

PART OF A SPECIAL ISSUE ON ECOLOGY AND EVOLUTION OF PLANT REPRODUCTION

Reproductive pathways in *Hieracium* s.s. (Asteraceae): strict sexuality in diploids and apomixis in polyploids

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Received: 28 February 2018 Returned for revision: 2 May 2018 Editorial decision: 18 June 2018 Accepted: 22 June 2018
Published electronically 18 July 2018

- **Background and Aims** Apomixis or asexual seed reproduction is a key evolutionary mechanism in certain angiosperms providing them with reproductive assurance and isolation. Nevertheless, the frequency of apomixis is largely unknown, especially in groups with autonomous apomixis such as the diploid–polyploid genus *Hieracium*.
- **Methods** Using flow cytometric analyses, we determined the ploidy level and reproductive pathways (sexual vs. apomictic) for 7616 seeds originating from 946 plants belonging to >50 taxa sampled at 130 sites across Europe.
- **Key Results** Diploid seeds produced by diploids were formed exclusively by the sexual pathway after double fertilization of reduced embryo sacs. An absolute majority of tri- and tetraploid seeds (99.6 %) produced by tri- and tetraploid taxa were formed by autonomous apomixis. Only 20 polyploid seeds (0.4 %) were formed sexually. These seeds, which originated on seven polyploid accessions of four taxa, were formed after fertilization of either unreduced embryo sacs through a so-called triploid bridge or reduced embryo sacs, and frequently resulted in progeny with an increased ploidy. In addition, the formation of seedlings with increased ploidy ($4x$ and $6x$) was found in two triploid plants. This is the first firm evidence on functional facultative apomixis in polyploid members of *Hieracium sensu stricto* (s.s.).
- **Conclusions** The mode of reproduction in *Hieracium* s.s. is tightly associated with ploidy. While diploids produce seeds exclusively sexually, polyploids produce seeds by obligate or almost obligate apomixis. Strict apomixis can increase the reproductive assurance and this in turn can increase the colonization ability of apomicts. Nevertheless, our data clearly show that certain polyploid plants are still able to reproduce sexually and contribute to the formation of new cytotypes and genotypes. The finding of functional facultative apomicts is essential for future studies focused on evolution, inheritance and ecological significance of apomixis in this genus.

Key words: Apomixis, apomeiosis, Asteraceae, diplospory, flow cytometry, *Hieracium*, parthenogenesis, polyploidy, residual sexuality, triploid bridge, unreduced gametes.

INTRODUCTION

Apomixis or agamospermy is asexual reproduction of plants by seeds, which are formed from unreduced female gametes parthenogenetically, i.e. without fertilization. In angiosperms, apomixis is relatively rare, being found in approx. 2.2 % of genera which mainly belong to three families: Asteraceae, Poaceae and Rosaceae (Carman, 1997; Hojsgaard *et al.*, 2014). Being tightly coupled with interspecific hybridization and polyploidization, the major evolutionary significance of apomixis lies in providing reproductive assurance to otherwise sterile hybrids (Darlington, 1939; Asker and Jerling, 1992). In addition, apomixis represents one of the most effective reproductive barriers in plants and therefore plays an important role in speciation (Rieseberg *et al.*, 2006). Because apomictic seed reproduction represents a deviation from the normal sexual pathway, which includes meiosis and double fertilization (Tucker and Koltunow, 2009), most apomicts still retain some proportion of sexuality (Hojsgaard *et al.*, 2014; Krahulcová *et al.*, 2014; Hand *et al.*, 2015). Facultative apomicts can generate new variation through residual sexuality, and in this way can cope with negative consequences of accumulation of deleterious mutations. On the other hand, frequent sexual reproduction might lower the level

of reproductive assurance and thus the colonization potential of apomictic plants (Hörandl, 2006). Despite current advances in understanding the mechanisms and consequences of apomictic reproduction (Hörandl *et al.*, 2007, and references therein), our knowledge about the frequency of apomixis and whether it is facultative or obligate in particular apomictic complexes is still very fragmentary.

Hieracium L. (excluding *Pilosella* Hill) belongs to the most diverse vascular plant genera in the world, with species numbers varying roughly between 500 and 5000, depending on the species concept (e.g. Zahn, 1921–1923; Schuhwerk, 2002; Tyler, 2006; Majesky *et al.*, 2017). These species numbers reflect taxonomic recognition of extensive morphological variation (Zahn, 1921–1923), now known to be accompanied by considerable molecular variation (Fehrer *et al.*, 2009; Krak *et al.*, 2013). It has been suggested that these patterns were probably caused by massive interspecific hybridization in the past with subsequent polyploidization (Fehrer *et al.*, 2009; Krak *et al.*, 2013). As a result of these processes, tri- and tetraploid taxa predominate ($2n = 3x = 27$ and $2n = 4x = 36$, respectively; based on $x = 9$), with a few pentaploids ($2n = 5x = 45$); a couple of diploids ($2n = 2x = 18$)

also occur (e.g. Merxmüller, 1975; Schuhwerk, 1996; Chrtek *et al.*, 2004; Tyler and Jönsson, 2009). Importantly, whole-genome multiplication in this group has been tightly coupled with a shift to apomixis (Asker and Jerling, 1992).

Apomictic reproduction in true hawkweeds was firstly discovered by Ostenfeld and Raunkiaer (1903) using Raunkiaer's castration method. This very simple and efficient treatment performed using a razor blade or scissors relies on cutting off the upper half of the capitulum (inflorescence in Asteraceae) just before its opening. Because the entire stigmas and upper parts of styles are removed, pollination and subsequent fertilization are excluded. If ripe achenes are formed after such a treatment, this is clear evidence for the parthenogenetic formation of seeds. The effectiveness of this method was subsequently confirmed by Murbeck (1904) who observed normal embryo development in 80–90 % of ovules after castration. Later on, castration has been subsequently used for testing apomictic reproduction in several tens of hawkweed taxa, usually within taxonomically oriented studies (summarized in Table 1). Despite its simplicity and effectiveness, which allows a large number of progeny to be tested simultaneously within the same capitulum, this method also has several disadvantages. One limitation is related to the method itself, as this invasive treatment may sometimes cause the abortion of the whole capitulum (Richards, 1997); others are related to the small amount of information obtained using this method. For instance, in the case of residual sexuality, the ploidy of the participating egg cells and pollen in mature embryos will remain unknown. Similarly, haploid parthenogenesis, when the

embryo directly develops from a reduced egg cell, will be undetected. Furthermore, in studies in which the castration procedure was applied, the reproductive output (seed set) and also the number of capitula/tested plants have only rarely been reported (Table 1). Such incomplete information precludes any reliable quantification of apomixis using these data.

The first detailed cytoembryological evidence on parthenogenetic development in *Hieracium sensu stricto* (s.s.) was published by Bergman (1935) who found that polyploid *Hieracia* reproduced by mitotic diplospory of the *Antennaria* type. This type is characterized by a direct transformation of the megaspore mother cell into a megaspore without meiosis which subsequently undergoes three rounds of mitotic divisions resulting in an eight-nucleate embryo sac (ES) of the *Polygonum* type. The mature apomeiotic ES consists of six cells, one of which is the egg cell, i.e. female gamete, and two polar nuclei which fuse together and form one central nucleus (Bergman, 1935). Because of a complete omission of meiosis, each cell has the unreduced somatic chromosome number, while the central nucleus has a doubled content of DNA. The subsequent development of the unreduced egg cell into an embryo (parthenogenesis) and of the central nuclei into the endosperm is independent of fertilization (autonomous apomixis). The resulting seed is therefore composed of a mature embryo of the same ploidy level as the mother plant, and endosperm whose ploidy is 2-fold higher compared with the embryo. Interestingly, apomictic members of *Hieracium* frequently initiate embryogenesis precociously before floret opening, thus making an

TABLE 1. An overview of studies which tested autonomous seed formation after castration (emasculation) treatment or diplosporic embryo sac (ES) formation using cytoembryological approaches in polyploid representatives of the genus *Hieracium* s.s.

