitosum* Dumort., is assumed by some authors to contribute to the genome of *H. rubrum* (ZAHN 1930; SELL and WEST 1976). As a stabilized hybridogenous species, *H. rubrum* is hexaploid ( $2n = 54$ ), and facultatively apomictic (SCHUHWERK and LIPPERT 1997, 2002; KRAHULCOVÁ and KRAHULEC 1999). It had been described by PETER (1881) only from the Krkonoše Mts, from where the plants used in our experiments originated. However, *H. rubrum* is distributed throughout the mountain grasslands of Central Europe.

The different colour of the inflorescences (red/orange in *H. rubrum*, yellow in both pollen parents), pubescence, shape and colour of leaves, and, above all, the different ploidy levels of parental species, enabled the detection of hybrids amongst the progeny from the

Table 1. Design of emasculation and crossing experiments carried out on *H. rubrum* (maternal species) in the years 2000 (white) and 2001 (coloured). Symbols of species: RU = *H. rubrum*, PI = *H. pilosella*. Asterisks indicate the appropriateness to different isozyme phenotypes.

Maternal species	Accession	Experimental treatment						
		Garden			Glasshouse			
		Emasculation	Cross		Emasculation	Cross		
			$\times H. lactucella$	$\times H. pilosella$		$\times H. pilosella$	$\times H. pilosella$	
				69/72PI*	120PI*		69/72PI*	120PI*
<i>H. rubrum</i>	8/3RU	2000	2000		2000			
	11RU	2000	2000		2000	2001	2001	2001
		2001		2001	2001			
	97RU	2000	2000		2000	2001	2001	2001
		2001		2001	2001			

putative crosses. The design of the experiments facilitated an unambiguous determination of reproductive pathways by which the particular cytotypes in the progeny were generated. The stainability of fixed pollen grains with cotton blue-lactophenol was used for a rough estimation of pollen viability in pollen plants (Table 3).

#### Emasculation and crossing experiments

The emasculation and crossing experiments were carried out during two years (2000 and 2001) in the experimental garden. This involved the emasculation and isolation of capitula of maternal plants of *H. rubrum* before anthesis, and the pollination of fully opened capitula of *H. rubrum* with *H. pilosella* and *H. lactucella*. The emasculation of capitula, to ensure only autonomously derived progeny were produced, was carried out by removing the upper part with a razor blade (GADELLA 1984; RICHARDS 1997). All inflorescences of each parent were isolated in nylon bags before anthesis (prior to the outer ligular flowers of the capitulum opening). The capitula were crossed in the stage of stigma receptivity (when bifurcate stigmas protruded from the flowers), by rubbing the whole parental capitula together. As the flowers in the capitulum open successively from the margins to the centre, the maternal capitulum was usually pollinated 2 to 3 times a week, on each occasion with a new capitulum of the same pollen plant. The ripe achenes were checked under a stereomicroscope, using an adequate pressure of tweezers for detection of well developed/aborted achenes. Consequently, the percentage of developed achenes obtained from emasculated versus crossed capitula was compared. Due to the greater efficiency of pollinations with *H. pilosella* compared to *H. lactucella*, accessions of *H. pilosella*

were used as the sole pollen donors in the second season (see below). Only two accessions of *H. rubrum* (11RU and 97RU), comprising one isozyme phenotype, were selected for experiments in 2001 (Table 1).

Different experimental designs were chosen for each of the two years, to reveal the variability in progeny composition at different levels: i) with respect to particular accessions of the maternal parent and to particular pollinating species as a whole (the year 2000), and ii) with respect to individual capitula of the two maternal accessions treated separately, including their pollination with two different clones of *H. pilosella* (the year 2001). In 2000, we compared the crosses of *H. rubrum* with *H. pilosella* and *H. lactucella* under garden conditions. In 2001, the results of both the emasculation of *H. rubrum* and its hybridization with *H. pilosella* under two environmental conditions (garden, an unheated glasshouse) were compared (Table 1). The two environments differed especially in the temperature range during 24 h, in the light intensity and quality and in air humidity.

