Origin, inheritance and ecological significance of apomixis in the genus *Hieracium* s.str.: The role of genetic and epigenetic mechanisms

Ph.D. Thesis

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Prague 2020

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Content

Declaration

I declare that I have written this thesis independently using the mentioned references. It has not been submitted elsewhere, in full or in part, to obtain the same or another academic degree.

In Prague on 24th May 2020 Jan Pinc

Acknowledgements

I would like to thank especially to my supervisor Patrik Mráz for scientific, financial, and all the support that makes this Ph.D. thesis possible. I would also like to thank Jindřích Chrtěk, Judith Fehrer and Vít Latzel for their numerous advice, Anna Koltunow and Susan Johnson for expertise/guidance in embryological methods and amazing opportunity to attend an internship in CSIRO, Adelaide. Moreover, I am also grateful to Lenka Macková, Viera Mrázová, Jan Ptáček, Lenka Flašková, Romana Bartošová, Tomáš Urfus, Štěpán Mareš, Michaela Jandová and Jiřina Josefiová for their help during the experiments and lab work. And finally, I would like to thank my family and friends for their emotional support.

This work was supported by the Czech Science Foundation (GAČR grant no. 14-0680, 17-11281S and 17-14620S) and Grant Agency of Charles University (GAUK grant no. 907218).

Author contribution statement

Paper I:

Jindřich Chrtek, Patrik Mráz, Alexander Belyayev, Ladislava Paštová, Viera Mrázová, Petra Caklová, Jiřina Josefiová, Danijela Zagorski, Matthias Hartmann, Michaela Jandová, Jan Pinc and Judith Fehrer. Evolutionary history and genetic diversity of apomictic allopolyploids in *Hieracium* s.str.: morphological versus genomic features. Published in American Journal of Botany (2020).

Total contribution 10% - Experimental cultivation and production of artificial hybrids, data collection, flow cytometry analysis, editing and review.

Paper II:

Jan Pinc, Alexander Belyayev, Ladislava Paštová, Michaela Jandová, Jiřina Josefiová Jindřich Chrtek, Judith Fehrer, Patrik Mráz: Interspecific hybridization and frequency of neopolyploidization in a predominantly polyploid plant genus (manuscript).

Total contribution 80% - Study design, experimental cultivation, data collection and data analysis, and manuscript preparation.

Paper III:

Jan Pinc, Jindřich Chrtek, Vít Latzel, Patrik Mráz: Negative effect of inbreeding on fitness of an arcto-alpine Hieracium alpinum (Asteraceae), a species with geographical parthenogenesis (in press, Plant Systematics and Evolution).

Total contribution 80% - Study design, experimental cultivation, data collection and data analysis, and manuscript preparation.

Paper IV:

Jan Pinc, Wilfred Rozhon, Patrik Mráz: Testing experimental demethylation in plants: Effect, efficiency and temporal stability (manuscript).

Total contribution 80% - Study design, experimental cultivation, data collection and analysis, and manuscript preparation.

Abstract

Apomixis (asexual reproduction by seeds) has the considerable potential in agriculture and crop breeding due to its ability to produce genetically identical progenies in a form of persistent propagules, i.e. seeds. However, the processes laying behind the origin of apomixis and connected molecular mechanisms are still unknown. Despite the fact, that apomicts are considered to be an evolutionary dead-ends, they are often more widely distributed than their sexual relatives (this phenomenon is commonly referred to as geographical parthenogenesis (GP). Although this phenomenon is studied for decades, its causes are still not fully understood. Importantly, several recent studies pointed out that apomicts with limited genetic variability can at some extent react to changing environment through changes in gene transcription by epigenetic modifications.

It is generally assumed, that hybridisation and polyploidization played a crucial role in the emergence of apomixis. For this reason, presented studies test the putative origin of selected polyploid apomicts and if the interspecific hybridisation between diploid sexuals triggers polyploidization using predominantly polyploid and apomictic genus *Hieracium* s. str. Surprisingly, the presented study demonstrates the hybrid origin of almost all investigated polyploid apomicts, some of which were originally considered as autopolyploids (based on morphology). Moreover, interspecific hybridisation between diploid taxa unexpectedly showed low production of neopolyploids in both, interspecific and intraspecific crosses. In contrast to parental diploid species (0.22%), neopolyploids produced higher amount of various polyploids (68.7%). Thus, it can be assumed that new unstable polyploid cytotype could be diploidised in following generations or that polyploidy can be fixed by bypassing meiosis and switch to apomictic reproduction. This pattern would correspond to strict sexuality in diploids and strict apomixis in polyploids of *Hieracium* s.str. observed in natural conditions.

Majority of the genus *Hieracium* s.str. is formed by polyploid apomicts while diploid sexuals are rare and geographically isolated (as suggested by GP theory). Presented study deals with Metapopulation hypothesis which can explain GP distribution pattern by the negative effect of inbreeding in the population of sexuals compared to genetically stable apomicts. In detail, the presented study showed inbreeding depression in sexuals of *H. alpinim*, which can limit their dispersal potential and thus contribute to GP in this species, according to Metapopulation hypothesis.

Despite the lack of genetic variation, clonal plants can in some extent react to changing environment by changes in DNA methylation. Importance of DNA methylation was already tested twice in *H. alpinum* using experimental demethylation with no/little effect on plants performance. For this reason, the presented study aims to determine the best methodological approach for experimental demethylation and test the temporal stability of induced changes in DNA methylation.

Future research should focus on unstable neopolyploids of *Hieracium* s.str. which can be produced by various types of crosses. Because new and unstable polyploid cytotype could be theoretically fixed by bypassing meiosis and apomictic reproduction, a close examination of this process could finally identify mechanisms laying behind apomixis. Understanding this mechanism can theoretically simplify the production of such crops as cowpea or sorghum by capturing hybrid vigour through apomictic reproduction. Furthermore, the best methodological approach of the experimental demethylation of *H. alpinum*, which is described in this thesis, can be used to close examination of the role of DNA methylation in the adaptability of clonal plants.

Abstrakt

Apomixie (nepohlavní rozmnožování pomocí semen) má velký potenciál v zemědělství a šlechtění zemědělských plodin, zejména díky schopnosti produkovat geneticky identické potomky ve formě semen. I přes početné pokusy, procesy stojící za vznikem apomixie jsou stále neznámé. Apomiktické druhy jsou navíc často rozšířenější než jejich sexuálně se množící příbuzní, a to i přes to, že apomiktické druhy jsou často považovány za slepou evoluční větev. Tento fenomén je v literatuře označován jako geografická partenogeneze (GP), a i přes desítky let výzkumu, nejsou jeho příčiny stále zcela známé. Kromě toho, několik studií z posledních let poukázalo na to, že klonální rostliny (včetně apomiktů) disponující omezenou genetickou variabilitou mohou reagovat na měnící se prostředí pomocí změn genové exprese způsobených epigenetickými modifikacemi.

Předpokládá se, že hybridizace a polyploidizace hrají klíčovou roli při vzniku apomixie. Z tohoto důvodu tato práce testuje původ vybraných polyploidních apomiktů a zda mezidruhová hybridizace mezi vybranými diploidními, sexuálně se rozmnožujícími taxony spouští polyploidizaci. K tomuto experimentu byly vybrány druhy z převážně polyploidního a apomiktického rodu *Hieracium* s. str.. Výsledky prvního experimentu překvapivě ukazují hybridní původ téměř všech studovaných polyploidních apomiktů, z nichž část byla na základě morfologie považována za autopolyploidy. Hybridizace mezi diploidními a sexuálně se množícími druhy navíc ukázala nízkou produkci neopolyploidů v obou typech křížení, mezidruhovém i vnitrodruhovém. V kontrastu s rodičovskými druhy, vzniklí neopolyploidi produkovali významně vyšší množství různých polyploidů. Na základě těchto dat můžeme předpokládat, že nově vzniklí polyploidi mohou být diploidizováni v následujích generacích anebo zafixováni apomiktickým způsobem rozmnožování, což by odpovídalo striktní sexualitě diploidních cytotypů a striktní apomixii polyploidních cytotypů pozorované v přírodních podmínkách.

Většina rodu *Hieracium* s. str. je tvořena polyploidními apomikty zatímco diploidní, sexuálně se množící cytotypy jsou vzácné a geograficky izolované (GP fenomén). Tato práce se mimo jiné zabývá Metapopulační hypotézou, která by mohla vysvětlit GP jako negativní vliv inbreedingu v populacích sexuálně se množících cytotypů v porovnání s geneticky stabilními apomikty. Konkrétně tato práce demonstruje inbrední depresi u sexuálně se množícího cytotypu *H. alpinum*, která může limitovat jeho kolonizační schopnost v kontrastu s geneticky stabilním apomiktickým cytotypem, a přispět tak ke GP tohoto druhu podle Metapopulační hypotézy.

I přes nedostatek genetické variability, klonální rostliny mohou reagovat na měnící se okolní prostředí pomocí změn v DNA methylaci. Význam DNA methylace u *H. alpinum* byl již dvakrát testován pomocí experimentální demethylace. Vzhledem k tomu, že tyto pokusy neměly velký efekt na zkoumané rostliny, má tato práce za cíl určit nejlepší metodu experimentální demethylace pro zkoumaný druh a otestovat časovou stabilitu získaných změn v DNA methylaci.

Budoucí výzkum by se mohl zaměřit na nestabilní neopolyploidní cytotypy *Hieracium* s.str., které jsou produkovány v různých typech křížení. Vzhledem k tomu, že nestabilní polyploidní cytotyp může být teoreticky zafixován apomixií v jedné z následujících generací, detailní výzkum tohoto procesu by mohl konečně objasnit mechanismus stojící za vznikem apomixie. Porozumění tohoto mechanismu by teoreticky mohlo výrazně zjednodušit produkci některých plodin díky fixování F1 hybridního charakteru pomocí apomixie. Určení nejlepší metody experimentální demethylace pro *H. alpinum*, která je popsaná v této práci, by dále mohlo být použito k bližšímu porozumění role DNA methylace v adaptabilitě klonálních rostlin.