No. of taxa	No. of plants/capitula per taxon	Seed set after castration (mean/range, %) or % of diplosporic ESs	References
Castration method			
15	NA	NA	Ostenfeld and Raunkiaer (1903)
3	NA	NA	Murbeck (1904)*
1	NA	NA	Ostenfeld (1921)
11	1–60/NA	NA	Christoff and Popoff (1933)
1	NA	72.8/NA	Guppy (1978)
5	NA	NA/84–100	Chrtek (1997a)
1	20/NA	NA/96.8–99.5	Chrtek (1997b)
8	NA	NA	Mráz (2001)
1	2/2	NA	Mráz (2002)
4	≥3 per population/NA	NA/78–97	Štorchová <i>et al.</i> (2002)
1	4/4	NA/85–98	Mráz (2003b)
8	NA	NA	Mráz and Szeląg (2004)
1	3/6	NA	Szeląg (2006)
6	10 per taxon/NA	77.3–84.9/33.1–100	Chrtek <i>et al.</i> (2007a)
1	3/3	NA	Rich <i>et al.</i> (2007)
1	2/4	NA	Rich <i>et al.</i> (2008)
42	215/NA	NA	Chrtek <i>et al.</i> (2009)
1	NA/6	NA	Szeląg (2010)
1	NA	NA	Musiał and Szeląg (2015)
1	NA	NA	Szeląg (2017)
Cytoembryological observations			
1	NA	NA	Bergman (1935)
11	NA	NA	Gustafsson (1935)
5	NA	NA	Gentcheff (1937)
12	NA	45–100/NA	Bergman (1941)
1	1/NA	NA	Battaglia (1947)
1	NA	54	Skawińska (1963)
12	15/NA	93–100	Hand <i>et al.</i> (2015)

*Viable embryos were observed after the castration experiment.

eventual fertilization of the unreduced gamete impossible (precocious embryony, Bergman, 1935; Skawińska, 1963; Hand *et al.*, 2015). Quantitative cytoembryological assessments have revealed that certain polyploid taxa are still able to produce a meiotic ES (Bergman, 1935; Skawińska, 1963; Table 1), with proportions sometimes reaching even up to 50 % (Bergman, 1935). However, later observations on 15 polyploid accessions showed that most of them produced the ES fully by apomixis, while the remaining accessions showed in addition a very low proportion of sexually derived ESs usually varying between 1 and 2 % (maximum 7 %) of examined ovules (Hand *et al.*, 2015). Because of the destructive nature of the cytoembryological approach, it is unknown whether or not the reduced egg cells could produce fully developed seeds after eventual fertilization. For instance, Skawińska (1963) reported very frequent abortion of ESs, suggesting serious developmental problems of probably reduced ESs in apomictic triploid *Hieracium alpinum*.

In contrast to polyploid *Hieracia*, diploid hawkweeds form seeds from tetrasporic ESs through meiosis and subsequent fertilization of the haploid egg cell and the diploid central nucleus – each by a haploid spermatid cell (Juel, 1905; Bergman, 1935; Yurukova-Grancharova *et al.*, 2006). These processes result in a seed containing a diploid embryo and triploid endosperm, i.e. the DNA content of the endosperm is 1.5-fold higher than that of the embryo. This embryological indication of sexuality in diploids has been confirmed by isolation experiments (Chrtěk, 1997a; Mráz, 2003a), which moreover showed that diploid taxa are strictly self-incompatible (sporophytic SI system), requiring pollination from a compatible partner to produce viable seeds.

In addition to the altered female function, polyploid taxa also differ from diploid taxa in pollen production. It has been observed that many polyploid apomicts are completely pollen sterile while others produce less pollen when compared with sexual diploids, and this pollen is heterogeneous in size (Chrtěk, 1997a; Slade and Rich, 2007; Mráz *et al.*, 2009). This is mainly because of serious developmental problems in the sporogenic tissue, and partly also due to irregularities with chromosome pairing during meiosis (Rosenberg, 1917, 1927; Gentcheff and Gustafsson, 1940).

The difference in endosperm:embryo DNA ratios between sexually and apomictically derived seeds has recently been used for the discrimination between both modes of reproduction using a flow cytometric approach called flow cytometric seed screening (FCSS) (Matzk *et al.*, 2000). In contrast to cytoembryology, FCSS uses mature, well-developed seeds which are putatively germinable and may therefore be evolutionarily relevant. As this method estimates the DNA content in cells, it provides important data on the ploidy of both embryo and endosperm within each individual seed. Therefore, in addition to the mode of reproduction and in combination with previous knowledge on the mechanism of apomixis in a given taxonomic group (e.g. diplospory, apospory or pseudogamy), the ploidy of the egg cell and that of the pollen participating in the formation of embryo and endosperm can also be deduced using FCSS (Matzk *et al.*, 2000; Krahulcová and Rotreklová, 2010). Despite the very easy and fast sample preparation and analysis and the reliability of the obtained results, this method, with the exception of work done by Mráz *et al.* (P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.), has not been used so far for the quantitative assessment of asexual reproduction in the genus *Hieracium*. This study based on very extensive FCSS analyses

of seed material collected throughout the distributional range of a single species, *H. alpinum*, revealed strict sexuality in diploids and strict asexuality in triploids (P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.). Triploid *H. alpinum* thus probably presents the first angiosperm taxon with proved obligate apomixis detected on such a high number of individuals collected across its distributional range. Because this pattern might be taxon specific and because there are several indications about production of unreduced ESs in several polyploid *Hieracium* taxa (Bergmann, 1935; Hand *et al.*, 2015), in the present study, we considerably extended our sampling taxonomically and spatially, and applied flow cytometric seed screening to answer the following questions. (1) Do diploid taxa produce seeds exclusively sexually? (2) Do polyploid taxa reproduce by strict apomixis and thus asexually? (3) If polyploid species occasionally reproduce sexually, how frequent and how widely distributed is facultative apomixis across polyploid taxa? (4) Does facultative apomixis involve both reduced and unreduced female gametes?

MATERIALS AND METHODS

Sampling

Sampling of seed material for FCSS was performed between 2014 and 2017 at 130 sites across Europe (Fig. 1; Supplementary Data Table S1). At each site we sampled seeds from fully mature, open and visually undamaged capitula, i.e. flower-like inflorescences composed of approx. 20–120 florets, depending on the taxon (Zďvořák, 2017). We sampled between one and nine capitula per plant originating from between one and 29 plants per taxon and locality. The number of collected capitula and plants depended on (1) the number of fully mature capitula present at the time of sampling; (2) taxonomic identity – the total number of capitula per plant varies from one in taxa from the sections *Alpina* or *Villosa* to several tens in taxa from the sections *Umbellata* or *Prenanthoidea* (Zahn, 1921–1923); and (3) the number of plants occurring at the site. The plants suitable for seed sampling were selected randomly with 2 m as a minimum distance between the plants. After sampling, seed samples were first kept for 1–2 months at room temperature and subsequently stored at 4 °C, with the exception of samples obtained from selected herbarium vouchers, which were kept at room temperature from the time of their collection (from 1 to 3 years). In addition, seed samples were also obtained from several plants collected in the field and cultivated in the experimental gardens at the Charles University in Prague and in Mnichovice (Czechia; private garden of the first author). If possible, the collected taxa were determined to the microspecies level by the first author, or by other *Hieracium* specialists (J. Chrtěk and J.-M. Tison). However, because of very complex morphological variation and confounding nomenclature, many microspecies were treated here within largely accepted species groups defined by a characteristic combination of morphological traits (Zahn, 1921–23). Therefore, the real number of microspecies we analysed in the present study was actually higher, especially in the following species groups: *H. murorum sensu lato* (s.l.), *H. nigrescens* s.l. and *H. prenanthoides* s.l. (Table 2; Supplementary Data Table S1). Voucher specimens are deposited in the herbarium collections of the Charles University (PRC).