#### Design of sowing and cultivation of progeny

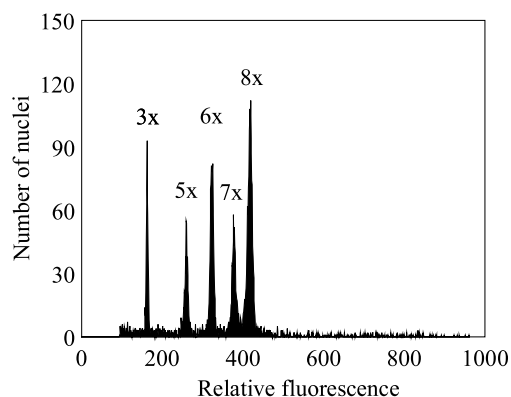
Well developed ripe achenes obtained from emasculated and crossed maternal plants were sown in the glasshouse during the summer of harvest. Achenes were sown into pots or boxes with sterilized garden soil. In 2000, nine groups of seeds were sown separately, representing the three accessions of seed parent (*H. rubrum*) and the three treatments (Table 1). Each group of seeds was prepared as a mixture of achenes harvested from seven maternal capitula, which underwent the emasculation or the pollination in the space of one month. A total of 200 seeds chosen from each mixture were sown for each of the

particular groups. The achenes obtained from 70 individual capitula were sown separately in 2001. Their progeny were analysed separately, and then summed into 12 groups, according to the accession of the maternal plant, type of experimental treatment, accession of the pollen parent, and environment (Table 1). The seedlings were cultivated during winter in a heated glasshouse, each plant being kept in a separate pot. When the rosettes were formed, parts of fresh leaves were removed for estimation of ploidy level, to determine the origin of each individual plant in the progeny.

#### Determination of ploidy level by flow cytometry

Two procedures were employed: i) approximately 20 mg ( $0.5 \text{ cm}^2$ ) of the fresh leaf tissue was chopped together with the same amount of leaf from the hexaploid maternal plant (internal standard) in a petri dish containing ca 0.5 ml ice-cold extraction solution (Partec DNA kit type P, No. 06-5-4004, supplemented by  $2 \mu\text{l ml}^{-1}$  mercaptoethanol). After 1–2 min incubation, the suspension was filtered through a nylon mesh ( $42 \mu\text{m}$ ), and 2 ml of staining solution with DAPI was added (staining solution of Partec DNA kit type P). ii) approximately one  $\text{cm}^2$  of the leaves of both the sample and the internal standard were chopped together in one ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) (OTTO 1990). The filtration through  $42 \mu\text{m}$  nylon mesh was followed by centrifugation at 150 g for 5 min. The supernatant was removed and 100  $\mu\text{l}$  of fresh Otto I buffer, added. Samples were resuspended and stored for 30 min at room temperature for incubation. Then, one ml of Otto II buffer (0.4 M  $\text{Na}_2\text{HPO}_4 \times 12 \text{H}_2\text{O}$ ) with the fluorochrome (DAPI in concentration  $4 \mu\text{g ml}^{-1}$ ) and  $2 \mu\text{l ml}^{-1}$  mercaptoethanol (as an antioxidant) were added.

Finally, in both procedures, the relative fluorescence of isolated nuclei was analysed after 1 min of staining using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany). The maternal hexaploid plants, which chromosomes have been counted ( $2n = 54$ ), served as internal standards in each analysis. The cytometer was adjusted to have the position of the peak of the internal standard between channels 350 and 400. Five thousand nuclei were analysed in each sample. The first, simpler procedure using Partec kits, was employed for screening of the progeny obtained in the year 2000. Only measurements with coefficients of variance (CV) less than 5% were taken into account, which was sufficient to distinguish the smallest difference between the ploidy levels expected among the progeny. The two-step procedure used in the year 2001 was more precise, and only CVs less than 2.5%



**Fig. 1.** Histogram of relative DNA content of DAPI stained nuclei from triploid, pentaploid, hexaploid, heptaploid and octoploid progeny of *Hieracium rubrum*. The nuclei of all five cytotypes were analysed simultaneously (the sample for flow cytometry was comprised of five pieces of leaves, each from a different individual). A total of 5000 nuclei were analysed. The coefficients of variance for the individual peaks ranged from 1.30% to 1.75%.

were taken into account (Fig. 1.). At first, the samples comprised of four seedlings with unknown ploidy, to which a hexaploid internal standard was added. This procedure allowed us to distinguish a particular individual with different ploidy within the analysed group of five leaf pieces. Only the non-homogeneous samples, which displayed more than one ploidy level, were analysed separately for each individual together with a hexaploid internal standard.

#### Statistical analysis

Chi-square tests were carried out to test the following hypotheses: i) the three accessions of *H. rubrum* differ in production of the particular types of progeny; ii) the frequency of particular types of hybrids produced by *H. rubrum* is different using different pollen donors; iii) the maternal species produce the particular types of progeny with different frequencies between years; iv) and the proportion of particular types of progeny is different under different environmental conditions (glasshouse and garden conditions). The significance level for hypothesis rejection was 5%. The composition of progeny was evaluated with respect to four individual categories (ploidy levels) corresponding to their different origin (Table 2).

