Heredity (2015) 114, 17–26

www.nature.com/hdy

ORIGINAL ARTICLE

Evolution of apomixis loci in *Pilosella* and *Hieracium* (Asteraceae) inferred from the conservation of apomixis-linked markers in natural and experimental populations

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The *Hieracium* and *Pilosella* (Lactuceae, Asteraceae) genera of closely related hawkweeds contain species with two different modes of gametophytic apomixis (asexual seed formation). Both genera contain polyploid species, and in wild populations, sexual and apomictic species co-exist. Apomixis is known to co-exist with sexuality in apomictic *Pilosella* individuals, however, apomictic *Hieracium* have been regarded as obligate apomicts. Here, a developmental analysis of apomixis within 16 *Hieracium* species revealed meiosis and megaspore tetrad formation in 1 to 7% of ovules, for the first time indicating residual sexuality in this genus. Molecular markers linked to the two independent, dominant loci *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*) controlling apomixis in *Pilosella piloselloides* subsp. *praealta* were screened across 20 phenotyped *Hieracium* individuals from natural populations, and 65 phenotyped *Pilosella* individuals from natural and experimental cross populations, to examine their conservation, inheritance and association with reproductive modes. All of the tested *LOA* and *LOP*-linked markers were absent in the 20 *Hieracium* samples irrespective of their reproductive mode. Within *Pilosella*, *LOA* and *LOP*-linked markers were essentially absent within the sexual plants, although they were not conserved in all apomictic individuals. Both loci appeared to be inherited independently, and evidence for additional genetic factors influencing quantitative expression of *LOA* and *LOP* was obtained. Collectively, these data suggest independent evolution of apomixis in *Hieracium* and *Pilosella* and are discussed with respect to current knowledge of the evolution of apomixis.
Heredity (2015) **114,** 17–26; doi:10.1038/hdy.2014.61; published online 16 July 2014

INTRODUCTION

The process of apomixis (asexual seed production) has been studied in various plant species, not only for its potential value to agricultural plant breeding, but also for its intriguing role in plant evolution. Apomixis is developmentally variant and has evolved independently many times across different angiosperm plant families (Carman, 1997). Although sexual seed formation generates variation in progeny through meiosis and fertilization, gametophytic apomictic processes produce chromosomally unreduced eggs in female gametophytes (or embryo sacs) through the avoidance of meiosis (apomeiosis). Embryos develop from the unreduced eggs in the absence of fertilization (termed parthenogenesis) and endosperm formation occurs with or without fertilization. The progeny resulting from apomictic reproduction always retain a maternal genotype (Bicknell and Koltunow, 2004; Ozias-Akins, 2006).

Gametophytic apomixis is separated into two types: apospory and diplospory, depending on the type of cell that gives rise to the chromosomally unreduced embryo sac. In diplospory, the diploid megaspore mother cell (MMC), which usually undergoes meiosis in sexual reproduction, develops into an embryo sac in the absence of meiosis (Tucker and Koltunow, 2009). During apospory, an unreduced embryo sac develops from an ovule cell, called an aposporous initial (AI) cell, positioned adjacent to sexual embryo sac precursors. In both forms of gametophytic apomixis, embryo development is fertilization independent via parthenogenesis, while endosperm formation may or may not require fertilization (Koltunow *et al.*, 2013).

The *Hieracium* and *Pilosella* genera of hawkweeds (previously treated as *Hieracium* subgenus *Pilosella*) contain closely related species of Eurasian origin, which have evolved via numerous hybridization and polyploidization events. Both genera contain mostly polyploid species and few diploid species (Fehrer *et al.*, 2007a; Krak *et al.*, 2013). Sexual and apomictic species are found in both genera (albeit at different frequencies), but the route taken to form chromosomally unreduced embryo sacs is diplosporous in *Hieracium* and aposporous in *Pilosella*. In both apomictic *Hieracium* and *Pilosella*, embryo and endosperm formation are fertilization independent. To date, apomictic *Hieracium* species have been considered near-obligate apomicts, appearing to produce only clonal maternal progeny.

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Received 28 January 2014; revised 30 May 2014; accepted 3 June 2014; published online 16 July 2014

However, apomixis has not been examined extensively in *Hieracium* species, having been documented in 12 species by Bergman (1941), and in a single species (*H. alpinum* L.) by Skawińska (1963). Some polyploid *Hieracium* are pollen fertile but abnormal pollen development has also been reported, resulting in male sterile plants (Mráz *et al.*, 2005; Slade and Rich, 2007). *Hieracium* experimental hybrids also set very few seed or are completely seed sterile (Mráz and Paule, 2006). As a result, *Hieracium* species have not been a major focus for genetic analyses of apomixis.

By contrast, *Pilosella* has been developed as a model system for the analysis of apomixis occurring by aposporous gametophyte formation coupled with fertilization-independent seed formation. A suite of characterized accessions, mutants and genetic and molecular tools have been developed to identify causal genes (Bicknell and Koltunow, 2004; Koltunow *et al.*, 2013). Unlike *Hieracium*, inter-cytotype and inter-specific crosses are common in nature for *Pilosella*, and experimental crosses confirm that reproductive barriers are almost completely absent (for example, Fehrer *et al.*, 2005, 2007b; Okada *et al.*, 2011).

Pilosella apomicts are facultative apomicts meaning that they retain a capacity to form a percentage of progeny via the sexual pathway. This results in three additional types of non-apomictic progeny termed 'off-type' or 'variable'. The nomenclature of Harlan and deWet (1975) designates the dominant apomictic progeny as $2n+0$ in relation to female and male gamete ploidy and parental contribution, respectively. Progeny also result from the fertilization-independent development of an embryo from a meiotically reduced egg and are designated as $n+0$ individuals, in addition to sexual progeny designated as $n+n$. The chromosomally unreduced eggs that develop into aposporous embryo sacs can also be fertilized to produce progeny of increased ploidy designated as $2n+n$ (Bicknell *et al.*, 2003). Facultative apomictic *Pilosella* species produce meiotically reduced fertile pollen and can act as both male and female parents in hybridizations (Fehrer *et al.*, 2007b). Therefore, if two or more *Pilosella* species/cytotypes occur in a population, they can form hybrid swarms in which the parental biotypes, their hybrids and the products of multiple hybridizations (including backcrosses) produce complicated population structures containing plants of differing ploidy levels (Krahulcová *et al.*, 2009).

Taxonomy of *Hieracium* and *Pilosella* is complex and the classifications of Nägeli and Peter (1885) and Zahn (1921–1923) are used to define basic and intermediate species. According to this classification system, the basic species have unique morphology, can be sexual or apomictic, and are considered as the fundamental evolutionary units or progenitors of the intermediate species. Some basic species can have ancient hybrid/allopolyploid origin (Fehrer *et al.*, 2007a, 2009). By contrast, intermediate species combine morphological characters of two or more basic species and are thought to be derived from hybridizations between basic species. They are usually polyploid and are considered as 'stabilized' taxa because of their predominant apomictic mode of reproduction. They often occupy sizeable areas that are independent of the parental species. Within *Pilosella*, continuous hybridization also generates recent hybrids that usually co-occur with their parents. They are often represented by only a few individuals within the hybridizing population and produce the highest number of 'variable' progeny, as apomixis appears less penetrant allowing higher frequencies of sexual seed formation (Fehrer *et al.*, 2007b; Krahulec *et al.*, 2008). Within *Pilosella*, chloroplast DNA (cpDNA) sequences are able to define two distinct and divergent haplotype groups, designated *Pilosella* I and *Pilosella* II (Fehrer *et al.*, 2007a; Koltunow *et al.*, 2011).