Chapter I: Introduction

1 Sexual versus asexual reproduction

Reproduction is a fundamental process common to all living organisms. During this process, the new organism (offspring) is produced by one or two parental organisms. There are two types of reproduction, sexual, which is accomplished by fusion of two (predominantly genetically distinct) gametes, and asexual, where the offspring arise from a single organism and thus inherit genetic information from only one parent. Moreover, asexual reproduction can be achieved by the production of more or less specialised vegetative propagules, which are eventually able to form independent plant, or by the production of primarily generative propagules (seeds) asexually. Comparing sexual and asexual reproduction, sexual reproduction is much more energetically demanding process than asexual reproduction (Lloyd, 1980). In detail, sexually reproducing organisms have to invest into the production of males, who do not produce the offsprings by themselves but only serves as gamete donors to females (cost of males, Lively, 2010). Furthermore, sexuals have to invest energy into often very complex reproduction structures (Williams, 1971; Smith and Maynard-Smith, 1978). Moreover, sexuals produce highly variable offsprings due to genetic recombination. As a result, recombinant offsprings may lack the genetic features of their well-adapted parents (cost of meiosis; Williams, 1971). On the other hand, asexuals lacking genetic recombination are theoretically not able to effectively purge deleterious mutations which are accumulated in the genome (i.e. Müller's ratchet; Müller, 1964). Furthermore, adaptability of asexuals is limited in changing environment due to their no or little genetic diversity (Hörandl, 2006). As a result, sex is the predominant reproduction type not only in plants but in all eukaryotes (Engelstädter, 2008). Yet, most of the plant species can reproduce asexually in some extent (Klimeš et al., 1997).

The process of asexual reproduction produces ramets and genets. While ramet refers to a more or less independent individual plant, genet refers to one genetically identical individual often formed by several ramets. In some cases is the identification of individual genet apparent because of the connection between different ramets for example in tufts or by stolons (Pitelka and Ashmun, 1985). In other cases, such as reproduction by specialised vegetative propagules or asexually

(apomictically) formed seeds, molecular method are needed to identify genets between apparently independent individuals often occupying a large area (Widén et al., 1994).

2 Apomixis

Apomixis, the asexual reproduction by primarily generative reproductive structures i.e. seeds, combine advantages of both, asexual and sexual reproduction. On the one hand, all offsprings are produced uniparentally, without the contribution of male, and thus genetically identical to maternal plant (Asker and Jerling, 1992). On the other hand, in contrast to other types of asexual reproduction, seeds can be dispersed over longer distances due to their relatively small size and seed persistency allows synchronizing of germination with the optimal environmental conditions (Stebbins Jr, 1950; Richards, 1997).

The first evidence of apomixis was recorded in 1841 from Kew Botanical Gardens, London, where female individual *Caelebogyne ilicifolia* (Euphorbiaceae) produced viable seeds without male individual available (Smith, 1841). This process was later described as apomixis i.e. agamospermy (Winkler, 1908; Tackholm, 1922). Since then, apomixis has been recorded in approximately 1% of all angiosperms (Burt, 2000). In detail, apomixis was found in 293 angiosperms genera mostly belonging to three families: Asteraceae, Rosaceae and Poaceae (Hojsgaard et al., 2014). Interestingly, apomictic reproduction is not limited only to flowering plants but it is much more widespread in ferns where up to 10% of species are considered to be apomictic (Walker, 1966). Moreover, there is a rare case of apomictic reproduction in gymnosperms like in *Cupressus duperziana* (Pichot et al., 2000).

In general, apomictic reproduction is not obligate, as the majority of apomicts combine apomictic and sexual reproduction (Richards, 1997). The proportion of sexual and asexual reproduction in apomictic species depends on the type of apomictic reproduction (see below). Functional apomixis combines two important processes in autonomous embryo development: apomeiosis and parthenogenesis. Apomeiosis is an avoidance of normal meiotic division resulting in the production of unreduced gamete. The second process, parthenogenesis is the autonomous development of embryo i.e. without fertilisation (Winkler, 1920; Koltunow and Grossniklaus, 2003). Also, two special cases when one of the processes essential for apomixis (apomeiosis and parthenogenesis) is not functional can occur. Non-functional apomeiosis (regular meiotic division)

and fully functional parthenogenesis lead to the production of reduced embryo sac which develops autonomously (without fertilisation) into the progeny containing reduced chromosome number compared to the maternal plant. This process is called haploid parthenogenesis (Krahulcová and Krahulec, 2000; Dobeš et al., 2013). Secondly, apomeiosis together with non-functional parthenogenesis leads to the production of unreduced embryo sac which needs to be fertilised to create progeny with higher ploidy level than maternal plant i.e. BIII hybrids (Rutishauser, 1948). Also, when neither of those two processes (apomeiosis and parthenogenesis) is functional, embryo sac develops sexually.

Moreover, autonomously developed embryo requires nutrition from endosperm which may or may not be developed autonomously in apomicts. In the first case, both, embryo and endosperm developed autonomously. As a result, two nuclei (out of eight nuclei present in mature embryo sac) fuse into a polar nucleus which subsequently develops into endosperm. This process leads to 1:2 ploidy ratio in mature seed where 1 is the ploidy of the embryo and 2 is the ploidy of the endosperm. In the second case, pseudogamy (see below), polar nucleus needs to be fertilised by pollen grain to form fully functional endosperm while the embryo is developing fully autonomously. This process is also displayed in embryo: endosperm ploidy ratio as 1: 2 + x, where x is the ploidy of the pollen grain. In contrast to apomictic seeds, sexually derived seeds arise after double fertilisation which is also reflected in embryo:endosperm ploidy ratio as 1 + x : 2 + x, typically as 2:3. Interestingly, described ratios between ploidy of embryos and endosperms can be used for relatively quick determination of reproductive pathways by flow cytometry seed screen, often abbreviated as FCSS (Matzk et al., 2000).

2.1 Types of apomixis

Based on the origin of the initial cell which eventually gives rise to an apomictic embryo, apomixis can be categorized into 3 main categories.

i) Adventitious embryony is classified as a sporophytic type of apomixis. In this case, embryo originates from sporophyte tissue (in most cases from nucellus integument) and later on invades meiotically derived embryo sac (abbreviated as ES) to use its endosperm. Furthermore, endosperm used by asexually derived embryo has to be fertilised, which means that adventitious embryony is dependent on the sexual process. Moreover, in this type of apomixis polyembryony can be often observed. In the case of

polyembryony, several apomictic embryos together with the sexual one can coexist within one ovule. Adventitious embryony is common mainly in tropical and subtropical plants including several widely diversified families such as Orchidaceae, Rutaceae, Cactaceae, Euphorbiaceae and some agriculturally important crops such as mango and citrus (Naumova, 1992; Czapik, 2000).

ii) Apospory is classified as a gametophytic type of apomixis. In the case of apospory, initial cell (aposporous initial) originates directly from the nucellus cell. Later, aposporous initial cell mitotically divides into an aposporous ES which coexists with sexual ES within the same ovule. Moreover, similarly to adventitious embryony, aposporous embryo sac also requires endosperm fertilisation i.e. pseudogamy. However, aposporous ES is usually developing significantly faster than sexual ES. As a result, aposporous ES usually outcompete the sexual one which eventually degenerates. In addition, the co-occurrence of sexual and aposporous embryo sacs ensure some level of sexuality in aposporous plants. Apospory is typical for Poaceae and Rosaceae (Asker and Jerling, 1992; Koltunow, 1993). Besides, apospory also occurs in the genus *Pilosella* (Asteraceae), which is closely related to our model genus, diplosporous *Hieracium* s.str (Rosenberg, 1907; Skawińska, 1963; Koltunow, 1993; Fehrer et al., 2009).

iii) Diplospory is together with apospory categorized as gametophytic apomixis. In the case of diplospory, embryo sac originates directly from the archesporial cell which is (partly or fully) omitting meiosis and gives rise to both, autonomously developed embryo and endosperm. Based on the further development of archesporial cell, three types of diplospory can be distinguished (Asker and Jerling, 1992; Koltunow, 1993). In diplospory of *Antennaria* type, meiosis is fully omitted and archesporial cell directly undergoes mitosis (Koltunow, 1993). In diplospory of *Taraxacum* and *Ixeris* types, meiosis of archesporial cell is not fully omitted and crossing over can occur. However, meiosis in diplospory of *Taraxacum* and *Ixeris* is restitutional and results in unreduced ES (Asker and Jerling, 1992; Koltunow, 1993). Moreover, diplosporous ES is usually developing precociously i.e. before flower opening (Richards, 1997; Noyes and Givens, 2013; Hand et al., 2015). Furthermore, in contrast to apospory, diplosporous apomixis is more obligate and residual sexuality is rare (Asker and Jerling, 1992; Mráz

and Zdvořák, 2019). Diplospory is typical especially for family Asteraceae including our study genus *Hieracium* s.str (Skawińska, 1963; Asker and Jerling, 1992).

As regards to the frequency of mentioned types of apomixis, adventitious embryony is the most frequent type of apomixis followed by apospory and diplospory (Hörandl and Hojsgaard, 2012; Hojsgaard et al., 2014).

2.2 Origin and inheritance of apomixis

Despite numerous attempts, origin and molecular mechanisms underlying apomixis are still not fully understood. The fact that apomixis was found in more than 40 families suggests its multiple origins (Koltunow and Grossniklaus, 2003; Van Dijk and Vijverberg, 2005). On the other hand, the majority of apomicts belongs to three families only (Asteraceae. Poaceae, Rosaceae) which suggests that some families might be preadapted (Quarin et al., 2001). Numerous authors suggest that apomixis is heritable and under genetic control (Van Dijk et al., 1999; Bicknell et al., 2000; Noyes and Rieseberg, 2000; Matzk et al., 2005). However, the fact that expressivity of apomixis can vary under different conditions suggests also the involvement of other mechanisms, among them environmental factors and genetic modifiers i.e. epigenetics (Koltunow and Grossniklaus, 2003; Grimanelli, 2012; Podio et al., 2014). Moreover, two important features are common for the majority of apomictic plants. Firstly, the majority of apomicts have hybridogenous origin (Carman, 1997). Secondly, all apomicts are polyploids with, as far as I know, only one well-documented exception of a diploid apomict, *Boechera holboellii* from Brasicaceae family (Böcher, 1954; Sharbel et al., 2009). Based on these findings, several authors have suggested that alleles control apomixis cannot be transmitted through haploid gametes or are lethal in the diploid state (Nogler, 1984). On the other hand, Quarin et al. (2001) showed that simple chromosome duplication in *Paspalum notatum* (Poaceae) triggers apomictic reproduction. Those authors are suggesting that apomictic allele (or alleles) are already present in diploids, but they can be expressed only in polyploids through some transcription factors or via secondary locus which requires higher allele dosage to affect the expression of the main locus. Genome collision hypothesis formed by Carman (1997) proposed that polyploidy when originating from hybridization between different species or between genetically distant ecotypes of the same species, can cause asynchronous expression of regulatory genes that control megagametogenesis i.e. development of female gametophyte. This process leads to the production of highly variable and mostly sterile progeny due to disturbed

meiosis. Subsequently, apomixis can occur in a subset of the progeny containing the right combinations of genes (Carman, 1997; Grimanelli et al., 2001).