## RESULTS

#### Variation in progeny obtained in two successive years of experiments

A total of 609 individuals were analysed for ploidy in 2000. The plants, which originated as hybrids or autonomously derived progeny of *H. rubrum*, dis-

Table 2. The four groups of cytotypes (ploidy levels) in the progeny of a hexaploid *H. rubrum*, evaluated separately with respect to their origin in emasculation and crossing experiments. An exceptional fusion of *n* egg cell and *2n* sperm cell is marked by an asterisk, in other cases only *n* sperm cells fertilized the female gametes of maternal plants.

Origin of progeny	Experimental treatment of maternal species (6x)		
	Emasculation	Cross	
		× <i>H. lactucella</i> (2x)	× <i>H. pilosella</i> (4x)
Autonomous			
Haploid parthenogenesis	3x	3x	3x
Somatic parthenogenesis	6x	6x	6x
Hybrids			
<i>n</i> gametes of maternal plant (3x)		not recorded	//5x, 7x*/
<i>2n</i> gametes of maternal plant (6x)		7x	8x

played five of the seven possible ploidy levels (Table 2 and 4). Neither the tetraploids, which could have arisen from fusion of *n*+*n* gametes of hexaploid *H. rubrum* and diploid *H. lactucella*, nor the decaploids, as potential hybrids between hexaploid *H. rubrum* and tetraploid *H. pilosella* (*2n*+*2n* gametes), were recorded. The triploids, generated by haploid parthenogenesis, occurred among the progeny of emasculated, as well as of the crossed maternal plants. A comparison of the seed-set between the emasculated and crossed capitula found that the percentage of developed achenes was significantly higher in the emasculated capitula ( $\chi^2 = 20.81 > \chi^2_{df=1} (0.005) = 7.9$ ). This suggests no damage of female reproductive tissues caused by emasculation, which could have influenced the experiments.

The seeds from 70 capitula of *H. rubrum* were sown separately in 2001, 62 of which gave rise to progeny available for FCM analysis. The remaining 8 capitula provided either seeds which did not germinate at all, or the seedlings grew poorly and perished at an early developmental stage. Among the total of 42 capitula crossed, half of them gave rise to hexaploid progeny only (derived autonomously by apospory). The

remaining 21 capitula produced a variable progeny, i.e. trihaploids and/or hybrids in addition to the hexaploids. Conversely, 17 of the 20 emasculated capitula produced a homogeneous hexaploid progeny, while both hexaploids and trihaploids were detected amongst the progeny of three emasculated capitula. Contrary to the previous year, no trihaploids were recorded amongst the progeny of emasculated *H. rubrum* growing under garden conditions (Table 4 and 5). A considerable number of capitula (26) had in their progeny only five or less individuals available for FCM analysis. A total of 486 seedlings were analysed for ploidy in 2001, within which the same five progeny classes were detected as in the previous year (Table 5).

#### The differences among maternal plants

The three accessions of *H. rubrum* (97RU, 11RU, 8/3RU) showed no difference in the proportion of hexaploids and trihaploids in their progeny following emasculation in 2000. Similarly, the frequencies of the four cytotypes in the progeny did not differ among the three accessions of *H. rubrum* when pollinated with *H. pilosella* or *H. lactucella* (Table 2). Nevertheless,

Table 3. Estimation of pollen viability (staining in cotton blue-lactophenol) in two pollinating species and their success in hybridization with *H. rubrum*. The pollen of three capitula was evaluated per accession (200 pollen grains from each capitulum). Asterisks indicate the isozyme phenotypes distinguished (one in *H. lactucella*, two in *H. pilosella*).

Pollinating species	Accession	Percentage of stainable pollen grains		Number of individuals in the progeny of <i>H. rubrum</i> pollinated in the garden (2000)		
		Garden	Glasshouse	Autonomous origin	Hybrids	Total
* <i>H. lactucella</i>	40 LA	96.7		143	1	144
	45 LA	97.5				
<i>H. pilosella</i>	*69/72 PI	75.5	89.2	204	12	216
	*120 PI	95.8	95.8			

some differences were found between two of the accessions (11RU and 97RU) of the maternal species, which displayed different tendencies in the formation of hybrids with *H. pilosella* (Table 4). While accession 11RU gave rise to octoploid  $2n+n$  hybrids only, indicating the fertilization of unreduced egg cells, the majority of hybrids formed with accession 97RU were from reduced eggs. Moreover, accession 97RU, whether emasculated or pollinated with *H. pilosella*, formed trihaploids more frequently than 11RU (Table 4), again indicating that meiotic embryo sacs participate more frequently in the formation of progeny in this accession. However, these differences in function of reduced versus unreduced egg cells between the two accessions of *H. rubrum* were not found to be statistically significant, regardless of whether crossed and emasculated maternal plants were evaluated together, or if only emasculated plants were considered.