Genetic and molecular analyses of apomixis in *Pilosella* have utilized apomicts producing >93% apomictic progeny. Two dominant independent loci required for functional apomixis have been identified in R35, a specific isolate of *P. piloselloides* ssp. *praealta* (formerly treated as *Hieracium praealtum*) maintained as a vegetative clone by micropropagation (Catanach *et al.*, 2006; Koltunow *et al.*, 2011). The *LOSS OF APOMEIOSIS* (*LOA*) locus in R35 is required for apospory, including differentiation of AI cells and suppression of the sexual pathway, while the *LOSS OF PARTHENOGENESIS* (*LOP*) locus enables both autonomous embryo and endosperm development. Both loci are transmitted via pollen, and markers linked to both loci have been developed. Markers linked to *LOA* are non-genic although unique to the *LOA* region, and both genic and non-genic markers linked to *LOP* have been developed (Catanach *et al.*, 2006; Koltunow *et al.*, 2011; Okada *et al.*, 2011). The *LOA* locus is subtelomeric on the long arm of a hemizygous chromosome and is associated with extensive repeats that extend along the long chromosome arm, although these extensive repeats are not essential for *LOA* function in R35 (Kotani *et al.*, 2014). The chromosomal location of *LOP* is unknown. Autonomous endosperm formation is controlled by the independent locus *AutE* in *P. piloselloides* isolate D36 (formerly *H. piloselloides*) and is genetically separable from *LOA* and fertilization-independent embryogenesis. Markers linked to *AutE* and its chromosomal location remain to be identified (Ogawa *et al.*, 2013). The genes responsible for apomixis function at all three loci are unknown.

An initial, limited survey in *Pilosella* identified that the repeat-rich *LOA*-carrying chromosome structure and *LOA*-linked repetitive markers were also conserved in an isolate of *P. caespitosa* (C36; formerly *H. caespitosum*) and in two isolates of *P. piloselloides* (tetraploid D36 and diploid, D18). All examined plants belong to the *Pilosella* II cpDNA haplotype group. In the R35 and C36 plants, the *LOA*-carrying chromosome is significantly elongated but this is not the case for the D36 and D18 plants (Okada *et al.*, 2011). The elongated *LOA*-carrying chromosome and linked markers were not detected in two apomictic *P. aurantiaca* (formerly *H. aurantiacum*) isolates (A35; A36, both containing *Pilosella* I cpDNA haplotype) investigated or a sexual *P. officinarum* (formerly *H. pilosella*) isolate (P36). This initial survey therefore revealed a tentative association between apospory-linked *LOA* marker presence and the *Pilosella* II cpDNA haplotype (Fehrer *et al.*, 2005, 2007a; Okada *et al.*, 2011).

In this study, we sought to determine whether sexuality also co-exists with apomixis in the *Hieracium* genus, as is the case for *Pilosella*. We cytologically examined apomixis in 16 *Hieracium* species. We then utilized a collection of molecular markers linked to apospory (*LOA*) and autonomous seed development (*LOP*) in *P. piloselloides* ssp. *praealta* to examine the extent of their association with apomixis and its penetrance in *Hieracium* and *Pilosella* individuals naturally sourced across Europe. In addition, *LOA* and *LOP* marker presence was assessed in experimentally derived progeny obtained from crosses between *Pilosella* species to study inheritance of the loci. All *Pilosella* individuals were characterized with respect to their reproductive mode, apomixis penetrance, ploidy level, cpDNA haplotype and species/hybrid origin. This study is one of the first to explore the incidence of apomixis-linked markers in natural populations and has provided insight into the inheritance, penetrance and evolution of apomixis components in *Pilosella* and *Hieracium*.

MATERIALS AND METHODS

Plant material

Seeds from a total of 20 individuals representing 16 different sexual and apomictic *Hieracium* species collected in Europe (Table 1) were grown under

Table 1 Analysis of embryo sac development and apomixis loci-associated molecular marker presence in *Hieracium* accessions

Species	*Individual*	*Ploidy*	*% Ovules undergoing meiosis (n)*[a]	*% Embryo sacs containing:*			*Mode of reproduction*	*Pollen morphology*	*Number of LOA markers present*	*Number of LOP markers present*	*Country*[b]	*Status*[c]
				Early embryo	*Globular embryo*	*Heart/ torpedo embryo*						
H. amplexicaule	1242	4×	2% (50)	0	69	21	Diplosporous	Degenerated	0/14	0/3	SW	BS
H. bifidum	885/2	4×	2.5% (80)	0	31	26	Diplosporous	Degenerated	0/14	0/3	SK	BS
	VZ 39/1	3×	0% (90)	0	37	59	Diplosporous	Degenerated	0/14	0/3	CZ	BS
	VZ 21/25	4×	7% (43)	0	51	33	Diplosporous	Degenerated	0/14	0/3	CZ	BS
H. caesium	1231[d]	4×	ND	ND	ND	ND	Diplosporous	Degenerated	0/14	0/3	SE	BS
H. humile	1064[d]	4×	1.5% (132)	0	22	54	Diplosporous	Degenerated	0/14	0/3	AT	BS
H. laevigatum	1031/11[d]	3×	0% (97)	0	62	32	Diplosporous	Degenerated	0/14	0/3	CZ	BS
H. murorum	1153/4	3×	1% (89)	21.5	33	43	Diplosporous	Degenerated	0/14	0/3	CZ	BS
H. nigrescens	1011	4×	ND	ND	ND	ND	Diplosporous	Degenerated	0/14	0/3	CZ	IS
H. olympicum	1206/5[d]	3×	2% (53)	0	19	75	Diplosporous	Degenerated	0/14	0/3	BG	BS
H. sabaudum	1098/2[d]	3×	ND	0	7	80	Diplosporous	Degenerated	0/14	0/3	DE	BS
	1254	3×	0% (89)	5	37	21	Diplosporous	Degenerated	0/14	0/3	CZ	BS
H. villosum	1036	4×	0% (78)	2	72	15	Diplosporous	Degenerated	0/14	0/3	SK	BS
H. virosum	1238/1[d]	3×	1% (97)	0	18	25	Diplosporous	Degenerated	0/14	0/3	RU	BS
H. saxifragum	843	4×	0% (90)	6	28	56	Diplosporous	Degenerated	0/14	0/3	CZ	IS
H. eriophorum	1223	2×	96% (79)	ND	ND	ND	Sexual	Intact	0/14	0/3	FR	BS
H. intybaceum	Int 25	2×	100% (92)	0	0	0	Sexual	Intact	0/14	0/3	AT	BS
H. tomentosum	1066/6	2×	98% (84)	0	0	0	Sexual	Intact	0/14	0/3	FR	BS
H. umbellatum	1162/2	2×	97% (67)	0	0	0	Sexual	Intact	0/14	0/3	CZ	BS
	1244	2×	93% (43)	ND	ND	ND	Sexual	Intact	0/14	0/3	DE	BS