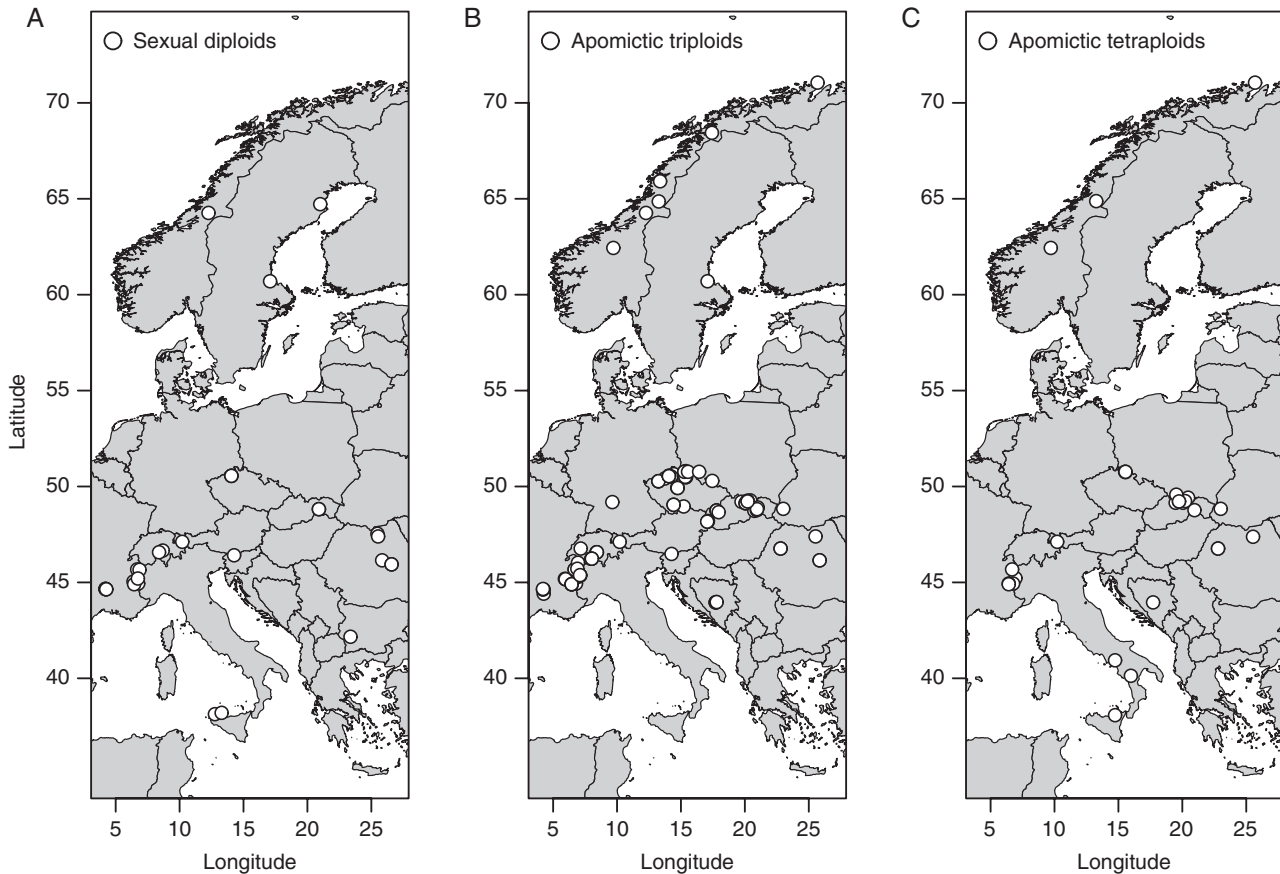


FIG. 1. Maps of sampling sites of representatives of the genus *Hieracium* s.s. for which the reproduction mode was assessed using flow cytometric seed screening (FCSS).

Ploidy level estimation and inference of reproductive pathways

The ploidy level of mother plants was deduced directly from the ploidy of embryos they produced based on knowledge of the reproductive mode by which the seed was formed. This is possible because seed progeny in polyploid apomictic taxa with autonomous diplospory are formed mitotically, while seed progeny in diploid taxa are formed by double fertilization (Matzk *et al.*, 2000; see also the Introduction). In particular, as diploid maternal plants were considered those which produced diploid embryos by the sexual pathway (ploidy of endosperm:ploidy of embryo $3x:2x$), as triploid those which produced triploid embryos by the apomictic pathway (ratio $6x:3x$) and, finally, as tetraploid those mother plants which formed tetraploid embryos by the apomictic pathway (ratio $8x:4x$). This theoretical assumption was confirmed on selected plants that originated from diploid, triploid and tetraploid taxa (Table 2), when the ploidy of both maternal plant and seeds was determined using conventional flow cytometry or by chromosome counting (cultivated mother plants) as described by Mráz (2001). In addition, the ploidy level, which is uniform within microspecies in *Hieracium* s.s., was compared with chromosome counts/ploidy level estimations published in numerous literature sources (approx. 2000 chromosome/ploidy level records for about 800 named taxa have been published so far in *Hieracium* s.s.; P. Mráz, CU, Prague, Czechia, unpubl. res.). Flow cytometry was also used to determine the ploidy level of

seedlings that originated from two triploid *H. nigrescens* s.l. accessions collected at Mt. Vlădeasa, where cytotype variation in seed progeny was found in other accessions of the same microspecies (Table 3).

In the course of FCSS, the total number of seeds (progeny) per plant usually varied from five to 30, with an average of eight. Because of high cytological homogeneity within plants and populations in the genus *Hieracium* s.s. (e.g. Chrtek *et al.*, 2004; Tyler and Jönsson 2009; P. Mráz, Charles University, Prague, Czechia, unpubl. res.), we used bulk samples usually consisting of five seeds from the same mother plant. Pilot analyses of artificial mixtures of five seeds with two different ploidies ($3x$ and $4x$; $2x$ and $3x$) and of varying frequencies showed that it was possible to assess correctly the number of seeds with corresponding ploidies in the case of a cytotype mixture. If cytotype variation was observed in a bulk sample, we analysed further seeds individually. As BII progeny we evaluated those seeds which arose from the fusion of reduced gametes (including partially reduced in the case of polyploids), whereas as BIII progeny were considered those seeds which were formed from fusion of unreduced eggs and either reduced or unreduced pollen (Rutishauser, 1948).

The samples were prepared using a two-step procedure (Otto, 1990; Doležel and Göhde, 1995) with co-chopped leaves of *Bellis perennis* from the Botanical Garden of the Charles University, Prague as internal standard. The genome size of the internal standard was $2C = 3.61$ pg of DNA (P. Mráz, CU, Prague, Czechia, unpubl. res.) and was estimated against *Pisum*

TABLE 2. List of taxa of the genus *Hieracium* s.s. tested for their type of reproduction using flow cytometric seed screening (FCSS)