#### *The influence of pollen parent on progeny*

No difference was found in the composition of progeny obtained from the three accessions of *H. rubrum* crossed in 2000, when the accessions were evaluated separately for each of the two pollinating species. The maternal species did hybridize more readily with *H. pilosella* than *H. lactucella*, although both pollen parents had high rates of pollen viability, comparable to each other (Table 3). Following pollination of *H. rubrum* in 2000, the profiles of both autonomously derived progeny (hexaploids and triploids) and hybrids combined, were found to be significantly different for each of the two pollinating species, *H. pilosella* and *H. lactucella* ( $\chi^2 = 5.866 > \chi^2_{df=1} (0.025) = 5.0$ ). Accession 11RU of *H. rubrum* formed its' hybrid progeny exclusively from unreduced egg cells in the year 2000 (Table 4). Its' tendency to generate  $2n+n$  hybrids continued in 2001, but only when pollinated with one of the two clones of *H. pilosella* (Table 5). Under glasshouse conditions in 2001, a significant difference was detected in the reproductive mode of *H. rubrum* following pollination with two different clones of *H. pilosella* ( $\chi^2 = 10.739 > \chi^2_{df=3} (0.025) = 9.4$ ). However, no corresponding influence of the clone of the pollen parent was found in the garden crosses.

#### *Comparison of progeny between years and between different environments*

Comparing the crosses between *H. rubrum* and *H. pilosella*, there was no difference in the progeny profiles between the two years sampled. The rate of trihaploid progeny formation following emasculatation under garden conditions was also stable over the two

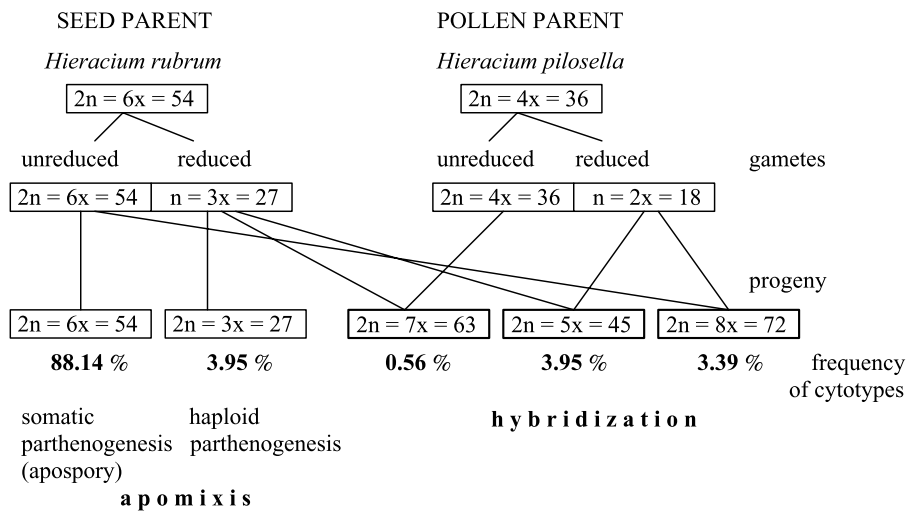
years. Although the garden versus glasshouse conditions influenced the rates of hexaploids and trihaploids in the progeny of emasculated *H. rubrum* ( $\chi^2 = 4.012 > \chi^2_{df=1} (0.05) = 3.841$ ), no significant influence of environment on the progeny of plants crossed in the garden compared with those in the glasshouse was detected.

A single capitulum of accession 97RU was found to produce an extreme range of variation in cytotype, following pollination with *H. pilosella* clone 120PI. 31 seedlings derived from this capitulum were analysed in total, 16 of which displayed a ploidy level different to the hexaploid maternal plant (3 trihaploids, 10 pentaploid  $n+n$  hybrids, 1 heptaploid  $n+2n$  hybrid and 2 octoploid  $2n+n$  hybrids). This suggests the successful functioning of reduced and unreduced egg cells, especially in hybridization, which may lead under some conditions to 50% frequency of non-maternal progeny within an individual capitulum. However, the ratio of capitula which generated the trihaploid and/or hybrid progeny as opposed to capitula with uniform hexaploid progeny was not significantly higher in the glasshouse (ratio 12:10) than in the garden (ratio 6:8). Only those capitula from which six or more individuals from the progeny were analysed by FCM were included in this comparison.