Numerous studies were trying to elucidate genetic control of apomixis. These studies pointed out that two processes ensuring apomixis (apomeiosis and parthenogenesis) are inherited together, either as a single locus or a cluster of linked loci (Grimanelli et al., 1998; Bicknell et al., 2000; Quarin et al., 2001) or two independent loci controlling apomeiosis and parthenogenesis independently (Tas and Van Dijk, 1999; Albertini et al., 2001; Matzk et al., 2005). However, most of those studies were performed on aposporous plants and studies dealing with the inheritance of diplosporous apomixis are still scare. In detail, experimental crosses between sexual and apomictic dandelions (*Taraxacum*, Asteraceae) revealed that two components of diplospory, apomeiosis and parthenogenesis, are segregating independently (Asker and Jerling, 1992; Van Dijk et al., 1999). Inheritance of diplosporous apomixis of *Antennaria* type was studied only in *Erigeron* (Asteraceae), where apomeiosis and parthenogenesis were inherited also independently (Noyes et al., 2007). In contrast to well-studied aposporous *Hieracium* subg. *Pilosella*, there is no information about the inheritance of apomixis in diplosporous *Hieracium* s.str.. Crosses between sexuals and apomicts were performed only once, but all progeny were either diploid hybrids or produced by mentor effect i.e. induced selfing. Moreover, diploid hybrids in this experiment did not produce any viable seeds without pollination (Mráz and Tomčíková, 2004).

2.3 Ecological and evolutionary significance of apomixis

Apomixis played a crucial role in plant diversification by creating agamic complexes. In detail, interspecific hybridization can produce a large number of morphologically highly variable and unstable genotypes. Those genotypes can be fixed by apomixis and spread to the appropriate niches as different microspecies with different combinations of parental features (Babcock and Stebbins, 1938; Hörandl and Hojsgaard, 2012). In addition, due to the accumulation of deleterious mutation and inability to environmental adaptation with limited genetic variability, apomictic microspecies are often consider as evolutionary dead ends (Stebbins Jr, 1950; Lynch et al., 1993). Yet, there are numerous examples of apomicts that are more widely distributed than their sexually reproducing relatives (Beaton and Hebert, 1988; Thompson and Whitton, 2006; Mráz et al., 2009; Cosendai and Hörandl, 2010; Lo et al., 2012).

2.3.1 Geographic parthenogenesis

The phenomenon, when asexuals occupy a larger area than sexuals is called geographical parthenogenesis (abbreviated as GP; Vandel, 1928). In addition, asexuals are often more successful in the marginal habitats, higher altitudes, higher latitudes or previously glaciated areas (Hörandl, 2006). Despite numerous attempts to explain this phenomenon, underlying factors of different distributional ranges between sexuals and asexuals are still unclear. Amongst most discussed hypotheses belongs:

(i) Advantages of uniparental reproduction ensure more stable reproduction of apomicts (Baker, 1967; Linder and Barker, 2014). In detail, apomicts can benefit from their independency on pollinators or availability of a mating partner, especially in the unfavourable environmental conditions on the edge of the distributional area or during colonizing of the new environment. Moreover, sexuals would be in the same environmental conditions even more disadvantaged because closely related sexual species are usually strict outcrossers. In addition, the described process is not functional in pseudogamous apomicts, because they also require fertilisation of the endosperm (Asker and Jerling, 1992).

(ii) Since almost all apomicts are polyploids, the advantage of polyploidy can provide a higher level of phenotypic and ecophysiological plasticity compared to their diploid relatives (Bierzychudek, 1985). The higher level of plasticity can ensure adaptability to broader spectrum of environmental conditions and thus favour polyploid apomicts.

(iii) Another two hypotheses are dealing with better niche exploitation by one fixed genotype of asexuals compared to genetically variable sexuals. On the one hand, General-purpose Genotype hypothesis assumes, that one asexually reproducing genotype has fixed broader environmental tolerance than their sexual relatives and thus occupies a larger geographical area (Lynch, 1984). On the other hand, the Frozen Niche Variation hypothesis suggests that selection can favour several highly specialised genotypes which are well adapted to particular niches than genetically variable and thus more generalized sexuals (Vrijenhoek, 1984).

(iv) Red Queen hypothesis suggests that the success of asexuals is context-dependent and crucial are different levels of biotic interactions (Van Valen, 1973). In detail, Red Queen hypothesis assumes that evolutionary arms race in host-parasite/pathogen

dynamics selects strongly against low genetic variability. As a result, asexuals are more successful than their sexual relatives only in environments with low level of biotic interactions, presumably in marginal habitats, at higher altitudes or higher latitudes.

(v) Metapopulation hypothesis presumes that populations of asexuals are advantaged due to avoidance of genetic load and inbreeding depression in small subdividing populations on the margin of distributional area (Haag and Ebert, 2004). It has been supposed that marginal populations often face to extinction and recolonization. In that population, the fitness of sexuals can be significantly lowered due to inbreeding. As a result, asexuals can establish marginal population with lower risk of extinction compared to their sexual relatives.

The fact that asexually (diplosporous apomixis of *Antennaria* type; Skawińska 1963) reproducing cytotypes of genus *Hieracium* s. str. are generally widely distributed than sexuals (Mráz and Szeląg, 2004), make this genus an excellent model for studying the phenomenon of geographical parthenogenesis. Several attempts to explain GP distributional pattern in this genus were made on alpine hawkweed *Hieracium alpinum.* This species displaying GP distributional pattern (Mráz et al., 2009) is, as far as we know, single species of this hybridogenous genus containing diploid sexual and autopolyploid asexual cytotype and thus the best candidate to study GP (Fehrer, pers. comm.). In detail, Hartmann et al. (2018) tested the advantage of polyploidy in eco-physiological traits and demonstrated that foliar carbon (C) and nitrogen (N) content differ more along latitudinal gradient than between sexual and asexual cytotypes. Moreover, by testing the Red Queen hypothesis, Hartmann et al (2017) showed no differences in seed herbivory or latitudinal and/or altitudinal pattern. Furthermore, Mráz et al. (2019) demonstrate that seed production did not differ between sexuals and asexuals under natural condition, however, asexuals produce more seeds in the greenhouse with limited availability of pollinators suggesting that apomixis can provide more stable and predictable reproduction. In summary, since those studies did not show any clear explanation of GP distributional pattern in *H. alpinum*, more studies testing the other hypothesis should be performed to better understanding this phenomenon.

2.3.2 Importance of epigenetic variation in asexuals

Asexuals are considered as evolutionary dead-end due to low genetic variability, accumulation of deleterious mutations and consequently their lower ability of reaction to environmental changes

(Lynch et al., 1993). However, asexual organisms can react to environmental changes in some extent through changes in gene expression i.e. epigenetic mechanisms, which can increase the adaptability of asexuals (Latzel and Klimešová, 2010; Dodd and Douhovnikoff, 2016).

Several types of DNA modifications affecting translation are participating on the epigenetic variation of which DNA methylation is well known for a response to environmental factors (González et al., 2016; Herman and Sultan, 2016). DNA methylation is limited on two bases, namely cytosine and adenine, from which methylation of cytosine residues at 5C position is widespread across both, plants and animals (Montero et al., 1992). Cytosine methylation is established and maintained enzymatically using different types of DNA-methyltransferases during whole ontogenetic development (Cokus et al., 2008). However, most (but not all) of those changes in the global DNA methylation i.e. methylome including those with adaptive potential is lost during meiosis (Jablonka and Raz, 2009; Boyko et al., 2010; Feng et al., 2010) . Consequently, DNA methylation probably cannot play such an important role in the adaptability of sexuals, since the epigenetic signal cannot be fully transferred to subsequent generations. On the other hand, two-thirds of vascular plants in central Europe are capable to reproduce asexually (Klimeš et al., 1997) and thus, without meiosis, epigenetic variation can play an important role in their adaptability and potentially buffer low/no variation on the genetic level (Feng et al., 2010; Latzel and Klimešová, 2010; Richards et al., 2012).

Numerous studies demonstrated epigenetically mediated phenotypic plasticity as cytosine hypo- or hyper-methylation, especially during plant stress responses. Particularly, responses to cold stress (Hashida et al., 2006; Steward et al., 2000) water stress (González et al., 2011) high salinity (Dyachenko et al., 2006; Song et al., 2012) and pathogen (Wada et al., 2004; Yu et al., 2013) are well known to be mediated by epigenetic mechanisms. Moreover, the epigenetically mediated environmental response can be transferred through asexual reproduction with an adaptive potential i.e. epigenetic memory and ensure higher fitness of a clonal progeny (Latzel and Klimešová, 2010; Latzel et al., 2016; González et al., 2017). Furthermore, epigenetically mediated adaptability has been shown in the field, especially on populations of clonal or/and invasive plants. Firstly, one apomictic lineage of common dandelion *Taraxacum officinale* s. lat. showed differentiation in DNA methylation profile manifested in phenology (Wilschut et al., 2016). Secondly, two mostly clonally reproducing and highly invasive species, Japanese knotweed (Richards et al., 2012) and *Alternanthera philoxeroides* (Gao et al., 2010) exhibited epigenetic

differentiation among different microhabitats with only a little genetic variability. In summary, epigenetic variation can play an important role in the adaptability of plants, especially in the case of asexually reproducing plants lacking genetic diversity.

3 Genus *Hieracium* s. str.

The presented thesis is focused on true hawkweeds, genus *Hieracium* s.str. Genus *Pilosella* (formerly *Hieracium* subg. *Pilosella*) is excluded because of phylogenetic relationship, types of apomixis and morphology (Fehrer et al., 2007; Suda et al., 2007; Chrtek et al., 2009; Krahulcová et al., 2014).

Genus *Hieracium* s.str containing from 500 to 5000 species (depending on the used taxonomical concept) is considered as one of the most diversified genera in Angiosperms (Zahn, 1921–1923; Majeský et al., 2017). Apomicts form the major part of the diversity of this highly diversified genus while sexuals are rare (Mráz and Szeląg, 2004). This pattern has presumably been caused by numerous hybridization events in the past followed by polyploidization, which is in *Hieracium* s.str closely linked with apomixis. As a result, new hybridogenous lineages were formed combining morphological features of two or more parental taxa, which were fixed by apomictic reproduction (Zahn, 1921–1923; Fehrer et al., 2009). Origin of majority hybridogenous species was described already at the beginning of the twentieth century by Zahn (1921–1923), based purely on morphology (Fig. 1). This assumption was largely confirmed by molecular methods (Fehrer et al., 2009; Krak et al., 2013; Fig. 2). Moreover, the role of hybridization, polyploidization and apomixis is also reflected in cytotype distribution when diplosporous polyploid taxa (mainly triploid, tetraploid and a couple of pentaploid cytotypes) exhibiting frequent signs of hybridization are the most common while diploid sexually reproducing taxa are scare (Merxmüller, 1975; Schuhwerk, 1996; Mráz and Szeląg, 2004; Tyler and Jönsson, 2009).