Taxon	Ploidy	Nsite	Nplant	Nseed	Emb	End	Rep
<i>intybaceum</i>	2x [‡]	5	48	375	2x	3x	Sex
<i>ludicum</i> *	2x [‡]	2	3	15	2x	3x	Sex
<i>pojoritense</i>	2x [‡]	1	2	50	2x	3x	Sex
<i>porrifolium</i>	2x [‡]	1	14	75	2x	3x	Sex
<i>prenanthoides</i> s.s.	2x [‡]	3	45	478	2x	3x	Sex
<i>stelligerum</i>	2x ^{‡,§}	5	39	275	2x	3x	Sex
<i>sparsum</i>	2x [‡]	1	1	20	2x	3x	Sex
<i>transsilvanicum</i>	2x [‡]	2	45	309	2x	3x	Sex
<i>umbellatum</i>	2x ^{‡,§}	9	64	541	2x	3x	Sex
<i>amplexicaule</i>	3x [‡]	2	10	188	3x	6x	Apo
<i>atratum</i> s.l.	3x [‡]	1	1	10	3x	6x	Apo
<i>caesium</i> s.l.	3x [‡]	1	1	10	3x	6x	Apo
<i>crinitum</i>	3x [‡]	1	2	10	3x	6x	Apo
<i>froelichianum</i>	3x	1	1	10	3x	6x	Apo
<i>glanduliferum</i>	3x [‡]	3	19	95	3x	6x	Apo
<i>halleri</i>	3x [‡]	8	22	125	3x	6x	Apo
<i>jaubertianum</i>	3x [‡]	2	14	200	3x	6x	Apo
<i>jurassicum</i> s.l.	3x [‡]	1	1	30	3x	6x	Apo
<i>laevigatum</i> s.l.	3x [‡]	6	27	251	3x	6x	Apo
<i>lachenalii</i> s.l.	3x [‡]	7	47	285	3x	6x	Apo
<i>macilentum</i> s.l.	3x [‡]	1	8	115	3x	6x	Apo
<i>murorum</i> s.l.	3x [‡]	17	108	676	3x	6x	Apo
<i>nigrescens</i> s.l.	3x ^{‡,§}	6	39	192	3x	6x	Apo
<i>pallidiflorum</i>	3x	3	21	160	3x	6x	Apo
<i>petiolare</i>	3x [‡]	2	20	250	3x	6x	Apo
<i>picroides</i> s.l.	3x	3	17	143	3x	6x	Apo
<i>piliferum</i>	3x ^{‡,§}	3	5	45	3x	6x	Apo
<i>pilosum</i> s.l.	3x [‡]	1	3	45	3x	6x	Apo
<i>praecurrens</i> s.l.	3x [‡]	2	27	195	3x	6x	Apo
<i>prenanthoides</i> s.l.	3x [‡]	10	50	360	3x	6x	Apo
<i>racemosum</i> s.l.	3x ^{‡,§}	3	17	155	3x	6x	Apo
<i>sabaudum</i> s.l.	3x [‡]	17	96	634	3x	6x	Apo
<i>scitulum</i> s.l.	3x	1	8	45	3x	6x	Apo
<i>telekianum</i>	3x ^{‡,§}	1	1	35	3x	6x	Apo
<i>valdepiosum</i> s.l.	3x [‡]	1	5	65	3x	6x	Apo
<i>villosum</i> s.l.	3x [‡]	1	4	60	3x	6x	Apo
<i>atratum</i> s.l.	4x [‡]	1	6	50	4x	8x	Apo
<i>barrelieri</i>	4x	1	10	150	4x	8x	Apo
<i>bupleuroides</i> s.l.	4x [‡]	2	6	40	4x	8x	Apo
<i>carpathicum</i> s.l.	4x [‡]	1	1	15	4x	8x	Apo
<i>caesium</i> s.l.	4x [‡]	1	1	10	4x	8x	Apo
<i>glaucum</i> s.l.	4x [‡]	1	6	45	4x	8x	Apo
<i>grovesianum</i> subsp. <i>rigoanum</i>	4x	1	1	25	4x	8x	Apo
<i>chloropsis</i>	4x [‡]	1	1	45	4x	8x	Apo
<i>intybaceum</i>	4x [‡]	1	9	85	4x	8x	Apo
<i>lachenalii</i> s.l.	4x [‡]	2	5	30	4x	8x	Apo
<i>murorum</i> s.l.	4x [‡]	1	4	20	4x	8x	Apo
<i>nigrescens</i> s.str.	4x ^{‡,§}	1	1	15	4x	8x	Apo
<i>nigrescens</i> s.l.	4x ^{‡,§}	5	10	53	4x	8x	Apo
<i>brachytrichellum</i> [†]	4x§	1	3	20	4x	8x	Apo
<i>rapunculoides</i>	4x§	1	5	25	4x	8x	Apo
<i>scitulum</i> s.l.	4x	1	6	30	4x	8x	Apo
<i>silesiacum</i>	4x [‡]	1	1	10	4x	8x	Apo
<i>stygium</i>	4x [‡]	2	8	55	4x	8x	Apo
<i>sudeticum</i>	4x [‡]	2	4	50	4x	8x	Apo
<i>vagneri</i>	4x [‡]	1	16	231	4x	8x	Apo

Only the accessions which produced ploidy-uniform progeny are included in this table. Taxa are arranged alphabetically within each of the three ploidies. Emb, ploidy of embryo; End, ploidy of endosperm; Rep, mode of reproduction (Apo, apomictic; Sex, sexual).

*Including *H. lucidum* subsp. *cophanense*.

[†]*nigrescens* subsp. *brachytrichellum*.

[‡]Confirmed by literature records.

[§]Those taxa in which seed plants were checked for their ploidy level.

sativum 'Ctirad' with $2C = 9.09$ pg DNA (Doležel *et al.*, 1998). We used 4',6-diamino-2-phenylindole (DAPI) as a fluorescent stain and a Partec Cyflow instrument equipped with a HBO

lamp as ploidy level analyser. Histograms were accumulated at a flow rate of approx. $10\text{--}30$ particles s^{-1} for a total count of 3000 nuclei. The mean values of coefficients of variation of fluorescent

TABLE 3. List of polyploid accessions of the genus *Hieracium* s.s in which intraindividual variation in reproduction mode (Sex, sexual; Apo, apomictic) was found using flow cytometric seed screening (FCSS)

Taxon	Pop code	Plant code	Nseed	Emb	End	Origin	Rep
<i>amplexicaule</i>	HAMPL_1	HAMPL_1_1 (3x)	12	3x	6x	3x + 0x/6x + 0x	Apo (80 %)
			3	4x	6x	2x + 2x/4x + 2x	Sex (BII)
<i>amplexicaule</i>	HAMPL_2	HAMPL_2_1 (3x)	19	3x	6x	3x + 0x/6x + 0x	Apo (79.9 %)
			1	3x	4x	1x + 2x/2x + 2x	Sex (BII)
			1	4x	6x	2x + 2x/4x + 2x	Sex (BII)
			3	4x	7x	3x + 1x/6x + 1x	Sex (BIII)
<i>macilentum</i>	H_5	H_5_7 (3x)	15	3x	6x	3x + 0x/6x + 0x	Apo (88.3 %)
			2	5x	8x	3x + 2x/6x + 2x	Sex (BIII)
<i>nigrescens</i> s.l.	C2_2015	HSUB_1_10 (3x)	4	3x	6x	3x + 0x/6x + 0x	Apo (66.7 %)
	(= HSUB_1)		2	4x	7x	3x + 1x/6x + 1x	Sex (BIII)
<i>nigrescens</i> s.l.	C2_2015	HSUB_1_11 (3x)	6	3x	6x	3x + 0x/6x + 0x	Apo (75 %)
	(= HSUB_1)		2	4x	7x	3x + 1x/6x + 1x	Sex (BIII)
<i>rapunculoides</i>	HPREN_sl_3	HPREN_sl_3_4 (4x)	7	4x	8x	4x + 0x/8x + 0x	Apo (53.8 %)
			4	5x	9x	4x + 1x/8x + 1x	Sex (BIII)
			1	5x	8x	3x + 2x/6x + 2x	Sex (BII)
<i>rapunculoides</i>	HPREN_sl_3	HPREN_sl_3_6 (4x)	2	4x	8x	4x + 0x/8x + 0x	Apo (66.7 %)
			1	5x	8x	3x + 2x/6x + 2x	Sex (BII)

Emb/End, ploidy of embryo and endosperm, respectively. Origin, interpreted origin of embryo and endosperm in terms of ploidy level: ploidy of egg cell + ploidy of pollen (if it participated)/ploidy of central nucleus + ploidy of pollen (if it participated). BII and BIII denote sexually formed progeny arising from pollination of a reduced or unreduced ovule, respectively. The proportion of apomictically derived seeds per accession is given in parentheses.

intensities were as follows: 3.2 % for internal standard,; 3.2 % for embryos; and 2.5 % for endosperms. The ploidy level of embryos and endosperms was inferred as the position of the sample G_1 peaks relative to that of the internal standard (*B. perennis*) based on our previous analyses of karyologically counted plants and published genome sizes (Chrtěk *et al.*, 2007b, 2009; Mráz *et al.*, 2009). From 1648 FCSS analyses, 71 (4.3 %) were discarded from the final evaluation as they did not allow us to determine unambiguously the ploidy of the embryo and/or mode of reproduction because of either poor quality of electrophoregrams or a missing peak corresponding to the endosperm. In the latter case, a new seed sample from the same mother plant was analysed.