#### *Frequency of particular reproduction pathways*

Due to the finding that the production of the different progeny classes was stable between years (see above), the following results are based on data summarized for the two years of experiments. The calculation of the rates of the five ploidy levels in the progeny, which correspond to particular reproduction pathways in *H. rubrum*, was from the results of crosses with *H. pilosella* as the pollen donor (Fig. 2). While the rates of formation of trihaploid, pentaploid and octoploid hybrids are comparable (approximately in the order of single percentages), the rate of heptaploid formation is several times lower (approximately tenths of a percent). This suggests that the unreduced egg cells of a facultatively apomictic seed parent more frequently give rise to hybrids than the unreduced sperm cells of a sexual pollen parent.

The percentage of trihaploid progeny produced by *H. rubrum* following emasculatation in the garden was 1.6 (5/312 individuals analysed over the two years) (Tables 4 and 5). The remaining 98.4% of progeny were hexaploids, produced via somatic parthenogenesis (apospory). In contrast to this, following pollination with *H. pilosella* under garden conditions, *H. rubrum* produced 4.29% trihaploid progeny (14/326 non-hybrid progeny). This difference was detected as significant ( $\chi^2 = 3.995 > \chi^2_{df=1} (0.05) = 3.842$ ). Thus,



**Fig. 2.** Ploidy variation in the  $F_1$  progeny of hexaploid *Hieracium rubrum* crossed with tetraploid *H. pilosella*. The frequencies of the particular cytotypes (a total of 354 individuals were analysed) are based on the results of crosses under garden conditions for the two years combined, and summed for all three accessions of the seed parent (Tables 4 and 5). The hybrid progeny are denoted by the bold frames.

haploid parthenogenesis might operate more frequently in pollinated, than in emasculated maternal plants. This effect was also significant when the rate of trihaploids formation was compared with the rates of other types of progeny as a whole, including both autonomously derived hexaploids and all hybrids ( $\chi^2 = 3.970 > \chi^2_{df=1} (0.05) = 3.842$ ).

## DISCUSSION

### *Factors influencing the composition of progeny*

The progeny of individual maternal accessions, within the experimental treatments, did not differ with respect to the rate of particular ploidy levels. This suggests a stable frequency of a particular reproduction pathway among the different accessions within one clone (isozyme phenotype) of *H. rubrum*. However, because only a single isozyme phenotype was included, the effect of interclonal variation is unclear. The influence of the male parent on reproduction mode in facultative apomicts is well known, e.g. in *Potentilla* (ASKER and JERLING 1992). In our experiments, the more successful hybridization in crosses of *H. rubrum*  $\times$  *H. pilosella* than of *H. rubrum*  $\times$  *H. lactucella* was evidently due to different compatibilities of the parental genomes involved. This result could be expected because *H. pilosella* is one of parental species of *H. rubrum*, and therefore the hybridization between *H. rubrum* and *H. pilosella* represents a backcross. Moreover, as suggested by the comparison of the rates of trihaploid formation

between the emasculated and pollinated maternal plants, the pollen of the male partner may influence indirectly the process of autonomous development of reduced egg cells, even in the non-pseudogamous *H. rubrum*. It is already known in sexual taxa that haploid parthenogenesis can be induced, not only by colchicine treatment or temperature shocks, but also by particular kinds of pollination, e.g. use of inactivated pollen, wide crosses or delayed pollination (ASKER and JERLING 1992). We demonstrate that this phenomenon also operates in a facultatively apomictic species.

The progeny profiles of *H. rubrum* were found to be stable over the two years of experiments. Similarly, the ratio of aposporous to sexual embryo sacs in a facultatively aposporous panicoid grass, *Brachiaria decumbens*, showed no difference over two vegetation seasons (NAUMOVA et al. 1999). Although different rates of hexaploid and trihaploid formation were detected in the progeny of *H. rubrum* emasculated in the glasshouse compared to those in the garden, the corresponding influence of environment was not found for crossed maternal plants. The choice of environmental factors in such experiments is likely to be critical to provide a demonstrable response of a facultatively apomictic seed parent. For example, the proportion of parthenogenetic embryos was significantly reduced in late autumn compared to early summer in *Brachiaria decumbens*, which was interpreted as an influence of different day lengths (NAUMOVA et al. 1999).