In contrast to rich hybridization history of the genus, evidence of recent hybridization events are rare (Mráz et al., 2005; Chrtek et al., 2006; Mráz et al., 2011) . Lack of recent hybridization events can be possibly explained by non-overlapping distributional areas of diploid taxa (Mráz and Szeląg, 2004). However, since experimental hybrids in cultivation show reduced fertility i.e. limited seed production, the rarity of recent hybridization events can be also explain by partial sterility of the hybrids (Mráz and Paule, 2006). In that case, apomixis in *Hieracium* s.str

could represent an elegant mechanism for “escaping sterility” and subsequently contribute to the diversification of the genus (Asker and Jerling, 1992).

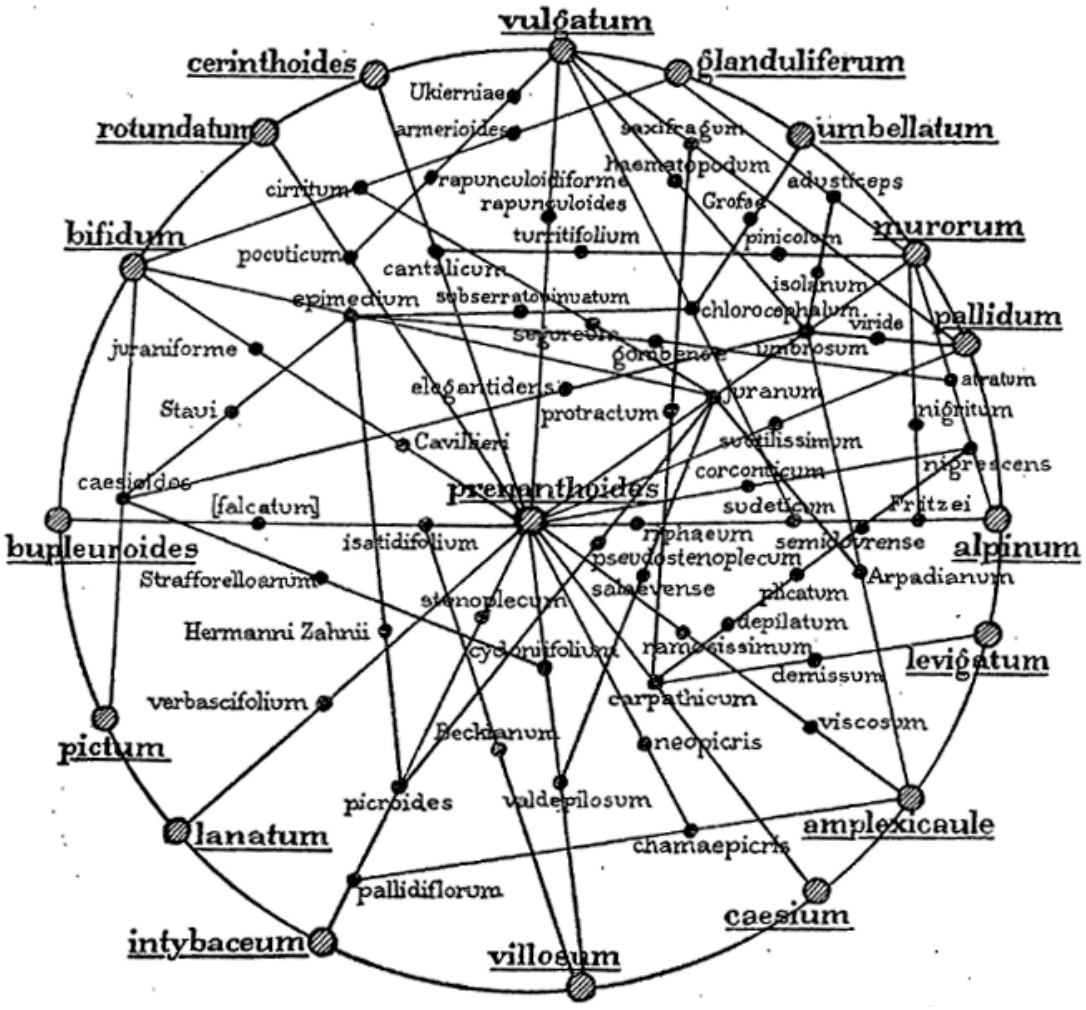

Fig. 1: Diagram of selected species from the genus *Hieracium* s.str. showing presumed relationships between supposedly non-hybridogenous i.e. basic species (big dashed dots) and supposedly hybridogenous i.e. intermediate species (small black dots). Relationships illustrated in this diagram are based purely on morphology. Figure is taken from Zahn (1921-1930).

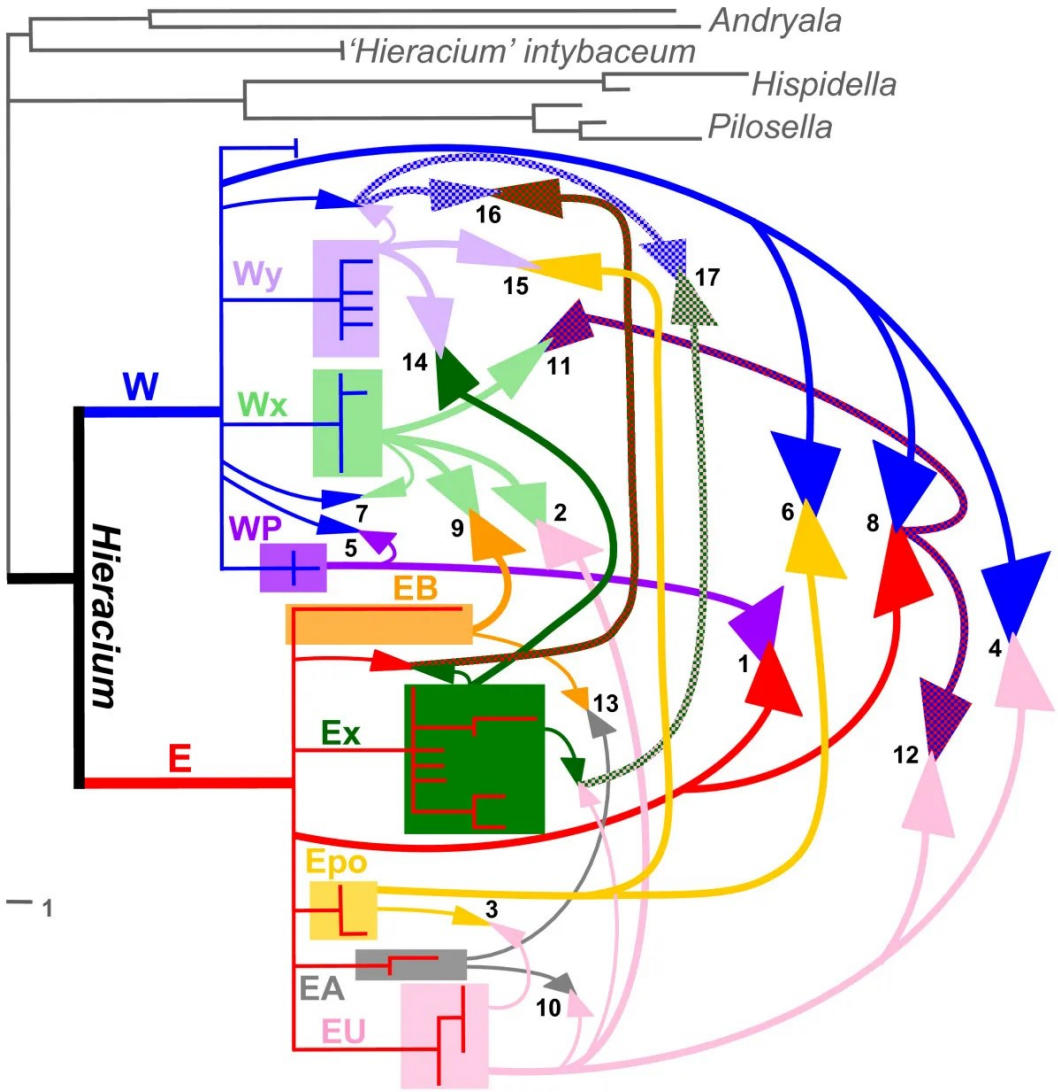

Fig. 2: Diagram showing hybridization history between selected species of *Hieracium* s.str. from different phylogenetic clades. The diagram is based on ETS features (and chloroplast capture in some cases) of 60 accessions (46 species). Diagram was taken from Fehrer et al. (2009).

4 Objectives of the thesis and a brief summary of results

Numerous plant species can reproduce by apomixis. However, the origin and molecular mechanisms behind apomictic reproduction are still unknown. Based on hybrid features and polyploidy of the major part of apomicts, it has been suggested that hybridization and polyploidization played a crucial role in the emergence of apomixis (Ernst 1918; Carman, 1997). Similarly to the origin of apomixis, it is still unclear why apomicts are commonly widely

distributed than their sexually reproducing relatives (geographical parthenogenesis phenomenon, abbreviated as GP) . In addition, several recent studies indicate that clonal plants (including apomicts) can perform some level of adaptability through epigenetic modification (Gao et al., 2010; Richards et al., 2012; González et al., 2016). In summary, many aspects of apomictic reproduction remain unclear. Therefore, this theses aims to elucidate processes behind the emergence of apomixis and its ecological significance using predominantly apomictic genus *Hieracium* s.str. as a model system for the following reasons:

i) Genus *Hieracium* s.str contains species/cytotypes of both reproductive strategies, sexual reproduction and apomixis which are closely linked to ploidy level. In detail, diploids of *Hieracium* s.str are strictly sexual while polyploids reproduce by diplosporous apomixis of *Antennaria* type (Skawińska, 1963; Mráz and Zdvořák, 2019).

ii) Polyploids commonly display a hybrid morphology which suggests that hybridization and/or polyploidization played a crucial role in the evolution of the genus and the emergence of apomixis. For example, hybridization history is reflected in the morphology of triploid *H. picroides* and *H. pallidiflorum* presumably derived from diploid *H. intybaceum* and *H. prenanthoides*. In addition, besides allopolyploid hybrids, *H. intybaceum* and *H. prenanthoides* contain both, sexual diploid and presumably autopolyploid triploid and tetraploid cytotypes (Zahradníček and Chrtek, 2015).

iii) Genus *Hieracium* contains the majority of widely distributed apomicts while sexuals are rare and thus, the whole genus illustrates a typical example of GP. At the level of species, GP was well documented in alpine hawkweed, *H. alpinum* (Figs. 3 and 4). This species contains diploid sexual and triploid apomictic cytotype (Mráz et al., 2009, Mráz et al., 2019).

iv) Rare incidence of autopolyploidy in *H. alpinum* (compared to the rest of the genus; Fehrer unpubl.) allows simpler results interpretation when comparing both ploidy levels and both reproductive modes. That is why the Papers III and IV are dealing with *H. alpinum* only.