RESULTS

In total, we were able to interpret ploidy level and reproductive pathways for 7616 seeds sampled from 946 plants belonging to >50 taxa originated from 130 sites throughout Europe (Fig. 1; Table 2; Supplementary Data Table S1).

All cytometrically analysed seeds formed on diploid taxa (2138 in total) and originated from 261 plants had diploid embryos and triploid endosperms, thus arising sexually via double fertilization of reduced ESs by two reduced haploid pollen sperms (Table 2; Fig. 2A). The remaining 5478 seeds from 685 plants had polyploid embryos. Of the plants with polyploid embryos, 4446 seeds were triploid, 1024 tetraploid and eight seeds had pentaploid embryos (Tables 2 and 3). An absolute majority of polyploid seeds (99.6 %) were formed by autonomous apomixis showing a mean value of 1.97 for the ratio of ploidy of endosperm to ploidy of embryo, thus very close to the expected ratio in apomictically derived seeds, i.e. 2 (Table 2; Fig. 2B, C, H, K, M). We did not find any pentaploid seed formed by the apomictic pathway and thus indicating a pentaploid maternal plant.

The sexual origin of embryos in polyploid taxa has been robustly confirmed in 20 seeds (approx. 0.4 % of all seeds with polyploid embryos) being produced by seven different plants

(approx. 1 % of 685 polyploid plants analysed in this study) representing four different taxa which were collected in the Alps (France and Italy) and Carpathians (Romania and Slovakia) (Table 3; Supplementary Data Table S1). From these 20 seeds, 13 were BIII progeny which arose from fertilization of an unreduced, apomeiotic, female gamete causing an increased ploidy in the resulting progeny (Table 3; Fig. 2I, L, O). Specifically, triploid taxa (*H. amplexicaule* and *H. nigrescens* s.l.) produced tetraploid (Fig. 2L) and very rarely also pentaploid BIII progeny (*H. macilentum* s.l., Fig. 2I) by fusion of an unreduced triploid ES and haploid and diploid pollen, respectively. Tetraploid *H. rapunculoides* formed pentaploid BIII progeny by fusion of an unreduced tetraploid ES with haploid pollen (Fig. 2O). In the case of BII progeny, where reduced ESs were involved, the ploidy of participating reduced female gametes indicated meiosis with a very small proportion of x and $2x$ gametes in triploid *H. amplexicaule* (Fig. 2E, F), and $3x$ gametes in tetraploid *H. rapunculoides* (Table 3; Fig. 2N). Despite the presence of residual sexuality, all these 'deviating' accessions produced seeds mostly apomictically (from 54 to 88 % of analysed seeds per accession), achieving a significantly highest proportion of apomictically derived seeds in those plants for which a higher number of seeds was analysed (Table 3, Spearman one-sided correlation test: $S = 18.7$, $P = 0.051$).

From 28 seedlings which we analysed cytometrically and which originated from seeds collected from two triploid *H. nigrescens* s.l. plants from Mt. Vlădeasa (C2_2015/HSUB_1 gatherings), i.e. the site where variation in reproductive pathways was found in two accessions (Table 3), 24 were triploid, three were tetraploid and one was hexaploid (Table 4; Fig. 3). Based on previous knowledge on formation of apomictic and BIII progeny on two plants of the same microspecies and population (Table 3), we suppose that triploid seedlings probably arose by autonomous apomixis, while tetraploids and hexaploids were probably formed from fusion of unreduced, i.e. triploid, ESs and pollen with haploid and triploid sets of chromosomes, respectively (Table 4).