Abbreviations: *LOA*, *LOSS OF APOMEIOSIS*; *LOP*, *LOSS OF PARTHENOGENESIS*; ND, not determined.
[a]Scored at developmental stage 4 (Koltunow *et al*., 1998).
[b]Country codes: FR, France; CZ, Czech Republic; SK, Slovakia; BG, Bulgaria; DE, Germany; AT, Austria; SW, Switzerland; SE, Sweden; RU, Russia; see also Supplementary Table 1 for more details.
[c]Species status: BS, basic species; IS, intermediate species/stabilized hybridogenous taxon.
[d]Accession used in Fehrer *et al*. (2009) and Krak *et al*. (2013).

quarantine conditions in Australia as described previously for *Pilosella* (Koltunow *et al*., 1998). A total of 65 *Pilosella* plants designated as basic and intermediate species were selected for analysis including recent and experimental hybrids. Species identifications followed the nomenclature of Bräutigam and Greuter (2007) and Bräutigam (2012). The parental origins of the hybrid individuals were inferred based on the recreation of the hybrid phenotype through experimental crosses. Plants were cultivated under identical conditions in the Experimental Garden of the Institute of Botany, Průhonice and details concerning nomenclature of the taxa, geographic origins and voucher information for all plants are provided in Supplementary Table 1.

Cytology, reproduction and penetrance of apomixis

The DNA ploidy level (relative genome size) of examined plants was estimated from leaf samples by flow cytometry as described in Krahulcová *et al*. (2004). Capitula of *Hieracium* plants were staged in terms of morphology as previously described for *Pilosella* species, and the ovaries were examined at a range of developmental stages as reported previously (Koltunow *et al*., 1998). Karyotypes in *Pilosella* were examined using root tip squashes as described by Krahulcová and Krahulec (1999) and chromosome numbers and the presence or absence of an elongated chromosome were scored.

The 65 plants of the *Pilosella* genus representing basic or intermediate species and recent hybrids were screened for apomixis by decapitation of at least three closed capitula to remove anthers and stigmas before floral opening. Under these conditions apomicts set seed, whereas sexual plants do not (Krahulcová *et al*., 2004). This test is an approximation for apomictically reproducing plants (Gadella, 1984) because fertilization-independent seed formation is predominantly coupled with apomeiosis (apospory) in wild-type apomicts of *Pilosella* (Bicknell and Koltunow, 2004). For a more precise determination of the reproductive pathway, the flow cytometric seed screen method was used (Krahulcová *et al*., 2011). Seeds were harvested from both decapitated capitula (to remove stigma and anthers) and also uncut capitula for comparative seed screening. This method was capable of distinguishing between apomictic plants that had undergone apospory and autonomous seed development ($2n+0$) from those which had formed a substantial number of their progeny (embryos) via either haploid parthenogenesis ($n+0$; meiosis with fertilization-independent embryogenesis), the sexual pathway ($n+n$) or following fertilization of a chromosomally unreduced egg ($2n+n$). This enabled an assessment of the penetrance of apospory. *Pilosella* plants were determined to have a low penetrance of intact, functional apospory when $<75\%$ of the progeny were of $2n+0$ origin. Significant efforts were made to obtain multiple apomictic and sexual plant samples per species type. This was not always possible because of the detailed phenotyping and ploidy analyses required for natural accessions over the period of collection.

Screening with *LOA* and *LOP*-linked apomixis markers

In total, 14 *LOA* and 3 *LOP*-associated markers derived from *P. piloselloides* ssp. *praealta* R35 were screened on the *Hieracium* species, while a smaller set of markers (6 *LOA* and 1 *LOP* associated) were used to screen the larger group of *Pilosella* plants. Marker identity, primer sequences and PCR conditions are specified in Supplementary Table 2. PCR products were scored on 2% agarose gels as presence or absence of a single strong band of the expected length. Positive and negative controls were included for each PCR (sample R35 and ddH_2O, respectively), and at least two technical repetitions were undertaken per sample.

CpDNA and *LOP* haplotype analysis

The *trn*T-*trn*L intergenic spacer of cpDNA was used to determine the maternal origin of the examined *Pilosella* plants. Amplification, purification and sequencing of this marker were performed as described in Fehrer *et al.* (2007a). Assignments of haplotype subgroups, as far as applicable, correspond to Fehrer *et al.* (2005). The LOP93 marker was examined for its sequence diversity among the different species because of its longer fragment size and genic nature. Sequence editing, alignment and indel coding were performed as described in Fehrer *et al.* (2007a). Median Networks for *trn*T-*trn*L and LOP93 sequences were constructed as implemented in Splitstree 4.11.3 (Huson and Bryant, 2006) with the default settings.

RESULTS

Apomictic *Hieracium* species show mitotic diplospory with residual sexuality

Reproductive events were examined in 4 sexual and 12 apomictic *Hieracium* species (Table 1) by ovule clearing. Figure 1 shows reproductive events in developing unfertilized ovules and anthers of sexual *H. umbellatum*, diplosporous *H. murorum* and, for comparison, aposporous *P. piloselloides*. Events of female gametophyte development in sexual plants were identical to those found in sexual members of *Pilosella*. A single MMC formed (Figure 1a), underwent meiosis (Figure 1b), megaspore selection and functional megaspore enlargement (Figure 1c), followed by mitotic events to form a 7-celled 8-nucleate embryo sac. Embryo and endosperm formation did not initiate in ovules of unpollinated sexual *Hieracium* plants. In the diplosporous species examined, the MMC (Figure 1e) did not undergo meiosis in most of the ovules examined, but underwent mitosis to form an embryo sac typical of the events of mitotic diplospory of the *Antennaria*-type. In 7 of the 15 diplosporous accessions, a small percentage of MMCs (1–7%) underwent meiosis forming a tetrad of megaspores, demonstrating that diplospory is not obligate (Figure 1f). The remaining MMCs underwent three rounds of mitosis (Figure 1g) and subsequent cellularization and differentiation, forming a mature embryo sac (Figure 1h). Embryo and endosperm formation was fertilization independent in diplosporous embryo sacs and occurred at floret opening in 95% of cases with the remainder occurring before floret opening.

In aposporous *Pilosella* species, the MMC progressed through meiosis with AI cells stochastically appearing near cells undergoing meiosis (Figure 1l). The AI cell expanded toward the sexual cells as it underwent mitosis and the sexual pathway ceased. Embryo and endosperm formation was fertilization independent and highly precocious with 35% of embryos initiating embryogenesis before floret opening (stage 6 of capitulum development; Figure 1m). Sexual *Hieracium*, sexual *Pilosella* and apomictic *Pilosella* accessions produced large quantities of pollen with regular morphology, while pollen formation aborted in most of the diplosporous accessions (Figures 1n–p).