Table 4. Frequency of cytotypes (ploidy levels) in the progeny of three accessions of *H. rubrum* (6x), emasculated and crossed under garden conditions, 2000. Symbols of species: RU = *H. rubrum*, PI = *H. pilosella*, LA = *H. lactucella*. For origin of particular cytotypes see Table 2.

Experimental treatment		Progeny of accessions of <i>H. rubrum</i>					
		11RU		97RU		8/3 RU	
		No.	%	No.	%	No.	%
Emasculatation	Total	63	100	78	100	108	100
	6x	63	100	75	96.2	106	98.2
	3x	0	0	3	3.8	2	1.8
Cross × PI (4x)	Total	54	100	120	100	42	100
	6x	50	92.6	109	90.8	39	92.9
	3x	1	1.8	4	3.3	1	2.4
	5x	0	0	5	4.2	1	2.4
	7x	0	0	1	0.8	0	0
	8x	3	5.6	1	0.8	1	2.4
Cross × LA (2x)	Total	33	100	62	100	49	100
	6x	33	100	58	93.6	46	93.9
	3x	0	0	3	4.8	3	6.1
	7x	0	0	1	1.6	0	0
Σ of individuals	Total	150	100	260	100	199	100
	6x	146	97.3	242	93.1	191	96.0
	3x	1	0.7	10	3.8	6	3.0
	Hybrids	3	2.0	8	3.1	2	1.0
Σ of individuals	Total	609	100				
	6x	579	95.1				
	3x	17	2.8				
	Hybrids	13	2.1				

In addition, the method used for the detection and quantification of progeny classes in facultative apomicts also influences the estimation of frequencies of particular reproduction mode. The frequency of progeny classes detected in a hexaploid *H. rubrum* following the cross with a tetraploid *H. pilosella* (Fig. 2), can be compared with a study of two other apomictic *Hieracium* species (BICKNELL et al. 2003): a hypertriploid *H. aurantiacum* (progeny profiles 96.80% of maternal progeny derived by apospory, 0.84% polyhaploids, 1.75% n+n hybrids, 0.54 2n+n hybrids, 0.05% n+n selfed progeny and 0.02% 2n+n selfed progeny) and a triploid *H. piloselloides* (progeny profiles 97.06% of maternal progeny, 0.94% polyhaploids, 1.91% n+n hybrids, 0.10% 2n+n hybrids, no progeny selfed). In *H. rubrum*, we detected somewhat higher frequencies in each non-maternal progeny class, to the detriment of the progeny generated by apospory. In addition to differences between the genotypes and ploidy levels of parental species used, the two methods

differ in several aspects from each other. The method using the germination of seeds on agar-solidified medium under a stable temperature and daylight regime (BICKNELL et al. 2003) increases the chance of germination and survival of the rare progeny classes (polyhaploids, some types of hybrids), which otherwise may not be detected. In spite of this, there were higher frequencies of non-maternal progeny recorded in our experiments following the germination of seeds in soil and cultivation of seedlings under less optimal conditions. The further advantage of Bicknell's method consists in the detection of progeny arisen from selfing. In our experiments, the hexaploid n+n progeny of *H. rubrum* which could originate in this way, was hidden in the maternal progeny class. Nevertheless, the frequency of selfed progeny is certainly expected to be low (in the order of hundredths of a percent). Conversely, BICKNELL et al. (2003) assumes the stability of progeny profiles regardless of the of pollination treatment. As we

Table 5. Frequency of cytotypes (ploidy levels) in the progeny of *H. rubrum* (6x), emasculated and crossed under both glasshouse and garden conditions, 2001. Symbols of species: RU = *H. rubrum*, PI = *H. pilosella*. The different clones (isozyme phenotypes) of pollen parent are marked by asterisks. For origin of particular cytotypes see Table 2.