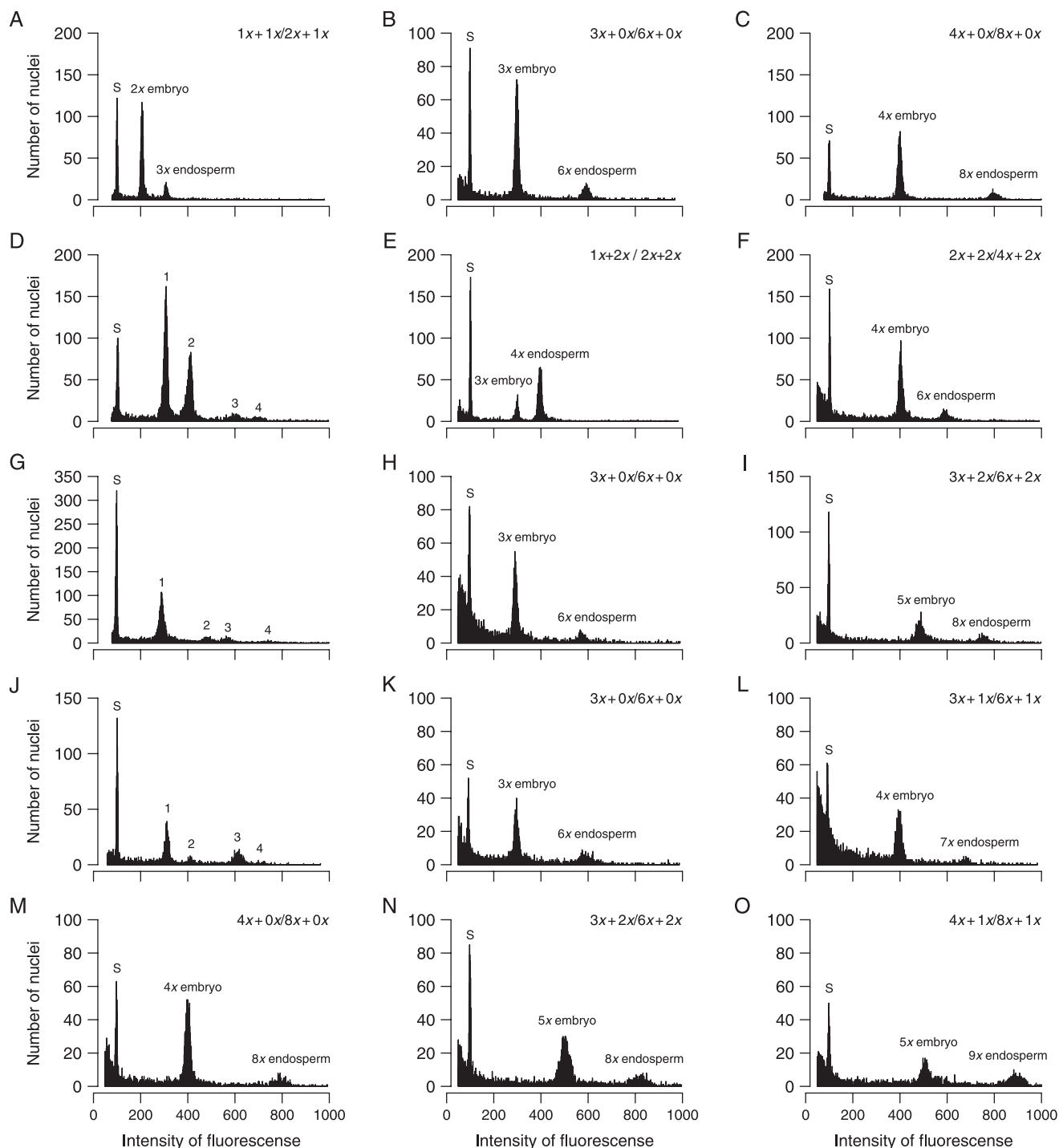


FIG. 2. Flow cytometric histograms showing relative fluorescence intensities (i.e. ploidy levels) of embryonic and endospermic nuclei isolated from seeds of representatives of the genus *Hieracium* s.s. and nuclei of leaves isolated from *Bellis perennis* used as an internal standard (S). Interpreted origin of the embryo and endosperm in terms of the ploidy level: ploidy of egg cell + ploidy of pollen (if it participated)/ploidy of central nucleus + ploidy of pollen (if it participated) in all single seed analyses and those bulk analyses which showed uniform origin, is given in the right-hand corner. (A) *H. intybaceum* – sexual reproduction of a diploid plant (accession HINT_5_12; a bulk sample of five seeds); (B) *H. laevigatum* s.l. – apomictic reproduction of a triploid plant (HLAEV_1_2, a bulk sample of five seeds); (C) *H. chloropsis* – apomictic reproduction of a tetraploid plant (HVAL_1_1_C3/3, a bulk sample of five seeds). (D–F) *H. amplexicaule* – apomictic and sexual modes of reproduction of a triploid plant [HAMPL_2_1_C1/2, D; a bulk sample of five seeds interpreted as follows: three seeds with a 3x embryo (peak #1) and 6x endosperm (peak #3), and two seeds with a 4x embryo (peak #2) and 7x endosperm (peak #4); (E and F) single seed analyses showing sexual reproduction]; (G–I) *H. macilentum* s.l. – apomictic and sexual modes of reproduction of a triploid plant [H_5_7, G; a bulk sample of five seeds interpreted as follows: four seeds with a 3x embryo (peak #1) and 6x endosperm (peak #3), and one seed with a 5x embryo (peak #2) and 8x endosperm (peak #4); (H and I); single seed analyses showing apomictic (H) and sexual reproduction (I)]; (J–L) *H. nigrescens* s.l. – apomictic and sexual modes of reproduction of a triploid plant [HSUB_1_11, J; a bulk sample of five seeds interpreted as follows: four seeds with a 3x embryo (peak #1) and 6x endosperm (peak #3), and one seed with a 4x embryo (peak #2) and 7x endosperm (peak #4); (K and L); single seed analyses showing apomictic (K) and sexual reproduction (L)]; (M–O) *H. rapunculoides* – apomictic and sexual modes of reproduction of a tetraploid plant [HPREN_s.l._3_4; M; a bulk sample of five seeds showing only an apomictic reproduction; (N and O); single seed analyses showing sexual reproduction].

TABLE 4. Ploidy-level variation in seedlings germinated from seeds collected on two triploid plants of *Hieracium nigrescens* s.l.

Taxon	Pop code	Plant code	Nseedling/ploidy	Putative pathway	Reproduction
<i>nigrescens</i> s.l.	C2_2015 (= HSUB_1)	C2_2015_20 (3x)	12 (3x) 1 (4x) 1 (6x)	3x + 0x 3x + 1x 3x + 3x	Apo (85.7 %) Sex (BIII) Sex (BIII)
<i>nigrescens</i> s.l.	C2_2015 (= HSUB_1)	C2_2015_22 (3x)	12 (3x) 2 (4x)	3x + 0x 3x + 1x	Apo (85.7 %) Sex (BIII)

Putative reproductive pathways of seedling formation (the first number means ploidy level of the egg cell, while the second is the supposed ploidy of participating pollen, if present) are based on prevailing formation of apomictic and sexually derived BIII hybrids found in other plants from the same population using flow cytometric seed screening (see Table 3 and the Results). The proportion of seedlings putatively derived by apomixis is given in parentheses.

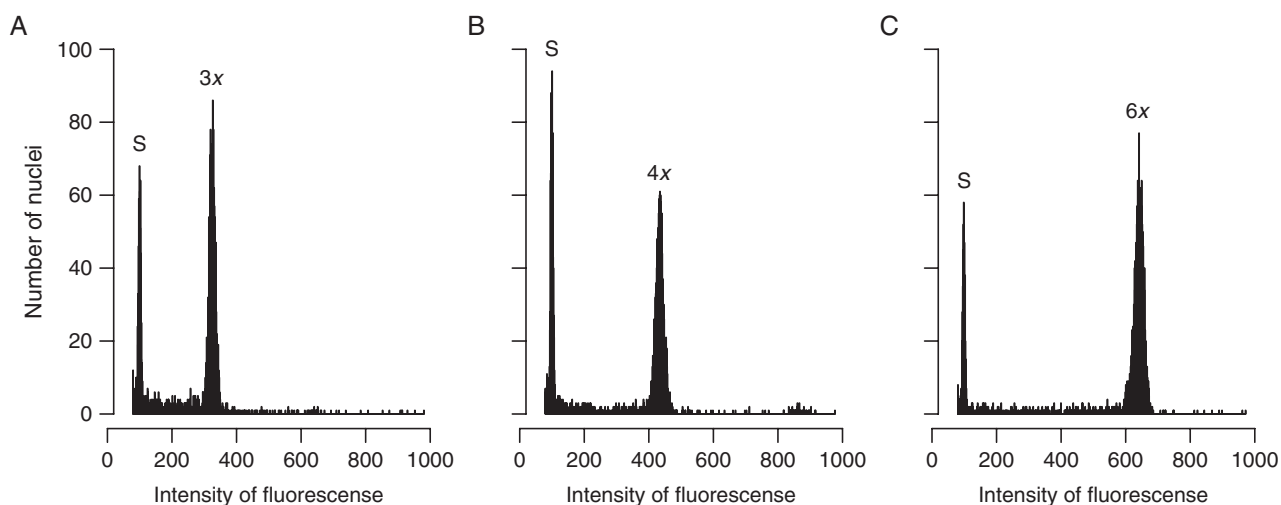


FIG. 3. Flow cytometric histograms showing relative fluorescence intensities (i.e. ploidy levels) of cell nuclei isolated from leaves of three seedlings representing three cytotypes, i.e. (A) triploid, (B) tetraploid and (C) hexaploid, all formed on the triploid *Hieracium nigrescens* s.l. (accession no. C2_2015_20), and leaves of *Bellis perennis* used as an internal standard plant (S).

DISCUSSION

In our study, using large-scale FCSS analyses, we found that diploid taxa formed seeds exclusively by the sexual pathway, while the vast majority of polyploid seeds produced by polyploid taxa arose by apomixis with autonomous formation of endosperm. Our results thus quantitatively corroborate a very tight link between mode of reproduction and ploidy in the genus *Hieracium* s.s. as previously suggested from castration/isolation experiments and cytoembryological observations (Table 1). However, despite predominant apomictic reproduction, our quantitative approach also revealed several rare sexual events in polyploids. To the best of our knowledge, this is the first clear evidence on functional, i.e. based on analyses of viable progeny, facultative apomixis in *Hieracium* s.s.