The penetrance of apomixis is variable within facultative *Pilosella*

Sampling of *Pilosella* was designed to represent a diverse range of accessions within the genus. Analyses of ploidy level and reproductive mode for each of the included individuals confirmed intra-specific diversity for both of these traits (Supplementary Table 3). The basic species sampled generally contained multiple cytotypes (up to four), and most basic species contain both apomictic and sexual individuals. This level of diversity observed in the basic species with regards to ploidy level and reproductive mode, indicates that intra-specific hybridization has been frequent.

Variability in the penetrance of apomixis was also observed. Twelve of the 13 apomictic accessions of basic and intermediate species displayed high penetrance of apomixis producing $>75\%$ of $2n+0$ clonal progeny. By contrast, most $2n+n$ recent hybrids (14 out of 15 accessions analyzed) had a lower penetrance of apomixis and thus produced more polyhaploid $(n+0)$ and sexual $(n+n)$ progeny in variable ratios with the frequency of apomictic $(2n+0)$ progeny being $<75\%$ of seed set (Supplementary Table 3).

Distribution of cpDNA haplotypes throughout *Pilosella* accessions

CpDNA haplotypes were determined for each included *Pilosella* individual to understand the maternal lineages of the natural and experimental hybrids, and to examine haplotype distributions across different species. Overall, eight distinct haplotype subgroups were observed (Figure 2a), three of them within the previously defined major group *Pilosella* I, and five within *Pilosella* II (Fehrer *et al.*, 2005). The majority of the basic species included belonged to the *Pilosella* II group, with only two basic species attributed to *Pilosella* I. Individuals of *P. aurantiaca* occurred in both of the haplotype groups; four displayed a *Pilosella* I haplotype (as was expected based on previous studies), and the remaining two belonged to the *Pilosella* II cpDNA group. Extensive sharing of cpDNA subtypes among species as well as the occurrence of different subtypes within a species is evident, reflecting their recent speciation and ease with which they can hybridize and introgress.

In those cases when the parental species were sufficiently uniform at the intra-specific level but sufficiently distinct from each other, the maternal progenitor of the intermediate species and recent hybrids could be inferred using the cpDNA sequence (Supplementary Table 3). This was the case for three of the five included intermediate species (*P. floribunda*, *P. iserana* and *P. rubra*), whose progenitor species belong to different haplotype groups. The maternal progenitor could also be inferred for all of the recent hybrids, with the exception of three *P. bauhini* × *P. officinarum* accessions (1433, 1647 and 1015), which have a cpDNA haplotype (II/7 and II/8) found within both progenitor species (Supplementary Table 3).

Extent of conservation of apomixis-linked markers in *Hieracium* and *Pilosella*

Sexual and diplosporous *Hieracium* species were screened with 14 markers that are non-genic, and specifically linked to the apospory locus *LOA*, in addition to three markers specifically linked to the autonomous seed initiation locus *LOP* in *P. piloselloides* ssp. *praealta* (Supplementary Table 2). None of the *LOA* and *LOP* markers were detected in the sexual and diplosporous *Hieracium* species (Table 1).

For *Pilosella*, a reduced subset of six *LOA*-linked markers was used because of the larger sample size, in addition to the LOP93 genic marker, which is most tightly linked to the *LOP* locus (Koltunow *et al.*, 2011). The inferred origins of intermediate species and recent hybrids from basic parental species in the *Pilosella* plants under examination are summarized in Figure 3. Screening of the *LOA*-linked markers revealed the expected marker patterns for the basic species, which have been examined in previous studies (Figure 3). All *LOA* markers were present as expected in the apomicts *P. piloselloides* ssp. *praealta* (R35) and *P. caespitosa*, but absent in the apomict *P. aurantiaca* and the sexual cytotypes of *P. officinarum*. *Pilosella glacialis*, a basic apomictic species collected from France, did not contain any of the tested markers, nor did the apomictic *P. officinarum* individuals. The *LOA* markers were only present in apomictic basic species of the *Pilosella* II cpDNA haplotype group, although not all apomictic *Pilosella* II individuals contained the *LOA*