Experimental treatment	Progeny of accessions of <i>H. rubrum</i>														
	11 RU						97 RU								
Emasculación garden	No.					No.									
	%					%									
	Total	31					32					100			
6x	31					32					100				
3x	0					0					0				
Emasculación glasshouse	Total	44					54					100			
	6x	42					50					92.6			
	3x	2					4					7.4			
Cross × PI (4x) garden			× 69/72 PI*				× 120 PI*								
			No.			No.									
			%			%									
	Total	41	100	29	100	Σ	70	100	44	100	24	100	Σ	68	100
	6x	36	87.8	21	72.4		57	81.4	34	77.3	23	95.8		57	83.8
	3x	2	4.9	1	3.4		3	4.3	5	11.4	0	0		5	7.4
	5x	0	0	5	17.2		5	7.1	2	4.5	1	4.2		3	4.4
	7x	0	0	0	0		0	0	1	2.3	0	0		1	1.5
	8x	3	7.3	2	6.9		5	7.1	2	4.5	0	0		2	2.9
	Cross × PI (4x) glasshouse	Total	39	100	38	100	77	100	60	100	50	100	110	100	
6x		31	79.5	29	76.3	60	77.9	53	88.3	34	68.0	87	79.1		
3x		6	15.4	1	2.6	7	9.1	2	3.3	3	6.0	5	4.6		
5x		0	0	4	10.5	4	5.2	5	8.3	10	20	15	13.6		
7x		0	0	0	0	0	0	0	0	1	2	1	0.9		
8x		2	5.1	4	10.5	6	7.8	0	0	2	4	2	1.8		
Σ of individuals			Garden		Glasshouse		Garden + glasshouse								
			No.			No.									
			%			%									
	Total	201	100	285	100	486	100								
	6x	177	88.1	239	83.9	416	85.6								
3x	8	4.0	18	6.3	26	5.3									
Hybrids	16	8.0	28	9.8	44	9-Jan									

have demonstrated above, pollination compared to emasculation increases the frequency of polyhaploid progeny, contradicting this assumption.

*The frequency of unreduced gametes participating in crosses*

Fusion of unreduced gametes ( $2n+n$ ,  $n+2n$  or  $2n+2n$ ), is considered to be a common cause of hybrid polyploids among angiosperms (HARLAN and DE WET 1975). GADELLA (1988) recorded such hybrids (1.26% of all individuals) amongst the progeny of crosses between nonhybrid species of *Hieracium* subgen. *Pilosella*, in which both unreduced pollen and/or eggs contributed. The ratio of unreduced fertilized egg cells to fertilizing unreduced sperm cells was 2.2. Gadellas' choice of parental types contrasts somewhat with our experiments, as most maternal plants in his crosses were tetraploid sexuals, and most pollen donors highly polyploid apomicts. The frequency of  $2n+n$  hybrids in our crosses (3.95%), is approximately three times higher than Gadellas'. This confirms a more successful formation of many-ploid hybrids via fertilization of  $2n$  eggs produced by polyploid apomictic maternal species, compared to a reciprocal order of crossing partners (sexual species as a seed parent, apomictic species as a pollen donor). In experiments performed by BICKNELL et al. (2003), the frequency of  $2n+n$  progeny of facultatively apomictic maternal plants was estimated as 0.54% in the hypertriploid *H. aurantiacum* and as 0.10% in the triploid *H. piloselloides*, respectively, following crosses with a tetraploid pollen donor *H. piloselloides*. We recorded an order of magnitude higher frequency of  $2n+n$  hybrids (3.95%) in the progeny of facultatively apomictic hexaploid *H. rubrum*, when crossed to tetraploid *H. pilosella*. This variation might be due to differing abilities of the maternal species to generate  $2n$  female gametes capable of fertilization; as well differing compatibilities between the pairs of parental plants used in crosses.

The frequency of  $2n$  gametes in hybrids is assumed to be higher compared to non-hybrids, which can be due, especially in interspecific hybrids, to meiotic irregularities resulting in inviable reduced gametes. In general, the production of unreduced gametes is influenced by both genetic and environmental factors and is often connected with polyploidy and a perennial growth form (RAMSEY and SCHEMSKE 1998). Both of these conditions that are expected to promote the formation of unreduced gametes are found in *Hieracium* subgen. *Pilosella*. The natural frequency of unreduced gametes is estimated to occur at a similar rate in megasporogenesis and microsporogenesis within sexual non-hybrid species (approximately

$10^{-4}$  to  $10^{-2}$ ). The corresponding data for hybridogenous species, particularly the rate of  $2n$  egg production, are missing in the literature (RAMSEY and SCHEMSKE 1998). The frequency of  $2n$  eggs participating in crosses with *H. rubrum* as a maternal parent corresponds to the upper range for non-hybrid sexuals.