Strict sexuality at the diploid level

All seeds analysed in the present study originating from nine diploid *Hieracium* taxa were formed after double fertilization of reduced ESs by reduced haploid pollen. The same pattern was found in the diploid cytotype of *H. alpinum*, where 1670 seeds from 358 plants were analysed (P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.). The quantitative results from FCSS analyses are in accordance with rare cytoembryological

observations (Juel, 1905; Bergmann, 1935; Hand *et al.*, 2015) and isolation experiments (Chrtek, 1997a; Mráz, 2003a) showing that *Hieracium* diploids reproduce strictly sexually. This is the rule in all sexual–apomictic complexes (Asker and Jerling, 1992), but there is a notable exception – the genus *Boechera* (Brassicaceae), where certain diploids are able to reproduce apomictically (Böcher, 1951; Aliyu *et al.*, 2010). Further rare cases of apomixis at the diploid level were also found in the aposporic genera *Paspalum* (Siena *et al.*, 2008) and *Pilosella* (as *Hieracium* subgen. *Pilosella*; Bicknell, 1997). However, the latter case is an artificially produced clone when a single diploid plant arose through haploid parthenogenesis from a tetraploid apomictic mother and was propagated under laboratory conditions (Bicknell, 1997). Interestingly, a relatively large number of diploid plants with the apomictic mode of reproduction has recently been found in the genus *Erigeron* which is characterized by the same diplosporic type of apomixis as *Hieracium* s.s. (Noyes and Wagner, 2014). Several diploid apomicts showing high frequency of an unreduced ES accompanied by high seed sets were found on a single, highly recombinant tetraploid arising from an experimental cross (Noyes and Wagner, 2014). In our FCSS analyses, however, we did not find any diploid peak putatively corresponding to diploid embryos produced by tetraploid taxa parthenogenetically derived from a reduced ES (Tables 2 and 3).

Given our large sampling size (3398 seeds from 619 plants; this and the study on *H. alpinum* by P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.), the absence of polyploid progeny in diploid *Hieracium* taxa is noteworthy and deserves attention. Polyploid progeny can be formed by diploids by two principal pathways: either within diploids through fusion of unreduced and reduced gametes (Ramsey and Schemske, 1998), or in intercytotype crosses with polyploids producing diploid or higher ploidy pollen. In both cases, the fusion of reduced and unreduced gametes might lead to the abortion of seed progeny because of disruption of the balanced 2:1 maternal to paternal genome ratio in the endosperm, which is required for normal seed development (triploid block effect; Marks, 1966; Köhler *et al.*, 2010; Dobeš *et al.*, 2017). However, our heteroploid crosses did not confirm the negative effect of unbalanced genome dosage on the viability of seeds in *Hieracium* (Mráz, 2003a; P. Mráz, CU, Prague, Czechia, unpubl. res.). Therefore, the possible effect of triploid block seems not to be a very straightforward explanation for such a pattern. Rather, the absence of polyploid progeny could be associated with either an extremely low frequency of unreduced gametes produced by diploids (<0.03 %, i.e. a maximum of one event from 3398 + 1 seeds analysed from diploid taxa) and/or a low production of functional pollen of co-occurring polyploid species. This very low rate or absence of unreduced gamete formation in diploids could also explain the extreme scarcity of autopolyploids in *Hieracium*. In fact, with the exception of *H. alpinum*, which contains an autotriploid cytotype (Mráz *et al.*, 2009), probably all remaining diploid taxa in the strict sense (approx. 25 in total) are uniformly diploid (Chrtěk *et al.*, 2004, 2009), while most of the polyploids analysed so far are of allopolyploid origin (Fehrer *et al.*, 2009; Krak *et al.*, 2013). Similarly to our data, only diploid progeny, from 347 checked in total, were recovered in diploid × diploid experimental crosses in the closely related genus *Pilosella* (Asteraceae; Peckert and Chrtěk, 2006). Very low frequency of neotetraploids (<0.03 %) was recorded in diploid *Tripleurospermum perforatum* populations (Čertner *et al.*, 2017). In contrast, a non-negligible contribution of unreduced gametes to formation of functional polyploid progeny, whose frequency ranged between 0.1 and 0.3 %, was found in experimental intra- and interspecific diploid × diploid crosses in the genus *Malus* (Considine *et al.*, 2012). It has to be noted, however, that in the latter case >27 000 F₁ seedlings have been checked (Considine *et al.*, 2012).

Concerning heteropolyploid crosses, many polyploid *Hieracium* taxa show serious problems with microsporogenesis or they are even completely pollen sterile (Chrtěk, 1997a; Slade and Rich, 2007; Mráz *et al.*, 2009), making cross-pollination between polyploids and diploids rare or even impossible. Moreover, if such heteroploid cross-pollination eventually happens, a mixture of self- and heterospecific pollen might cause the breakdown of the otherwise strict self-incompatibility system, possibly resulting in self-fertilization, thereby eliminating the chance for the formation of neopolyploids (Mráz, 2003a; Mráz and Paule, 2006). Similarly to our results, sexual diploids of *Taraxacum* sect. *Taraxacum* occurring sympatrically with pollen producing diplosporic triploids, or pollinated by a mixture of pollen from diploids and triploids, formed only diploid progeny supposedly because of conspecific pollen preference, but also due to induced autogamy (Mártonfiová, 2006, 2015).

Obligate apomixis vs. residual sexuality in polyploids

Almost all seeds with polyploid embryos (99.6 % of 5478) analysed in the present study arose by the apomictic pathway with autonomous formation of endosperm, i.e. without fertilization of the central nucleus. If we consider in addition 2463 seeds produced apomictically by triploids of *H. alpinum* (P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.), the proportion of apomictic seeds formed by polyploid plants increases to 99.75 %. At the level of individuals, a solely apomictic mode of reproduction was found in 99 % of the analysed plants. When accounting also for *H. alpinum* triploids (502 plants, P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.), obligate apomixis was found in 99.4 % of so far tested polyploid plants (1183 in total). Our results thus show that apomixis is by far the prevailing mode of reproduction in polyploid representatives of the genus. This is in concordance with the cytoembryological observations by Hand *et al.* (2015) where most of the polyploid accessions produced only unreduced ESs, and with observations based on castration treatments (summarized in Table 1). Similarly, a very high proportion of unreduced ESs has been observed in *Erigeron* polyploids showing the same type of diplospory as *Hieracium* s.s. (Noyes and Givens, 2013). Though strict asexuality via seeds is considered to be disadvantageous from a longer evolutionary perspective due to accumulation of deleterious mutations (Asker and Jerling, 1992), it might provide obligate apomicts with greater reproductive assurance and consequently with better colonization ability as can be observed from current distributional patterns of representatives of diplosporic genera such as *Erigeron*, *Hieracium* or *Taraxacum* (Menken *et al.*, 1995; Noyes *et al.*, 2006; Mráz *et al.*, 2009).

The high frequency of obligate apomixis found in the present study should, however, be treated with caution, especially at the level of individual plants, given the relatively low number of analysed seeds (eight on average) per plant. This number thus represents only a small fraction of well-developed seeds produced by the plant and, therefore, rare sexual events might remain undetected. Taking into account huge interspecific variation in the number of florets (= potential seeds) per capitulum (20–110), the number of capitula per individual plant (1–100; the upper limit is a very rough estimate) and the mean seed set of plants from the field being around 75 % per capitulum (Zďvořák, 2017), we analysed roughly 0.1–30 % of well-developed seed progeny per plant, with an average of approx. 1–5 %. In a similarly conducted study focused on the frequency of apomixis in tetraploid *Hypericum perforatum* (Molins *et al.*, 2014), the estimated mean proportion of analysed progeny was, however, much lower than 0.1 %, considering 24 seeds analysed per plant (Molins *et al.*, 2014) which produces between 15 000 (the most conservative estimate) and 34 000 seeds on average (Tisdale *et al.*, 1959). Despite this, Molins *et al.* (2014) were able to show that, in addition to apomictically derived seeds (63 % of approx. 5900 seeds analysed in total), the tetraploid plants also produced seed sexually via double fertilization of reduced or unreduced ESs (21 and 10 % of seeds, respectively). While the frequency of apomixis at the individual level was not specified (Molins *et al.*, 2014), it is clear that facultative apomixis in *Hypericum perforatum* at the overall seed level is much more frequent than in *Hieracium* s.s. This is probably because *Hypericum perforatum* has an aposporic type of apomixis

combined with pseudogamy (Noack, 1939). As a consequence, tetraploid *Hypericum perforatum* has a very versatile mode of reproduction (Mártonfi *et al.*, 1996) due to the co-existence of reduced and unreduced (aposporic) ESs within the same ovule and their dependence on pollination of the central nucleus for the production of functional endosperm (Asker and Jerling, 1992; Koltunow, 1993). Unsurprisingly, relatively frequent facultative apomixis was detected using the FCSS approach also in various aposporic complexes such as the closely related genus *Pilosella* (Krahulcová *et al.*, 2014), *Crataegus* (Talent and Dickinson, 2007), *Poa* (Kelley *et al.*, 2009), *Potentilla puberula* (Dobeš *et al.*, 2013), *Ranunculus kuepferi* (Cosendai and Hörandl, 2010) and *Rubus* (Šarhanová *et al.*, 2012), but also in diplosporic *Taraxacum* sect. *Erythrosperma* (Mártonfiová *et al.*, 2010). In contrast, no traces of sexual reproduction were recorded in tetra- and pentaploids of *Pilosella alpicola* (Šingliarová *et al.*, 2011) or in several polyploid taxa in *Sorbus* (Hajrudinović *et al.*, 2015).