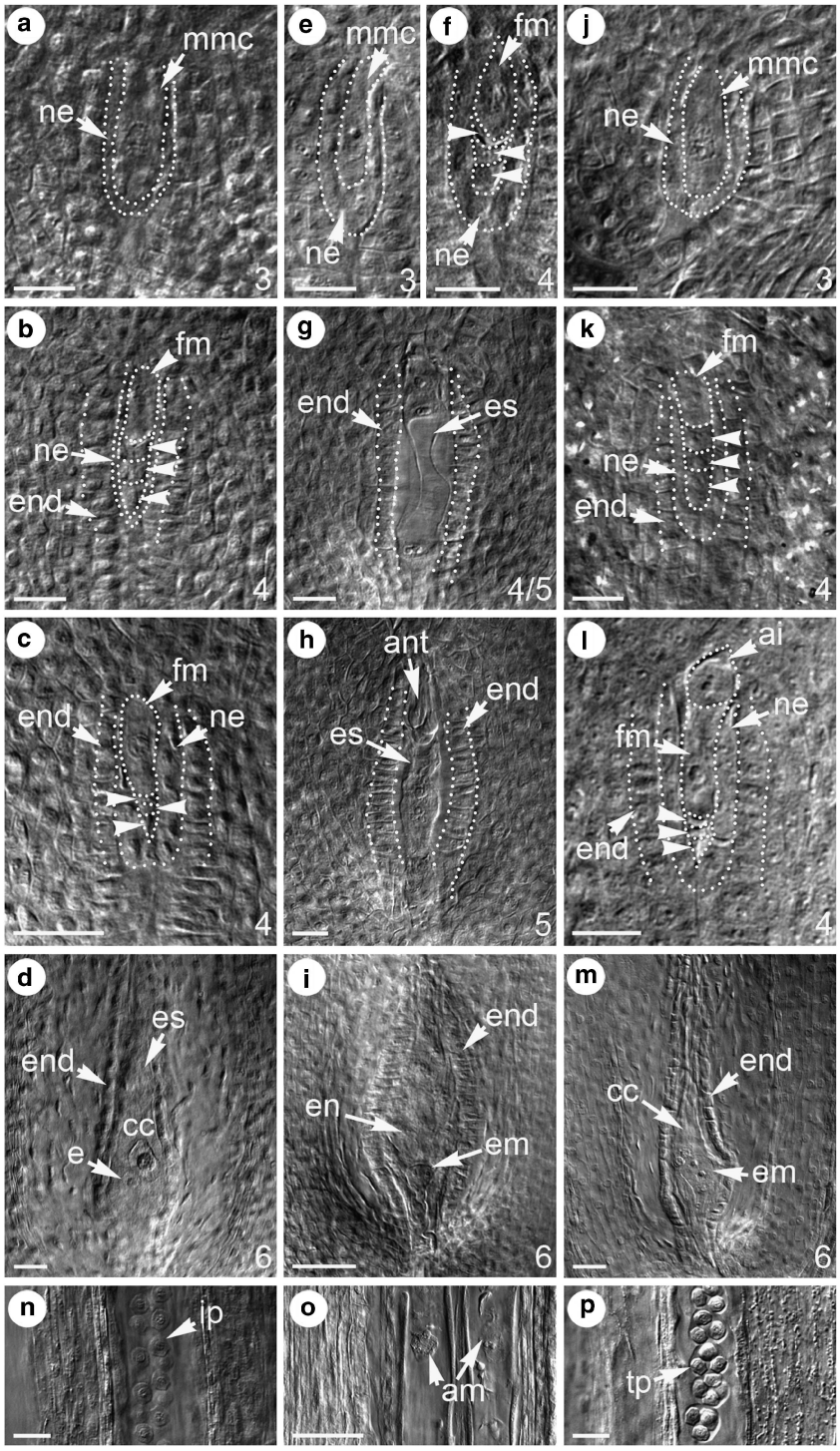

Figure 1 Female gametophyte development in ovules of sexual, diplosporous and aposporous species. Figures **a** to **m** show female gametophyte development in ovules of sexual *Hieracium umbellatum* (**a**–**d**), diplosporous *Hieracium murorum* (**e**–**i**) and aposporous *Pilosella piloselloides* (**j**–**m**). Figures **n**, **o** and **p** show pollen typical of *H. umbellatum*, *H. murorum* and *P. piloselloides*, respectively. Sexual mmc (**a**) undergoes meiosis to form four megaspores (**b**), three of which degrade leaving a functional megaspore (**c**). This functional megaspore undergoes mitosis to form the mature female gametophyte at stage 6 (**d**). In the diplosporous plant the mmc (**e**) usually avoids meiosis and undergoes two mitotic divisions to form a four nucleate embryo sac (**g**), followed by a further division to form the mature gametophyte (**h**) by stage 5. At stage 6, a precocious embryo and endosperm have formed (**i**). In some ovules of *H. murorum*, the mmc follows the sexual pathway and undergoes meiosis to form four megaspores (**f**). In the aposporous plant, the mmc (**j**) undergoes meiosis to form four megaspores (**k**). A cell adjacent to the functional megaspore develops into an aposporous initial (**l**), which then undergoes mitosis to form the female gametophyte and displaces all the products of meiosis. This has formed a precocious embryo at stage 6 (**m**). ai, aposporous initial; am, aborted microspores; ant, antipodal; cc, central cell; e, egg cell; em, embryo; en, endosperm; end, endothelium; es, embryo sac; fm, functional megaspore; ip, immature pollen; mmc, megaspore mother cell; ne, nucellar epidermis; tp, tetrad pollen. Unlabelled arrowheads indicate degrading megaspores. The ovule stage appears in the bottom right of each figure. Scale bars represent 20 μm with the exception of sections **l**, **m** and **o**, which correspond to 50 μm.

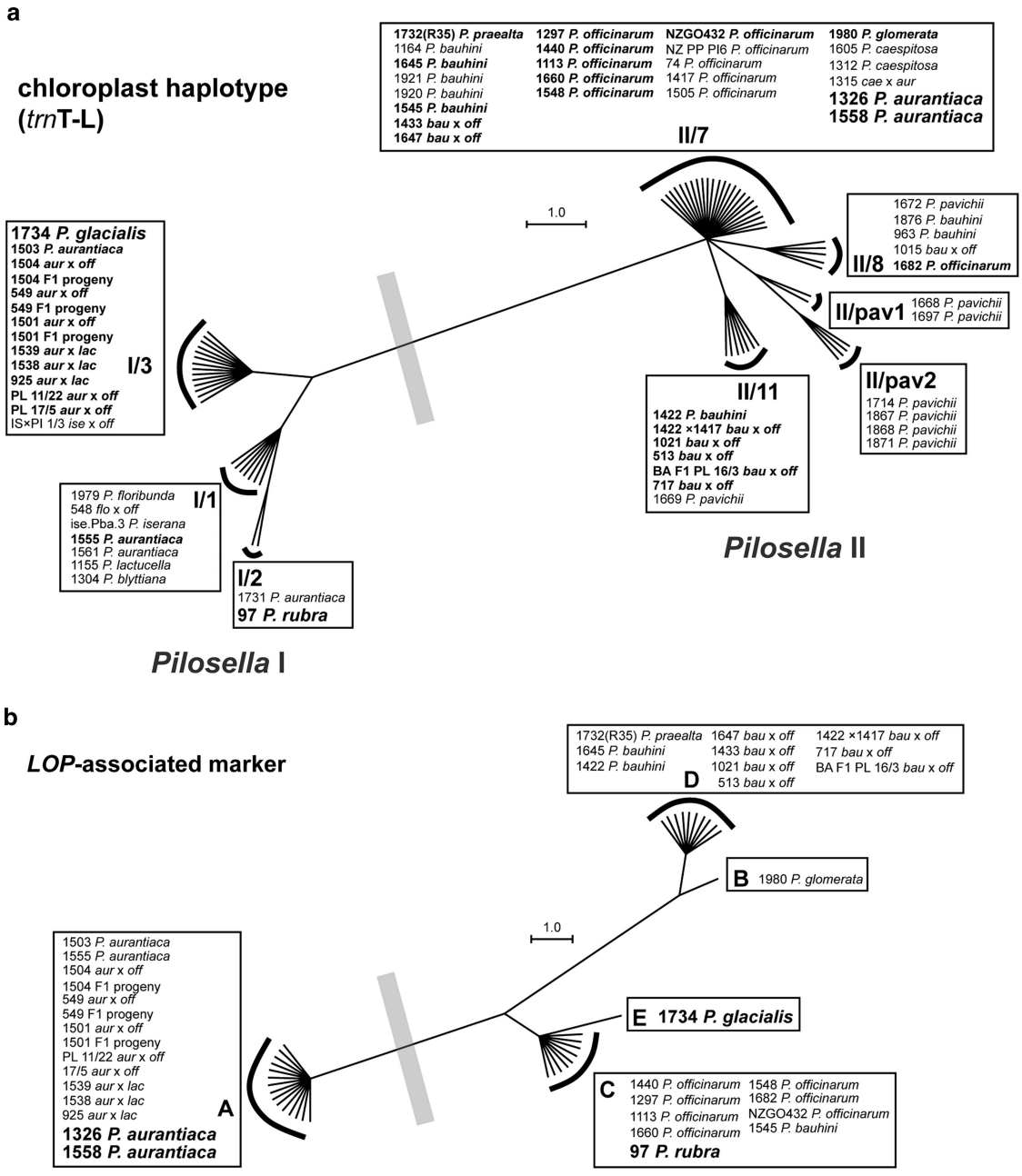

Figure 2 Median networks of chloroplast haplotype (**a**) and LOP93 marker (**b**) sequences. (**a**) The major cpDNA haplotype groups *Pilosella* I and *Pilosella* II are shown along with an assignment of individual plants to subtypes within each group according to Fehrer *et al*. (2005) (see also Supplementary Table 3). Those that are LOP93 positive are indicated in bold. (**b**) Five haplotypes (A–E) of the *LOP*-associated marker can be distinguished. (**a**, **b**) The grey bar indicates a general correspondence of taxa possessing the major cpDNA haplotype group *Pilosella* I and LOP93 haplotype A versus *Pilosella* II and LOP93 B–E. Four individuals not matching this pattern (*P. glacialis* 1734, *P. aurantiaca* 1326 and 1558, *P. rubra* 97) are shown in bold and larger font in both networks. They indicate transmission of *LOP* via pollen.

markers (Figure 3). No correlation between *LOA* marker presence and cpDNA haplotype was therefore observed.