In detail, this thesis aims to answer several questions which are grouped into two main topics:

Origin of apomictic reproduction and apomictic species in *Hieracium* s.str.

It has been suggested, that hybridization can trigger polyploidization and subsequently apomictic reproduction (Carman, 1997). For this reason, I decided to test the origin (autopolyploid or allopolyploid) of triploid *H. picroides* and *H. pallidiflorum* showing morphological characteristics of both, *H. intybaceum* and *H. prenanthoides*, and origin of tetraploid *H. intybaceum*, triploid and tetraploid *H. prenanthoides* which morphology does not show signs of hybridization (Zahradníček and Chrtek, 2015). Furthermore, I decided to test, whether experimental hybridization between several diploid sexual taxa triggers the production of unreduced gametes in hybrids displayed in production of neopolyploids. Specifically, concerning this topic, the presented thesis aims to answer the following questions:

1. Is the presumable hybrid origin of polyploid apomictic species *H. pallidiflorum* and *H. picroides* (presumably derived from two basic diploid species *H. intybaceum* and *H. prenanthoides*) reflected in plant morphology, shared alleles, haplome constitutions and genome size?
2. Does morphological (incl. pollen size), a genetic and cytogenetic variation of polyploid apomicts *H. pallidiflorum* and *H. picroides* indicating their single or multiple origins?
3. Do polyploid cytotypes of *H. intybaceum* and *H. prenanthoides*, with similar morphology as their diploid relatives, shows auto- or allopolyploid origin?
4. Does interspecific hybridization between diploid sexual taxa trigger higher production of unreduced gametes which could contribute to the increased production of neopolyploids?
5. If viable neopolyploids arise from homoploid crosses, are they fertile and able to produce the next generation of polyploids?
6. Do interspecific hybrids have reduced fertility displayed in megasporogenesis, megagametogenesis and seed production, presumably caused by non-homology of pairing chromosomes during meiosis?

To answer the questions 1-3 origin and variation of polyploid apomicts *H. pallidiflorum* and *H. picroides*, presumably derived from *H. intybaceum* (with two ploidy levels, 2x and 4x) and *H. prenanthoides* (with three ploidy levels, 2x, 3x and 4x), was evaluated using morphometric analysis, phylogenetic analysis (three unlinked molecular markers), cytogenetic analyses (*in situ*

hybridization) and genome size screening (see the Paper I). The presented study proved hybrid origins of two apomictic polyploid species, *H. pallidiflorum* and *H. picroides*, with the same parental combination of *H. intybaceum* and *H. prenanthoides*. Interestingly, the genomic contribution of both apomictic hybrids, *H. pallidiflorum* and *H. picroides*, corresponded with morphology, similarly like in diploid artificial hybrids *H. prenanthoides* x *intybaceum*. Moreover, considerable variation in all used molecular markers (ETS, *trn*T-*trn*L, *gsh1*) was detected in *H. pallidiflorum* and *H. picroides*, indicating their multiple polytopic origins. Furthermore, almost all polyploids of *H. intybaceum* (4x) and *H. prenanthoides* (3x and 4x) originally assumed as autopolyploids exhibited hybrid i.e. allopolyploid origin. In summary, these results presented in the Paper I indicate the importance of interspecific hybridization in the evolution of predominantly apomictic genus *Hieracium* s.str.

To answer the questions 4-6, large hybridization experiment between eight diploid taxa of *Hieracium* s.str and their experimental hybrids was performed (see the Paper II). Fertility was assessed as a seedset i.e. the proportion of well-developed seeds per capitulum, ploidy of both parental plants and their progeny was estimated by flow cytometry and hybridogenous origin of the progeny was evaluated using morphological traits. Moreover, cytoembryological approach was used to examine irregularities of megasporogenesis and megagametogenesis in hybrids compared to parental species. In agreement with our expectations, we found reduced seed production in plants whose both parents or at least the maternal one were interspecific hybrids compared to crosses were maternal or both parents were not hybrids. Irregularities during megasporogenesis and megagametogenesis possibly leading to unreduced embryo sac formation in hybrid progeny were not observed. In total, eight neopolyploid progeny out of 3,739 analysed progeny (0.2%) arisen in six out of 432 homoploid crosses (1.4%) was found. We did not observe an increased production of neopolyploids in diploid interspecific hybrids and introgressants when compared to parental diploid species. Importantly, fertile neotriploids originated from homoploid crosses produced the majority of triploid offsprings (63%) followed by diploid (31%) and hexaploid offsprings (6%), though the maternal neotriploids were in general highly sterile. In conclusion, the results of the hybridization experiment suggest that neopolyploidization in *Hieracium* s.str occurs in relatively low rate independently on the plant origin (hybrid or non-hybrid). However, neopolyploids produce a polyploid progeny more frequently than their diploid parents when crossed to diploids

Ecological significance of apomixis

Despite the fact, that apomicts are usually considered as an evolutionary dead end, apomictically reproducing species occupy often larger areas than their sexually reproducing relatives (geographical parthenogenesis phenomenon, abbreviated as GP). Several hypotheses were already tested to explain GP in *H. alpinum* (Figs. 3 and 4) from which only greater reproductive assurance of apomictic triploids partly explains this phenomenon (Mráz et al., 2019). Potential role of Metapopulation hypothesis, i.e. the negative effect of inbreeding on the fitness of sexuals, has been the topic of Paper III. Specifically, this thesis aims to answer the following questions:

1. Are seed production and seed germination of self-incompatible diploid cytotype of *H. alpinum* reduced in inbred crosses?
2. Do inbred plants have lower fitness than non-inbred plants?
3. Is the effect of inbreeding enhanced in the presence of interspecific competition, similarly like in natural conditions?

To answer these questions experimental crosses using sexual diploid *H. alpinum* were performed to produce progeny showing different levels of inbreeding (see the Paper III). Furthermore, a subset of inbred and outbred progeny was exposed to competition with natural competitor *Nardus stricta* (Poaceae). Subsequently, experimental plants were assessed for multiple fitness traits. We did not found reduced seed set and germination in inbred crosses when compared to outbred, and interpopulation outbred ones. Nevertheless, inbreeding depression was demonstrated in several fitness traits like the amount of biomass or survival rate. Interspecific competition reduced all fitness traits regardless levels of inbreeding suggesting that inbreeding depression was not enhanced in the presence of environmental stress provided by the competitor. In conclusion, presented results showed that sexual diploids of *H. alpinum* are susceptible to inbreeding depression which might suggest disadvantage in comparison to apomictically reproducing triploid cytotype. Subsequently, this trend could contribute to the lower colonizing potential of sexuals compared to asexuals and possibly explain GP distribution pattern according to Metapopulation hypothesis.

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Fig. 3: Diploid plant of Hieracium alpinum in the Munții Bucegi Mts, Romania, 2014 © Patrik Mráz

Fig. 4: *Hieracium alpinim* in its natural habitat in the Malá Studená Dolina. © Peter Paleček

Several recent studies showed that asexually reproducing plants with limited genetic variability can respond to changing environment through epigenetic mechanisms, from which DNA methylation is well known to be involved in environmental response (González et al., 2016; Herman and Sultan, 2016). In detail, the importance of DNA methylation was already tested twice in *H. alpinum* using experimental demethylation with, in contrast to other studies, no/little effect on plants performance (Pinc unpubl, Vlk 2017). Based on this results, I decided to determine the best approach of experimental demethylation for alpine hawkweed, *Hieracium alpinum*, and help to elucidate the sources of inconsistency in the literature dealing with experimental demethylation as a part of this thesis (Paper IV). Specifically, presented thesis aims to answer the following questions:

4. Which application method of which demethylation agent reduces global DNA methylation the most with the smallest negative impact on plants viability?
5. Are induced changes in global DNA methylation stable in time or DNA will be methylated back i.e. remethylated after cessation of demethylation agent application?
6. Is the efficiency of experimental demethylation, i.e. decrease of the global amount of DNA methylation, species-specific, and if so, is there any association between efficiency of experimental demethylation and genome size and amount of global DNA methylation of untreated plants?

To answer these questions, germinating seeds and plants belonging to one triploid clone of *Hieracium alpinum* was experimentally demethylated using two demethylation agents (5-azacytidine and zebularine) with different concentrations applied with different frequencies (see the Paper IV). Selected fitness traits, leaf carbon and nitrogen contents were measured. In addition, we evaluated the amount of global methylation using HPLC two times during the experiment, the first time at the cessation of demethylation treatment and the second time 10 weeks after this cessation. Application of 50μM 5-azacytidine on germination seeds followed by spraying induced the largest drop in global DNA methylation without significant negative impact on plants viability and thus it was considered as the most efficient demethylation agent. We found also that experimental demethylation is not stable in time and without a constant supply of demethylation agent DNA can be remethylated to its original level. In the comparison study which included all so far analysed vascular plants, we found that the total amount of methylated cytidine in non-demethylated plants significantly influences the efficiency of experimental demethylation. In

detail, the presented study shows that the total amount of methylated cytidine of untreated plants was significantly negatively correlated with the efficiency of experimental demethylation i.e. decrease in global cytidine methylation. In other words, the bigger and more methylated plant genome is, the smaller is achieved a decrease in global DNA methylation. In summary, the best approach of experimental demethylation for *H. alpinum* is described in Paper IV, however, it must be pointed that that pilot study probably needed for different species. Otherwise, the unsuitable approach of experimental demethylation can result in an insufficient decrease in DNA methylation or death of experimental plants.