Hieracium accessions showing a mixed reproductive strategy belonged to four different taxa (microspecies) out of >50 analysed in this study. Importantly, a solely apomictic mode of reproduction has been found in other individuals co-occurring together with these ‘deviating’ individuals and belonging to the same species; specifically in *H. amplexicaule* at the HAMPL_2 site (30 apomictically derived seeds on one plant), in *H. macilentum* at the H5 site (115 apomictically derived seeds on eight plants), in *H. nigrescens* s.l. from population HSUB_1/C2_2015 (42 apomictically derived seeds on eight plants) and finally in *H. rapunculoides* at HPREN_sl_3 (25 apomictically derived seeds on five plants) (all these accessions were included in the summary in Table 2). Although this rareness of facultative apomixis at the level of plants could be biased by the relatively small number of seeds analysed per plant (see above), overall we found no traces of sexuality in several taxa, which were studied more intensively. Besides *H. alpinum* (502 triploid plants/2463 triploid seeds; P. Mráz *et al.*, CU, Prague, Czechia, unpubl. res.), only the apomictic mode of reproduction was also found in *H. laevigatum* s.l. (27 plants/251 seeds), *H. murorum* s.l. (108 plants/676 seeds) or *H. sabaudum* s.l. (96 plants/634 seeds), all collected at multiple sites (Table 2; Supplementary Data Table S1). Such a pattern might thus indicate taxon/population- or even genotype-specific patterns which would not be detected without a large-scale approach as applied in the present study. Similarly, a very strong taxon- or genotype-dependent pattern of reproduction/apomeiotic formation of the ovule was revealed in the closely related genus *Pilosella* (Krahulec *et al.*, 2008) or in the genus *Boechea* (Aliyu *et al.*, 2010).

Using FCSS, we convincingly proved facultative apomixis in seven individuals (Table 3), and possibly also in two other triploid plants which produced seedling progeny with an increased ploidy (Table 4). Overall, this rare sexual offspring was mostly formed as BIII progeny arising from pollination of unreduced female gametophytes (65 %), while participation of reduced ESs (BII hybrids) was rarer (35 %). Supposing that the first process could also be involved in the origin of four seedlings with an increased ploidy level (Table 4), this might indicate that fertilization of unreduced gametes could be more frequent/successful than the sexual process involving reduced female gametes, putatively suggesting problems with meiosis. However, given the very small number of sexual events recorded in total, this

pattern should be confirmed by further more targeted studies. The evidence for formation of so-called additive, or BIII hybrids in *Hieracium* s.s. is, however, of very high importance, as it helps us to understand the origin of higher ploidy levels in *Hieracium*, especially tetraploid and pentaploid levels. Both ploidies were produced by triploid and the latter also by tetraploid individuals. Therefore, the origin of two pentaploid taxa and a few singular pentaploid counts can be explained by this triploid/tetraploid bridge (Stace *et al.*, 1995; Chrtek, 1996; Tyler and Jönsson, 2009; Musiał and Szeląg, 2015). Moreover, because these pentaploid taxa/counts have been reported from geographically isolated regions (the Scottish Highlands, the Krkonoše Mts, the Harz Mts or Southern Scandinavia), this fact and the possibility of facultative reproduction revealed by this study suggest their *in situ* origin from plants able to reproduce sexually. As for potential pollen donors involved in the origin of both BII and BIII hybrids, they probably encompassed co-occurring pollen-producing plants belonging to the same or other polyploid or diploid taxa. Therefore, these seed progeny could represent intra- or interspecific hybrids, which was not assessed (phenotypically or molecularly) because of the destructive flow cytometric method. Previously, the formation of BIII progeny was suggested for triploid *H. aragonense*, which produced, in addition to triploid progeny of likely apomictic origin, also tetraploid progeny probably originated from fusion of unreduced triploid ovule and haploid pollen (Castro *et al.*, 2007).

Frequency of spontaneous natural hybridization in *Hieracium* s.s.

Previously, only hybridization between sexual diploid taxa, and between sexual diploids as seed parents and pollen producing polyploid apomictic taxa have been considered (Mráz *et al.*, 2005). While recent natural hybridization between diploid taxa has been recorded in only a few cases (Mráz *et al.*, 2005, 2011; Chrtek *et al.*, 2006), the diploid \times polyploid hybridization has never been proved in the field despite the success of both types of hybridizations in control crosses (Mráz, 2003a; Mráz and Paule, 2006). The rarity of interspecific hybridization in the field has been explained by several mutually non-exclusive phenomena which include: (1) frequent geographical and/or ecological allopatry of diploid taxa; (2) induced autogamy in otherwise strictly self-incompatible diploids; and (3) frequent male sterility and precocious embryony suggesting the prevalence of obligate apomixis in polyploids (Mráz *et al.*, 2005). Our finding that certain apomictic polyploid lineages are able to reproduce sexually as seed parents suggests that precocious embryony might not be so strict. The lack of precocious embryony alongside rare meiotic events during production of female gametophytes detected in several polyploid accessions thus widens the possibility for both, intra- and interspecific hybridization in polyploids.

Conclusions and further perspectives

Our quantitative data revealed that diploid taxa reproduced exclusively by sexual reproduction, whereas asexual reproduction only occurred in polyploids. Strict asexuality via seeds might provide obligate apomicts with higher reproductive

assurance and therefore with better colonization ability. Facultative apomixis has been found only in a few polyploid plants. Nevertheless, despite its rarity and apparent genotype-specific pattern, the detection of facultatively apomictic accessions is extremely important from two points of view: first, it helps us to better understand evolutionary pathways leading to the formation of cytotype and genotype diversity in the genus; and, secondly, it opens up new perspectives to studies of the evolution and ecological importance of apomixis. Specifically, newly detected genotypes/populations with facultative apomixis will allow us to (1) estimate the proportion of reproductive pathways more accurately because of more targeted effort encompassing more progeny and seed plants; (2) study the role of precocious embryony as an important reproductive barrier in apomictic polyploids in more detail; (3) assess the fate of higher ploidy (BIII) hybrids; (4) unravel the transmission and inheritance of apomictic elements using experimental crosses which will include facultatively apomictic lineages; and, finally (5) to infer gene expression changes associated with hybridization at the polyploid level.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of Table S1: geographical origin of *Hieracium* s.s. taxa analysed in the present study.

ACKNOWLEDGMENTS

We thank J. Chrtek, E. Di Gristina, P. Kučera, V. Mrázová, A. Nowak, M. Štefánek, Z. Szélag and J.-M. Tison for help with sampling of seed material or determining the plants; R. Bartošová, M. Hartmann, V. Mrázová, J. Pinc, H. Ryšavá and L. Vlk for help with flow cytometric analyses; J. Chrtek, J. Fehrer and M. Hartmann for valuable comments on an earlier version of manuscript; T.C.G. Rich for revision of the English text; and J. Karron, T.C.G. Rich and one anonymous reviewer for their useful comments on the submitted version. The study was financially supported by the Grant Agency of the Czech Republic (projects # 14-02858S & 17-14620S).

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