LOA-linked markers were absent in sexual *Pilosella* plants, with the exception of two individuals that possessed a single marker (14-T7, Supplementary Table 3). These two individuals belong to the *P. piloselloides* aggregate species *P. bauhini* and *P. pavichii*. *LOA* marker presence in these two species is variable and may indicate that recombination at the *LOA* locus has accompanied intra-specific hybridization. Apomictic *P. bauhini* individuals have either all, some (one to three) or none of the *LOA* markers. Similarly, all apomictic *P. pavichii* individuals contained only two to four *LOA* markers, with an inconsistent pattern observed between the individuals (Supplementary Table 3).

An elongated chromosome carries the *LOA* locus in *P. caespitosa* and *P. piloselloides* ssp. *praealta* (Okada *et al*., 2011). Karyotyping revealed that an elongated chromosome was present in four out of seven of the apomictic basic species, all of which belong to *Pilosella* II (Figure 3). The elongated chromosome can be stably transmitted

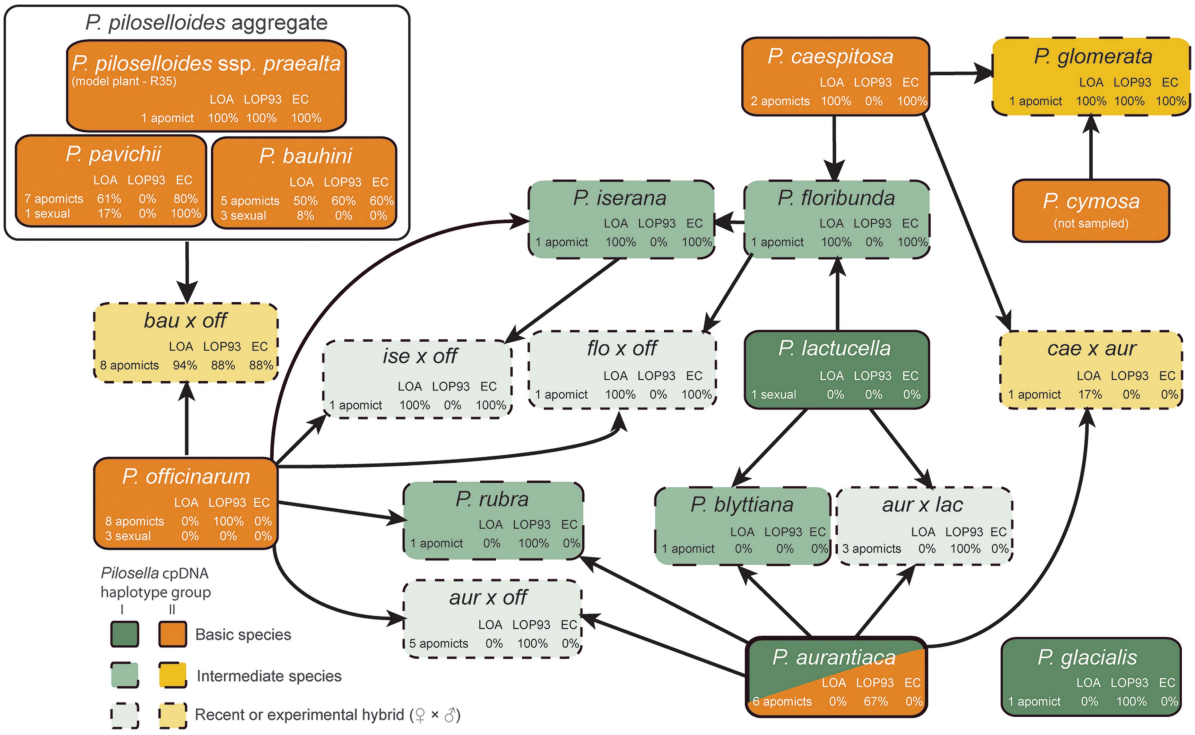

Figure 3 Summary of *Pilosella* species, cpDNA haplotype group, reproduction and apomixis-associated markers. This diagram depicts the included *Pilosella* species. Putative hybridization events between various basic species that gave rise to intermediate species and phenotypically corresponding recent hybrids are represented by arrows. The major chloroplast haplotype group for a given species is represented by either green (*Pilosella* I) or orange (*Pilosella* II) shading. If both haplotype groups were identified within a species, they are shaded with both colours. For each taxon, the number of individuals sampled is given along with their reproductive mode, the conservation of *LOA* and *LOP*-associated markers and the occurrence of the hemizygous elongated chromosome (EC). For *LOA*-associated markers, markers within the core region were included and calculated as a weighted average of each taxon. Detailed information for each individual including ploidy, cpDNA subtype, particular *LOA*-associated markers, *LOP* haplotype, penetrance of apomixis and geographic origin is given in Supplementary Table 3. For explanations concerning the *P. piloselloides* species aggregate, see Supplementary Table 1.

within *Pilosella* following hybridization because intermediate species, as well as recent and experimental hybrids can inherit the elongated chromosome along with the apomixis phenotype, when derived from an elongated chromosome-carrying progenitor species (Figure 3). Transmission of the elongated chromosome occurs in $2n+n$ hybrid progeny as maternal meiosis is absent, thus the entire unreduced ($2n$) maternal genome is inherited together with paternal chromosomes in nuclei following fertilization. Using this knowledge in conjunction with karyotype patterns, *P. bauhini* can be inferred as the maternal progenitor of two *P. bauhini* × *P. officinarum* hybrids (1433, 1647) for which maternal determination was inconclusive using cpDNA. Within *P. bauhini* and *P. pavichii*, not all apomictic individuals contained the long chromosome (Supplementary Table 3). This is indicative of potential recombination and coincident loss of elongated chromosome integrity as observed in rare recombinants in experimental cross populations (Kotani *et al.*, 2014).

Within *Pilosella*, the LOP93 marker was absent in all of the sexually reproducing individuals. Only 60% of the apomicts possessed the LOP93 marker indicating that it does not show perfect correlation with autonomous seed development (Supplementary Table 3). LOP93 was present in five of the seven apomictic basic species included in the study. An association of LOP93 with *LOA* markers was not always found. For example, *P. caespitosa* had all *LOA* markers, but lacked LOP93 and while no *P. aurantiaca* individuals carried *LOA* markers, four out of six had LOP93 (Figure 3).