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Chapter II: Papers

Paper I:

Evolutionary history and genetic diversity of apomictic allopolyploids in *Hieracium* s.str.: morphological versus genomic features

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Keywords: Asteraceae; gamma-glutamylcysteine synthetase; genome size; in situ hybridization; multivariate morphometrics; pollen size; polyploid evolution; repeatome

Abstract:

The origin of allopolyploids is believed to shape their evolutionary potential, ecology and geographical ranges. Morphologically distinct apomictic types sharing the same parental species belong to the most challenging groups of polyploids. We evaluated the origins and variation of two triploid taxa (*Hieracium pallidiflorum*, *H. picroides*) presumably derived from the same diploid parental pair (*H. intybaceum*, *H. prenanthoides*).

We used a suite of approaches ranging from morphological, phylogenetic (three unlinked molecular markers) and cytogenetic analyses (*in situ* hybridization) to genome size screening and genome skimming.

Genotyping proved the expected parentage of all analyzed accessions of *H. pallidiflorum* and *H. picroides* and revealed that nearly all of them originated independently. Genome sizes and genome dosage largely corresponded to morphology whereas the maternal origin of the allopolyploids had no discernable effect. Polyploid accessions of both parental species usually contained genetic material from other species. Given the phylogenetic distance of the parents, their chromosomes appeared only weakly differentiated in GISH as well as in overall comparisons of the repetitive fraction of their genomes. Furthermore, the repeatome of a phylogenetically more closely related species (*H. umbellatum*) differed significantly more.

We proved (i) multiple origins of hybridogeneous apomicts from the same diploid parental taxa, and (ii) allopolyploid origins of polyploid accessions of the parental species. We also showed that the evolutionary dynamics of very fast-evolving markers such as satellite DNA or transposable elements do not necessarily follow patterns of speciation.

Introduction

Polyploidization, the multiplication of complete chromosome sets, is a major force in plant evolution, and an important mechanism of sympatric speciation. Approximately 15% of speciation events in angiosperms have been estimated to be associated with polyploidy (Otto and Whitton, 2000; Wood et al., 2009; Wendel, 2015; Soltis et al., 2016). There are two main types of polyploids (although intermediates exist between these; Soltis et al., 2003). In allopolyploids, genome duplication is connected to hybridization, i.e. the merger of the nuclear genomes of two separate lineages (usually species) into a new lineage. In contrast, autopolyploids arise within a single species by genome doubling and produce structurally similar homologous genome. Autopolyploids may thus suffer from drastic changes in the functionality of meiosis due to multivalent formation and consequent unbalance in chromosome segregation, whereas allopolyploids predominantly form bivalents, as nonhomologous chromosomes do not pair (Ramsey and Schemske, 2002; Oxelman et al., 2017).

Polyploid evolution can sometimes be followed by a shift to apomixis – asexual seed production. As a result, clonal progeny retaining a maternal genotype is formed because meiosis and syngamy are completely circumvented (Bicknell and Koltunow, 2004). The switch to apomictic reproduction thus might avoid the penalties of meiotic disturbances caused by the polyploidization of the genome, and represent a possible strategy to escape from hybrid sterility as hypothesized by Darlington (1939). Furthermore, apomixis provides a very strong reproductive barrier (Rieseberg et al., 2006). Both evolutionary mechanisms, i.e. reproductive assurance and isolation, thus ultimately contribute to the establishment of newly formed apomictic species. Surprisingly, the evolutionary history of many polyploid apomicts remains to be unravelled, although it is of crucial importance for several reasons.

Firstly, the distinction between auto- and allopolyploid origins of apomicts is important as it provides a clue about the significance of interspecific hybridization in the evolution of apomixis. Because the evidence for autopolyploid origins of apomicts is scarce (Thompson and Whitton, 2006; Hojsgaard et al., 2008; Cosendai et al., 2011), and most apomicts were shown to be of hybrid origin (Asker and Jerling, 1992; Paun et al., 2006; Palop-Esteban et al., 2007; Sharbel et al., 2009; Šingliarová et al., 2011), it has been hypothesised that hybridization might be a trigger of apomixis (Barke et al., 2018).

Secondly, the detection of multiple origins of apomictic lineages might considerably change our view about apomicts as evolutionary 'dead ends' (Maynard-Smith, 1978). This is because increased genetic variation due to fixed heterozygosity will provide apomicts with increased adaptive potential (Bierzychudek, 1985). Furthermore, recent analyses of polyploid genomes have strengthened the idea that multiple independent origins are more common in generating polyploids than a single origin (Soltis and Soltis, 1993; Leitch and Bennett, 1997; Soltis and Soltis, 1999; Soltis et al., 2003; Ainouche et al., 2004; Guggisberg et al., 2006). Multiple origins has been also mentioned among the factors accounting for genetic diversity within polyploid apomictic taxa (e.g., Asker and Jerling, 1992; Hörandl and Paun, 2007).

The genus *Hieracium* L. (Asteraceae) is an excellent model system to study the evolutionary history of apomictic polyploids. It consists of only ca 25 sexual diploids ($x = 9$) and hundreds to thousands (depending on the taxonomic concept, cf. Zahn, 1921–1923; Majeský et al., 2015) of mostly triploid or tetraploid ($2n = 3x = 27$, $2n = 4x = 36$) apomictic taxa (Chrtek et al., 2004; Mráz and Zdvořák, 2019). Concerted events of hybridization, polyploidization and shifts to apomixis have played a principal role in *Hieracium* evolution (Fehrer et al., 2009). Importantly, all diploid taxa are well differentiated morphologically and, together with several polyploid species, form so called 'basic' species characterized by unique combinations of morphological traits. In contrast, most of the currently recognized apomictic polyploid taxa have been treated as 'intermediate' species as they combine morphological traits of two or more basic species (Zahn, 1921–1923). Often a distinction between truly intermediate accessions and those morphologically close to one or other parent is made (see Zahn, 1921–1923 and his 'morphological formulas'); each of those variants is traditionally classified as separate taxa, even when a broad species concept is applied. Such a 'morphological system' provides excellent evolutionary hypotheses assuming not only a putative parentage, but also hinting at the extent to which particular parental basic species may have contributed to their origin. Here we study one such system in detail.

As a parental combination, we chose the basic species *H. intybaceum* All. and *H. prenanthoides* L., well suited to decipher evolutionary history of the morphologically intermediate allopolyploids, because (i) diploid cytotypes of these species are to a limited extent interfertile, diploid F_1 progeny is viable and partly fertile, which mimics the past evolutionary processes; (ii) small, but consistent differences in genome size between *H. intybaceum* and *H. prenanthoides* have been reported (Chrtek et al., 2009), which might support the evolutionary scenarios based on

molecular markers, and (iii) with the exception of one [accession of a morphologically recognizable] subspecies, no other species are involved in the origins of the morphologically intermediate hybridogeneous allopolyploids in this group, as confirmed in a phylogenetic reconstruction involving nearly all *Hieracium* basic species (see below).

Hieracium intybaceum is genetically and morphologically most divergent from all other species of *Hieracium* (Fehrer et al., 2007a), which greatly facilitates the morphological, genetical and cytogenetic recognition of hybridization. The species comprises diploid sexual and tetraploid apomictic cytotypes (Zahradníček and Chrtek, 2015; Mráz and Zdvořák, 2019). Diploids occur throughout the Alps, tetraploids are restricted to the Western Alps and the Vosges Mts (Zahradníček and Chrtek, 2015). Populations corresponding to the tetraploid cytotype were included by Zahn (1921–1923) within the morphological variation of diploid populations, which could suggest their autopolyploid origin. A recent molecular study involving AFLP markers was rather inconclusive in respect of the auto- or allopolyploid origin of tetraploid *H. intybaceum* as a certain level of admixture between both ploidies and at the same time a clear separation of most tetraploid individuals were recorded (Zahradníček and Chrtek, 2015). The second basic species, *Hieracium prenanthoides*, occupies a large geographic range spreading from Greenland and the British Isles throughout the European mountains eastwards to Siberia and Central Asia (Bräutigam, 1992). It consists of diploid sexuals (restricted to the Southwestern Alps) and polyploid (3x, 4x) apomicts (throughout the range; Chrtek et al., 2007). Phylogenetic delimitation of the parental species was elaborated by three unlinked molecular markers. *Hieracium intybaceum* is well separated from all other species with all markers (ETS, *trn*T-*trn*L, *gsh1*) and is genetically quite uniform as shown previously by AFLP data (Zahradníček and Chrtek, 2015). *Hieracium prenanthoides* is more difficult to separate genetically from some other *Hieracium* species, a general feature of the core group of *Hieracium* which is characterized by allele and haplotype sharing (see Methods). *Gsh1* was chosen because in a pilot study diploid and triploid accessions of *H. prenanthoides* grouped together and were distinguishable from all other *Hieracium* species. The combination of these molecular markers allowed us to infer the origins of the allopolyploids.

Two triploid accessions of *H. prenanthoides* were previously found to have different allopolyploid origins, one of them originated by a subsequent hybridization with *H. umbellatum* L. or another closely related species (´*H. umbellatum*´ clade; Fehrer et al., 2009). *Hieracium*

umbellatum was thus included in the part aimed at the evolutionary history of polyploid accessions of *H. prenanthoides*.

Diversity in ploidy levels and multiple allopolyploid origins are reflected by high intraspecific morphological variation. Three species morphologically intermediate between *H. intybaceum* and *H. prenanthoides* have been described, namely *H. pallidiflorum* Jord. ex Aschers. (morphologically closer to *H. intybaceum*), *H. picroides* Vill. (intermediate or closer to *H. prenanthoides*), and *H. stenoplecum* Arv.-Touv. and Huter (morphologically closer to *H. prenanthoides*; Zahn, 1921–1923). Recently, *H. pallidiflorum* and *H. picroides* were proved to be triploid and to reproduce apomictically (Mráz and Zdvořák, 2019) while no information about *H. stenoplecum* is available.

In contrast to sexual allopolyploids, in general, evolutionary trajectories of apomictic allopolyploids are little known. To fill this gap, we use a suite of approaches ranging from morphological, phylogenetic and cytogenetic analyses to genome size screening and genome skimming. The genetic approaches employ plastid and nuclear DNA markers previously used in phylogenetic reconstructions of *Hieracium* (Fehrer et al., 2007a, 2009; Krak et al., 2013) as well as a new low-copy nuclear marker with high variation at species level (Krak et al., 2012). In combination, these markers allow us to assess the type of polyploidy, to determine the direction of the cross in allopolyploids and to detect potential introgressions events distinct from polyploidization. Pollen size heterogeneity can be used as a proxy for meiotic disturbances, frequently reported in *Hieracium* polyploids (Rosenberg, 1927; Gentcheff and Gustafsson, 1940; Chrtek, 1997; Slade and Rich, 2007). Cytogenetic investigations (Genomic and Fluorescence *In Situ* Hybridization, GISH, FISH) are powerful tools in verifying the origin of polyploid taxa as well as the genome dosage of the respective parents (Heslop-Harrison and Schwarzacher, 1996). Genome size is usually stable within morphologically defined species and often differs even between closely related species (e.g., Bräutigam and Bräutigam, 1996; Prančl et al., 2018; Róis et al., 2018; Zahradníček et al., 2019). This may help resolving conflicting hypotheses about the origin of polyploids (auto- vs. allopolyploidy) and identifying putative parental combinations in hybridogenous species (e.g., Mahelka et al., 2005). To better understand the observed patterns, we applied low coverage Next Generation Sequencing (hereafter genome skimming) to assess overall differences of parental repeatomes that are underlying cytogenetic patterns and genome size variation.