*Haploid parthenogenesis as a part of a versatile reproduction mode*

Seed production in *H. rubrum* is realized by means of four different pathways: i) somatic parthenogenesis, ii) haploid parthenogenesis, iii) hybridisation involving reduced egg cells, iv) hybridisation involving unreduced egg cells. Such a highly variable breeding system, operating within one generation, is rather rare among angiosperms. Haploid parthenogenesis is there a less common reproduction mode than hybridisation via  $2n$  gametes, however, the influence of the pollinator and of developmental and environmental factors on the expression of haploid parthenogenesis in sexual plants is accepted (ASKER and JERLING 1992). Whereas this reproduction mode is rarely observed in some sexual taxa (e.g. in Poaceae and Orchidaceae families), in some facultative apomicts it is commonly detected as a part of the cycle in which diploids and tetraploids alternate in consequent generations (e.g. in *Potentilla*, *Panicum*, *Bothriochloa* or *Dichanthium*) (ASKER and JERLING 1992; RICHARDS 1997). The two embryo types, polyhaploid and aposporous, can also coexist in one seed, as has been shown in some aposporous species of *Poa* (KIMBER and RILEY 1963; RICHARDS 1997). This is also the case in *Hieracium* subgen. *Pilosella*, where the meiotic embryos, including the polyhaploids, occurred with 7 fold greater likelihood in polyembryonic than single-embryo seeds of a hypertriploid *H. aurantiacum* (BICKNELL et al. 2003). An extreme versatility in breeding system, comparable with that recorded in *Hieracium*, is found e.g. in the aposporous *Ranunculus auricomus* group (NOGLER 1984). Unlike *Hieracium* subgen. *Pilosella* which has autonomous formation of polyhaploids, the above mentioned genera are pseudogamous, and therefore require pollination for polyhaploid production.

The classification of haploid parthenogenesis into apomixis, as used here (e.g. Fig. 2), might be subject to discussion. An autonomous formation of the embryo is only one of two substantial processes comprising apomixis (ASKER and JERLING 1992, CZAPIK 1994). In haploid parthenogenesis however, the second principal apomictic feature, avoiding of meiosis, is not realized. In spite of this, although it is a meiotic process, some authors include haploid parthenogen-

esis into broadly classified apomixis (MAHESHWARI 1950, JOHRI and SRIVASTAVA 2001).

#### *The role of residual sexuality*

The frequency of progeny generated by apomixis (both somatic and haploid parthenogenesis) was recorded as 92.1% in *H. rubrum*, when crossed as a seed parent with *H. pilosella* (Fig. 2). This is comparable to the frequency of apomictically derived progeny from a hypertriploid *H. aurantiacum* (93.8%) following artificial pollination (KOLTUNOW et al. 1998). A different method, based on the inheritance of introduced heterologous marker genes and screening several thousand seedlings (BICKNELL et al. 2003), estimated a somewhat higher rate of progeny generated by apomixis in a facultatively apomictic *Hieracium* (see above). While the frequency of sexually generated progeny, produced in crosses within pseudogamous facultatively apomictic *Potentilla* section *Niveae* did not exceed 2% (NYLÉHN et al. 2003), the rate of sexually derived progeny formed by non-pseudogamous *H. rubrum* following pollination is relatively high (7.9% of the total progeny), in spite of the predominantly apomictic nature of this maternal species. This supports the indications of the importance of residual sexuality in other facultatively apomictic representatives of the subgenus *Pilosella*, as has already been shown in field experiments in New Zealand where most populations are of recent origin (HOULISTON and CHAPMAN 2001, 2004). The range of hybridization in facultatively apomictic *H. pilosella* (pentaploid and tetraploid) was estimated to range from 0.2 to 2.7%, at three field localities over three years (HOULISTON and CHAPMAN 2004). Moreover, the first molecular studies on several natural stabilized interspecific polyploid hybrids from the Central Europe revealed their origin from fertilized 2n egg cells, which had been produced by a facultatively apomictic maternal species (unpublished results). Sexual reproduction, as an alternative to apomixis, plays an important role not only in aposporous apomicts as is shown here, but also in diplosporous taxa, such as *Taraxacum* (VAN BAARLEN et al. 2002).

It is evident that the capability for fertilization persists until the stage of floret opening and anthesis, at least for the eggs of *H. rubrum*, whether they are reduced or not. The enhanced induction of haploid parthenogenesis in crossed capitula, compared to emasculated ones, indicates the same phenomenon. The studies on embryogenesis in aposporous *Hieracium* species describe the degeneration of the majority of sexual embryo sacs before this stage (TURESSON 1972; KOLTUNOW et al. 1998, 2000). Nevertheless, the variation in timing of the rise of aposporous initials,

and also their site of differentiation, may allow a coexistence of both meiotic and ameiotic embryo sacs, increasing the chance of producing n + n hybrids in the progeny of an aposporous seed parent (POGAN and WCISLO 1995; KOLTUNOW et al. 2000).

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