Inheritance of *LOA* and *LOP*-linked markers in *Pilosella*

The inheritance of *LOA*-linked markers could also be studied using the naturally occurring stabilized intermediate species and the recent and experimental hybrids (Figure 3). Apomictic intermediate species inherited the same *LOA* genotype as their apomictic progenitor; that is, *P. caespitosa* intermediate species (*P. floribunda*, *P. glomerata* and *P. iserana*) were *LOA* positive, whereas *P. aurantiaca* intermediate species (*P. blyttiana* and *P. rubra*) were *LOA* negative. This was also the case for the recent hybrids, including those with low penetrance of apomixis. No difference in *LOA*-associated marker pattern was therefore observed between plants with high and low levels of penetrance. In the single sample that is inferred to be a recent hybrid of two apomicts (*P. caespitosa* (*LOA* positive) × *P. aurantiaca* (*LOA* negative)), only one of the *LOA*-linked markers was present. In this instance, the apospory locus has probably been inherited from *P. aurantiaca*.

When present, the LOP93 amplicon was sequenced to investigate the level of nucleotide diversity and possible evolutionary relationships within *Pilosella*. Sequencing revealed five different haplotypes (Figure 2b), which were distinguished by 13 substitutions and one 1-bp indel. For each sample sequenced, no variation was observed within the individual, as all reads provided a clear signal and no visible single-nucleotide polymorphisms in direct sequencing. In general, a single haplotype was associated with each species group, with the exception of *P. bauhini* ssp. *bauhini* for which two different haplotypes (C and D) were observed from the two individuals tested.

This species specificity is notable given the frequent hybridization known to occur within *Pilosella*. Analysis of the LOP93 haplotype relationships showed a strong correlation with the cpDNA haplotypes (Figure 2b), where the LOP93 A haplotype was found in *Pilosella* I individuals, and LOP93 haplotypes B–E were restricted to *Pilosella* II. Exceptions to this association include *P. rubra* (97) and *P. glacialis* (1734), which contained *Pilosella* I cpDNA and LOP93 haplotype C. Also, the two *P. aurantiaca* individuals (1326 and 1558), which contained unexpected *Pilosella* II cpDNA do not fit the correlation as they had LOP93 A haplotypes.

DISCUSSION

Residual sexuality within apomictic *Hieracium*

Hieracium and *Pilosella* genera are very closely related, yet are characterized by different mechanisms of gametophytic apomixis, diplospory and apospory respectively. Early cytological studies in *Hieracium* focused on a few species identifying apomicts as diplosporous often with non-viable pollen resulting from abnormal pollen meiosis (Rosenberg, 1927; Gentcheff and Gustafsson, 1940; Bergman, 1941; Skawińska, 1963). Diplosporous *Hieracium* species have been widely regarded as obligate apomicts and the genetic inheritance of apomixis is unknown in *Hieracium*. All apomictic plants identified here displayed mitotic diplospory of the *Antennaria* type.

We observed meiosis and tetrad formation in 1–7% of ovules in seven of the apomicts (Table 1). Skawińska (1963) also observed meiotic tetrads, which subsequently aborted in *Hieracium alpinum*. These observations of trace capacity for sexual female gamete formation in these diplosporous *Hieracium* are consistent with a potential facultative reproductive nature; however, it is unclear if viable chromosomally reduced eggs are produced in these plants. If so, it would be expected that a small fraction of polyhaploid $(n+0)$ progeny could form from the parthenogenetic development of a reduced egg cell, as is known to occur in *Pilosella* (Bicknell *et al.*, 2003; Krahulcová *et al.*, 2004). However, as the majority of the *Hieracium* species are triploid (in contrast to *Pilosella*), embryo development and seedling survival would depend upon egg viability following meiosis. Further analyses such as large-scale flow cytometric seed screens of diplosporous species (Matzk *et al.*, 2000) are needed to examine this possibility.

Assumed obligate apomixis in *Hieracium* has been proposed as a contributing factor in the lack of recent hybridization seen within this genus, and subsequently as one of the key reasons for population structure differences between *Hieracium* and the facultative *Pilosella* (Mráz *et al.*, 2011). Given the potential for residual sexuality of *Hieracium* suggested in this study, the population structure of *Hieracium* is possibly best explained by a combination of factors including reduced gene flow due to limited pollen viability in some species, self-fertilization following breakdown in self-incompatibility (Mráz, 2003) and precocious embryony, whereby some embryo formation initiates before anthesis.

Apospory in *Pilosella* and diplospory in *Hieracium* have probably evolved independently

The development of markers physically linked to the *LOA* and *LOP* loci of *P. piloselloides* ssp. *praealta* has provided the opportunity to assess their conservation throughout a larger sample of naturally sourced *Hieracium* and *Pilosella* species. The absence of *Pilosella*-derived apomixis-linked markers in *Hieracium* species may be an indication that apospory and diplospory have evolved independently in *Pilosella* and *Hieracium*, respectively. Based on phylogenetic distributions across angiosperm species, apomixis is thought to have evolved independently many times including multiple times within some genera (Carman, 1997; Hörandl and Hojsgaard, 2012). Carman (1997) documented 19 genera where both diplospory and apospory are evident. Our findings are therefore consistent with the polyphyletic description of apomixis. The lack of apospory markers within diplosporous *Hieracium* also agrees with the generally accepted idea of apospory and diplospory being non-homologous, given the different developmental pathways that define them (Van Dijk and Vijverberg, 2005). As both *Hieracium* and *Pilosella* have the capacity to undergo autonomous seed development, absence of *LOP*-associated markers in *Hieracium* species suggests that this process may also have evolved independently in *Hieracium* and *Pilosella* species.

The possibility remains, however, that *Hieracium* species do possess the same apomixis loci as *Pilosella* but they are undetectable using the markers used in this study, as they are not causal for apomixis. Genome divergence between *Hieracium* and *Pilosella* may also have interfered with cross-species marker amplification. To conclusively investigate whether the *Pilosella*-derived apomixis-linked sequences are present or rearranged within the genome of *Hieracium* species, future experiments could involve DNA hybridization of current linked sequences or amplification of genic sequences responsible for both apospory and autonomous seed formation following their identification within *Pilosella*.

Patterns of conservation and inheritance of *LOA* and *LOP*-linked markers in *Pilosella*

LOA-linked markers were absent in all but two sexual *Pilosella* accessions that each contained only one marker, while the *LOA* markers predominated in basic species of the *Pilosella* II cpDNA haplotype group. This supported a strong association with apospory as indicated by the prior limited survey (Okada *et al.*, 2011). Absence of *LOA*-linked markers in some apomictic basic species, and their corresponding stabilized and recent hybrids also substantiate previous work and may be indicative of the markers being non-genic and imperfect. Given the facultative nature of apomixis in *Pilosella*, another possibility is that the apomictic *LOA* marker negative species observed here have lost the *LOA* flanking marker sequences through recombination. The sporadic *LOA* marker patterns observed in the basic *Pilosella* species *P. bauhini* and *P. pavichii*, for example, could be interpreted as evidence of recombination at the *LOA* locus or alternatively as a series of fixed point mutations preventing marker amplification. Further analysis of more individuals per species will help clarify whether marker absence is due to meiotic recombination.