Here we endeavour to (1) prove that the polyploid apomictic hybridogeneous species *H. pallidiflorum* and *H. picroides* originated from the same parental species. Based on morphological affinities, we suppose that *H. pallidiflorum* is closer to *H. intybaceum* and that *H. picroides* is intermediate or closer to *H. prenanthoides*, not only in terms of morphology (assessed by multivariate morphometrics), but also concerning shared alleles, haplome constitution and genome size; (2) assess their morphological, genetic and cytogenetic variation and thus demonstrate their multiple or single origins; (3) test allo- versus autopolyploid origins of polyploid cytotypes of the parental species *H. intybaceum* and *H. prenanthoides*; (4) compare the repeatomes of the parental species and *H. umbellatum* that was found to have contributed to some polyploid genomes of *H. prenanthoides* and to relate these results to phylogenetic relationships in the genus *Hieracium* as well as to cytogenetic patterns revealed by GISH; and (5) test whether apomictic allopolyploids produce on average larger and more heterogeneously sized pollen compared to their diploid parental species, which would indicate meiotic disturbances.

Materials and methods

Plant material

Samples of *H. intybaceum* (2*x*, 4*x*), *H. prenanthoides* (2*x*, 3*x*, 4*x*) and two of their putative hybridogeneous species (*H. pallidiflorum* and *H. picroides*, both 3*x*) (Fig. 1) were collected between 2003 and 2017 in the Alps with a focus on geographically contrasting parts of the Western (French) and Eastern (Austrian) Alps (Appendix 1, Fig. 2). We did not find plants of the very rare third intermediate taxon *H. stenoplecum*, which was thus omitted from our study. Living plants were collected and transferred to the Experimental garden of the Institute of Botany in Průhonice and the Experimental Garden of the Faculty of Science, Charles University, Prague. Parts of the same individual plants were used for various analyses (Table 1); additional plants from the same populations were pressed/dried for morphometric studies and as herbarium vouchers. In addition, F_1 experimental hybrids between diploids of *H. intybaceum* and *H. prenanthoides* as well as two accessions of *H. umbellatum* (2*x*) were included in some analyses. Voucher specimens are

deposited in the herbarium of the Institute of Botany CAS in Průhonice (PRA) and in the herbarium of Charles University in Prague (PRC).

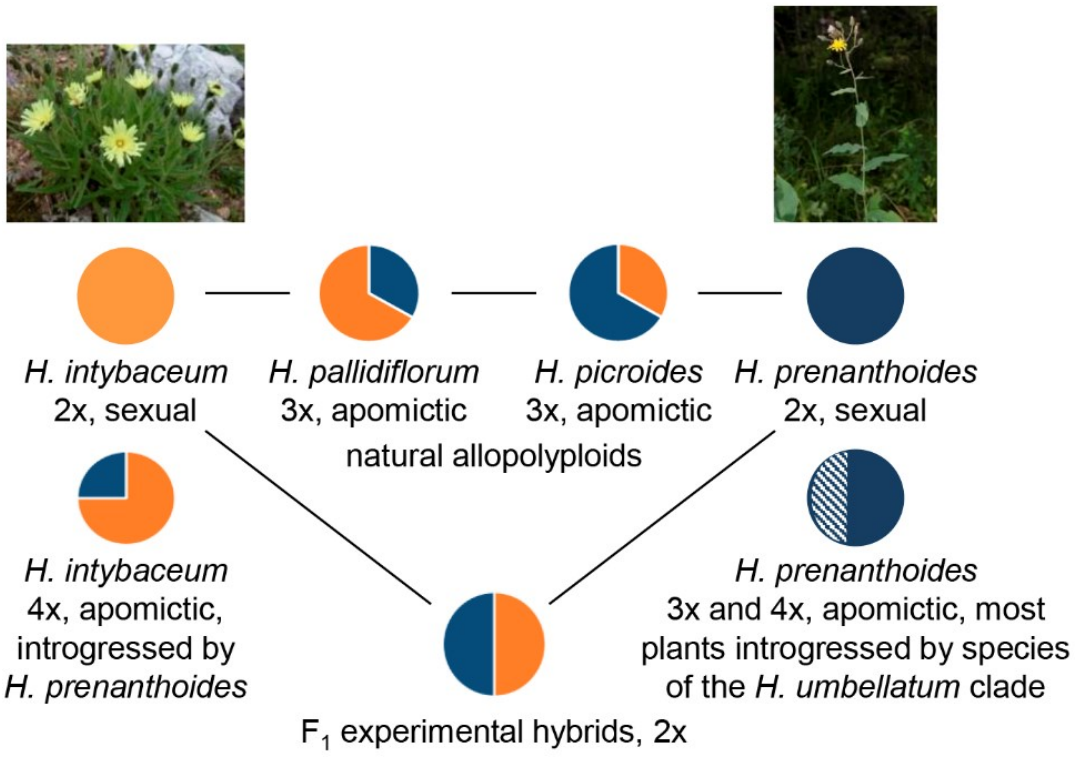

Fig. 1. Hypothesized evolutionary relationships among all species/cytotypes involved in the study. Different colors in *H. pallidiflorum*, *H. picroides* and the tetraploid apomictic cytotype of *H. intybaceum* indicate their genome composition as determined by *in situ* hybridization. The parental species, *H. intybaceum* and *H. prenanthoides*, clearly differ from each other in the height. While the former is a short, 0.1–0.3 m high plant, the latter is a tall, 0.4–1.2 m high plant.

Table 1. Overview of plant material investigated in various kinds of analyses. Either population labels (plants used only for morphological analyses are marked by an asterisk) or accession labels are provided. Ploidy level was checked using flow cytometry.

Species, ploidy, population / accession label(s)	Locality, voucher information	morpho-metrics (no. of plants)	pollen (no. of plants)	genome size	geno-typed	GISH	FISH	NGS
H. intybaceum* 2*x								
int_1709*	Austria, Kärnten: Kötschach	+ (5)						
inb.Kaer	Austria, Kärnten: Turracher Höhe				+ (1)			
H1622	Austria, Tirol: Sölden, Mautstelle	+ (5)			+ (1)			
1531/8	Austria, Tirol, Arlberg Massif: Arlbergpass I		+ (1)	+ (1)				+ (1)
Arlbergpass*	Austria, Tirol, Arlberg Massif: Arlbergpass II	+ (2)						
int_1809*	Austria, Tirol: Obergaimberg near Lienz	+ (4)						
H1626*	Austria, Tirol: Obergurgl	+ (3)						
H1321*	Austria, Tirol: Untergurgl (Poschach)	+ (3)						
Petit Bernard*, int_6/9/4, int_6/14/25	France, Savoie: Col du Petit Saint-Bernard	+ (5)	+ (1)	+ (1)				+ (1)
1069/1	Italy: Rhaetian Alps			+ (1)	+ (1)			
Timmelsjoch	Italy, Trentino-Alto Adige: Timmelsjoch saddle	+ (1)						
int_1610*	Switzerland, Ticino: Airolo	+ (2)						
int_3/2/7, int_3/2/8	Switzerland, Wallis: Furkapass		+ (2)					
int_1910*	Switzerland, Wallis: Oberwald	+ (5)						
H. intybaceum* 4*x								
INT1_Pel, 16439/5	France, Hautes-Alpes: massif des Écrins		+ (3)	+ (1)	+ (1)	+ (1)		
H. prenanthoides* 2*x								
H1632*	France, Hautes-Alpes: Ailefroide	+ (9)						
1252	France, Hautes Alpes: La Grave	+ (2)			+ (1)			
H1634*	France, Savoie: Avrieux	+ (7)						
H1631*	Italy, Piedmont: Claviere I	+ (15)						
pre_6*, pre_6/5/5, pre_6/8/5	Italy, Piedmont: Claviere II		+ (6)	+ (1)				+ (2)

H. prenanthoides* 3*x							
1187/1	Andorra: Canillo				+ (1)	+ (1)	
1161/2	Poland, Województwo dolnośląskie, Karkonosze Mts: Mały Kocioł Śnieżny				+ (1)		
PM2050, PM2051, PM2052	Slovakia, Banskobystrický kraj: Muránska planina Mts: Nižná Kľaková		+ (3)				
pre_1/4/4	Slovakia, Prešovský kraj, Belianske Tatry		+ (4)		+ (1)		
pre_sl_1/4/4, pre_sl_1/4/5	Mts: Ždiar						
pre_sl_1/3/1, pre_sl_1/3/9							
H. prenanthoides* 4*x							
pre_1/3/2, R1/8/1	Czechia, Královéhradecký kraj, Krkonoše Mts: Rýchory I		+ (2)		+ (1)	+ (1)	
R4.3.1	Czechia, Královéhradecký kraj, Krkonoše Mts: Rýchory II			+ (1)	+ (1)	+ (1)	
pre_3/9/3, rap_3/8/6/5, rap_3/8/7	France, Hautes-Alpes: Ailefroide		+ (1)	+ (1)	+ (1)	+ (1)	
H. pallidiflorum* 3*x							
H1609	Austria, Bundesland Salzburg: Muhr	+ (6)		+ (1)	+ (1)	+ (1)	
H1508	France, Savoie: Bonneval-sur-Arc I		+ (1)	+ (1)		+ (1)	+ (1)
H1616	France, Savoie: Bonneval-sur-Arc II	+ (14)	+ (1)		+ (1)		
H1614	France, Savoie: Col du Petit Saint-Bernard I		+ (1)	+ (1)	+ (1)		
pal_1*, pal_1/4/2, pal_1/4/5	France, Savoie: Col du Petit Saint-Bernard II	+ (11)			+ (1)	+ (1)	+ (1)
Col Iseran*	France, Savoie: Val-d´Isère	+ (3)					
H. picroides* 3*x							
H1608	Austria, Bundesland Salzburg: Muhr	+ (2)		+ (1)	+ (1)	+ (1)	+ (1)
H1637	Austria, Tirol: Sölden, Gaislach				+ (1)		
H1603	Austria, Tirol: Wirl			+ (1)			
H1604	Austria, Tirol: Wirl, Alte Kopsstraße			+ (1)			
H1605	Austria, Vorarlberg: Zeinissee	+ (3)		+ (1)	+ (1)		
H1613	France, Hautes-Alpes: Col du Lautaret	+ (3)	+ (2)	+ (1)	+ (1)	+ (1)	+ (1)