It is also possible that the *LOA*-linked marker negative taxa, such as apomictic *P. aurantiaca*, *P. glacialis* and *P. officinarum*, may represent lineages where apospory is caused by another genomic region distinct in function from *LOA*. This would imply that an alternate apospory pathway may have evolved within *Pilosella* that is responsible for regulating apospory in these *LOA*-negative species. The notion of multiple apospory pathways within *Pilosella* may be supported by the observation of subtly different phenotypes when apospory is studied at the cytological level. *P. piloselloides* (*LOA* marker positive) and *P. aurantiaca* (*LOA* marker negative) differ with respect to the timing and mode of aposporous embryo sac formation (Koltunow *et al.*, 2000, 2011). Furthermore, experimental *P. piloselloides* × *P. aurantiaca* hybrids display a range of developmental alterations in apospory compared with the parental species (Koltunow *et al.*, 2000). The developmental alterations seen in these hybrids may possibly be due to the co-expression of different apospory-inducing genes or pathways that exist in the parental species. Apospory appears to have arisen independently at least three times in *Ranunculus* (Van

Dijk and Vijverberg, 2005). Thus, the same may have occurred in *Pilosella*.

As with apospory-associated markers, the genic LOP93 marker also showed variable conservation throughout *Pilosella* (Supplementary Table 3), which demonstrated that it is also a useful, but not perfect marker for autonomous seed development. Absence of the marker may be the result of the range of possibilities already discussed above for the *LOA* markers. Alternatively, the *LOP* locus may genuinely be absent, which may suggest different routes to autonomous seed development within *Pilosella*. In any case, the lack of a strict association between *LOA* and *LOP* markers throughout the population is interesting and supports the proposition that apospory and autonomous seed formation are independently inherited and evolved as separate traits.

Sequencing of the LOP93 amplicon, when present, provided the opportunity to examine how this locus is inherited and also how it has evolved within *Pilosella*. LOP93 sequence revealed intriguing findings, including low sequence divergence across species, only a single haplotype per species (irrespective of ploidy level) and species specificity of the *LOP* haplotypes. As a result of the ease of hybridization between *Pilosella* species and cytotypes, species-specific markers are thought to be rare. Certainly, cpDNA is not strictly species specific (Fehrer *et al.*, 2007a). The LOP93 haplotypes, however, show almost complete species specificity for the accessions analyzed (Figure 2b).

Furthermore, the association seen between the LOP93 and cpDNA haplotypes indicates that *LOP* is often transmitted maternally. The exceptions to this trend include four individuals, which have apparently inherited *LOP* via pollen. Thus, comparison of LOP93 and cpDNA haplotypes revealed in many cases the parent from which *LOP* was obtained, confirming that it can be inherited both maternally or paternally in natural populations. *LOP* confers the essential capability to form a viable seed independent of the mode of female gametophyte formation and ploidy of the egg (that is, n or $2n$). Therefore, *LOP* transmissibility via male and female gametes maximizes seed formation and fecundity in populations. It would be interesting to determine if there is a stronger selection pressure for *LOP* in apomictic populations. As chromosome walking continues along the *LOA* and *LOP* loci, the development of perfect causal gene markers will help clarify this and determine the degree of conservation of apomixis-inducing pathways within *Pilosella*.

Paternal modifiers influence expressivity of apomixis in $2n+n$ *Pilosella*

We have discovered that at least three loci are required for the qualitative traits of apomixis in *Pilosella* to confer apospory and fertilization-independent embryo and endosperm formation. Analyses of crosses between sexual and apomictic species demonstrate the variable penetrance of apomixis in progeny suggesting that other, quantitative factors or modifiers may influence the final reproductive behaviour in hybrids (Koltunow *et al.*, 2000; Krahulcová *et al.*, 2011; Tucker *et al.*, 2012). However, to date, apomixis penetrance has not been correlated to the presence of the *LOA* and *LOP* loci.

Low penetrance or expressivity of apomixis has been previously reported within naturally sourced and experimental $2n+n$ hybrids arising from crosses between a range of *Pilosella* apomicts as the maternal parent (*P. bauhini*, *P. floribunda* and *P. aurantiaca*) and sexual *P. officinarum* as the pollen donor (Krahulec *et al.*, 2008; Krahulcová *et al.*, 2009; Krahulcová *et al.*, 2011). These hybrids arise from the fertilization of a chromosomally unreduced egg. This study analyzed a greater range of $2n+n$ hybrids from crosses between apomicts (*P. bauhini*, *P. floribunda*, *P. iserana* and *P. aurantiaca*) and sexual *P. officinarum*, all of which (except one) displayed low penetrance of apospory (Supplementary Table 3). Each $2n+n$ hybrid contained the same *LOA* marker patterns as their maternal apomictic parent confirming inheritance of the primary apospory locus in the inherited maternal genome. The low expressivity of apomixis in such hybrids indicates that the penetrance of apomixis can be affected by parental genome interactions following hybridization. These may involve factors such as modifiers, dosage effects and epistatic interactions arising from the incoming paternal genome. Interestingly, in polyhaploid progeny $(n+0)$ arising from multiple $2n+n$ *P. aurantiaca* × *P. officinarum* plants, apomixis was highly penetrant (Supplementary Table 3). This suggests that the suppressive modifiers may have been lost following meiotic reduction, or genome dosage and epistatic factors have been removed. Recovery of apomixis penetrance via a meiotic event clearly suggests that these modifiers are unlinked and can be lost over various meiotic cycles. Future cytological screening of those plants with low expressivity of apomixis will help to determine exactly which reproductive processes are being affected.

Conclusions and future directions

This study demonstrated that meiosis can occur at low levels in diplosporous *Hieracium*, indicating the potential for progeny to arise via the sexual pathway. The absence of the *LOA* and *LOP*-linked markers developed from aposporous *Pilosella* species in the examined diplosporous *Hieracium* plants tested, suggests independent evolution of apomixis in these two genera, although further analyses with perfect, causal gene markers would be required to conclusively determine this. Within *Pilosella*, *LOA* and *LOP* markers were essentially absent within the sexual plants, and conserved in many but not all apomictic species, which may imply multiple origins of apomixis even within *Pilosella*. Marker presence also had no association with the penetrance of apospory, and overall, many associations tested relating to phenotype and genotype were only weakly correlated, which indicates a complex evolutionary history. Evidence for independence of *LOA* and *LOP* loci was suggested in natural populations, which supports previous experimental observations. To further understand the evolution of apomixis loci within *Pilosella*, perfect markers for the causal genes at *LOA* and *LOP* loci are actively being sought to screen across natural populations. A molecular phylogeny of apomictic *Pilosella* species based on neutral nuclear markers is still lacking, mostly because of complications due to the high ploidy of most apomicts ($4\times - 7\times$) and their almost unlimited potential to hybridize. Knowledge of species relationships within *Pilosella* would contribute to the understanding of apomixis evolution within this complex genus.

DATA ARCHIVING

Sequence data have been submitted to GenBank: accession numbers KF196158–KF196254. Voucher specimens of plants analyzed are deposited in the Herbarium PRA (Průhonice, Czech Republic).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Siegfried Bräutigam for expert determinations of the plant material and help with the taxonomic background for this paper. This research was supported by a Science and Industry Endowment Fund grant to AMGK and funding from the Czech Science Foundation (206/08/0890, P506/10/1363) and the Academy of Sciences of the Czech Republic (RVO 67985939).

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