H1621	France, Savoie: Bonneval-sur-Arc				+ (1)			
H1509/2	France, Savoie: Bonneval-sur-Arc, L´Écot I				+ (1)			
H1619	France, Savoie: Bonneval-sur-Arc, L´Écot II	+ (7)			+ (1)			
H1615	France, Savoie: Col du Petit Saint-Bernard I	+ (16)		+ (1)	+ (1)	+ (1)	+ (1)	
pic_1/1/2, pic_1/1/3, pic_1/1/4, pic_1/2/1	France, Savoie: Col du Petit Saint-Bernard II		+ (2)	+ (1)	+ (1)	+ (1)	+ (1)	
H1620	Italy, Aosta: Gran San Bernardo	+ (2)			+ (1)			
H1623	Italy, Piedmont: Limone Piemonte				+ (1)			
H1618	Italy, Trentino-Alto Adige: Passo del Tonale	+ (2)			+ (1)			
Experimental 2*x* F_1 hybrids *H. intybaceum* x *H. prenanthoides*								
pre_6/8/5 x int 1531/8 (17038)	experimental garden			+ (1)				
91preint_2, 91preint_3, 92preint_1, 92preint_5, 16116_3	experimental garden		+ (5)			+ (1)		
pre1507/2 x int 1110/2	experimental garden	+ (2)						
H. umbellatum* 2*x								
H1617/1	Czechia, Praha: Troja			+ (1)				+ (1)
umbellatum_8/9/2	Slovakia, Košický kraj: Prakovce			+ (1)				

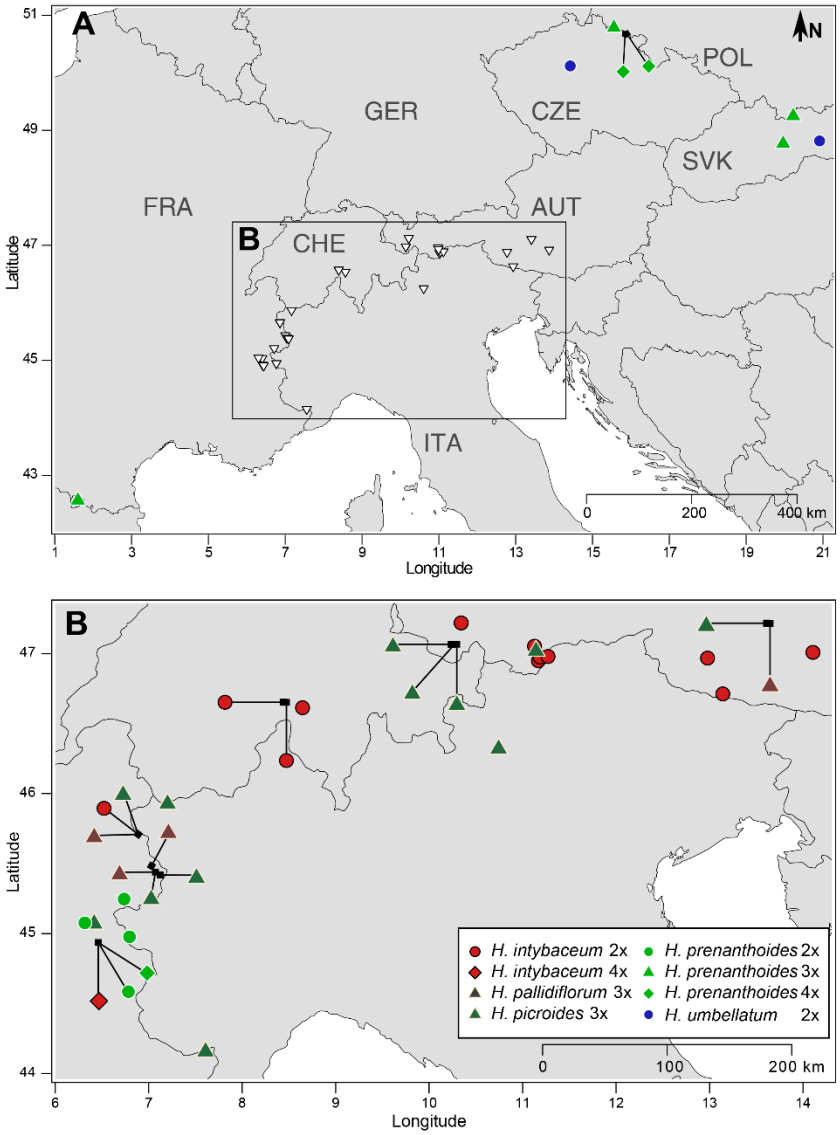

Fig. 2. Sampling locations of accessions investigated. Locations across Europe (top) and, in detail, across the Alps (bottom) are given.

Country abbreviations: (AUT) Austria, (CHE) Switzerland, (CZE) Czech Republic, (FRA) France, (GER) Germany, (ITA) Italy, (POL) Poland, (SVK) Slovakia. The figure was produced within the R environment using the package rworldmap (South, 2011)

Multivariate morphometrics

In order to examine the overall pattern and morphological differentiation between the taxa studied, 16 characters (Table 2) were measured/scored on herbarium material from our own collections (Table 1, Appendix 1). We also included experimental diploid F_1 hybrids between diploid cytotypes of *H. intybaceum* and *H. prenanthoides*. The morphological characters include those reported as diagnostic in determination keys and Floras (e.g., Zahn, 1921–1923; Fischer et al., 2008; Tison and Foucault, 2014) as well as characters that appeared variable based on our field

observations. Three datasets were used in the analyses: (1) a dataset that included all plants from the *H. intybaceum – H. prenanthoides* complex (dataset 1), (2) a dataset that included plants of *H. picroides* attributed to either *H. intybaceum* or *H. prenanthoides* as a maternal parent based on ptDNA sequencing (dataset 2), (3) a dataset of exclusively *H. pallidiflorum* accessions, divided into two groups according to their provenience (Western vs. Eastern Alps; dataset 3). The data were transformed by the common logarithm.The correlations among the characters were investigated using the non-parametric Spearman's rank coefficients to detect high correlations (>0.95) and to avoid distortion of the multivariate analysis. Principal component analysis (PCA) based on a correlation matrix of characters and individual plants as OTUs (operational taxonomic units) was conducted to reduce the multidimensional nature of the character space and to show the overall pattern of morphological variation. Randomization-based statistics (rnd-LambdaF and rndF) were calculated to estimate the number of PCs (number of permutations = 1000, $\alpha = 0.05$). The estimated number of PCs was the last point at which the p-value of the statistic of interest greater than the observed one is at least the threshold significance level. Canonical discriminant analyses (CDA) and classificatory discriminant analysis (classificatory DA) were performed based on individual plants and groups defined on the basis of genome composition (GISH results). In CDA, both marginal effect of particular characters and their conditional effects in a stepwise selection of the most informative characters were assessed using a Monte Carlo test with 1000 permutations. The analyses were performed using a set of R functions contained in MorphoTools version 1.01 (Koutecký, 2015), PCDimension (Coombes and Wang, 2019) and ade4 package (Dray and Dufour, 2007).

Table 2. Characters used in the morphometric study.

Character acronym	Detailed definition of the characters and comments
PLH	plant height (cm)
SHR	stem height from the base up to the first ramification
LAC	length of the acladium, i.e. the peduncle of the middle (principal) capitulum (cm)
LLB	length of the longest lateral branch (cm); measured from the ramification to the base of the principal capitulum of the longest lateral branch
LPC	length of involucrum of the middle (principal) capitulum
LML	length of a middle stem leaf (cm)
WML	maximal width of a middle stem leaf (cm)
LGT	length of the longest glandular hair on the acladium (mm)
NCA	total number of capitula on the plant; all capitula, including capitulum buds bigger than 3 mm were counted
NFC	number of florets per principal capitulum (if possible, otherwise the biggest capitulum was used)
NSL	number of stem leaves; counted from the stem base up to the acladium (including all bract-like leaves), withered leaves (their traces are visible on the stem) were included
NLB	number of leaves in the basal part of the stem; measured from the stem base up to the first ramification, withered leaves (their traces are visible on the stem) were included
NMG	number of mucronate glands (including tooth apex) on one side of the middle stem leaf margin
NTL	number of teeth on one side of the middle stem leaf margin; as tooth we considered protuberances bigger than 3 mm
NGT	number of glandular trichomes on the middle stem leaf; counted on the leaf margin in the middle part on a length of 1 cm
NCT	number of simple eglandular hairs on the middle stem leaf; counted on the leaf margin in the middle part on a length of 1 cm

Pollen size variation

The pollen equatorial and polar lengths were measured on samples collected from plants cultivated in the experimental gardens. To prevent possible pollen cross-contamination, we sampled capitula at the stage just before their opening. The capitula were sampled and stored in plastic tubes filled with Calberla´s solution containing glycerol, 95% ethanol and distilled water in proportions 1:2:3 and a sufficient amount of basic fuchsin to create transparent pink colour (Ogden et al., 1974). At least four florets were dissected from each capitulum, placed onto a slide with a drop of Calberla´s solution, and broken up with tweezers to release the pollen. The samples were covered by a cover slip, examined and photographed using a Zeiss Axio Imager.Z2 microscope system (Carl Zeiss, Jena, Germany) at a magnification of 200×. Pollen equatorial and polar lengths, including exines, were measured using the ZEN blue 2.5 software (Carl Zeiss Microscopy, Jena, Germany). Because polar and equatorial lengths were strongly correlated (r = 0.96, Pearson correlation test), only the latter parameter was used to show variation among the predefined groups, i.e. species by ploidy combinations. To illustrate the level of homogeneity of pollen equatorial length within each group, we firstly calculated the coefficient of variation for each individual accession separately, and then we constructed boxplots based on these individual data. In total, we measured ca 100 pollen grains per plant if possible, and 3–9 plants per species and ploidy (Table 1). Because of the small sample size at the level of accessions (from 3 to 9 replicates per group), we did not perform formal statistical tests.

Genome size and ploidy level estimations

Genome size was estimated in a total of 21 plants (fresh leaves) of *H. intybaceum*, *H. pallidiflorum*, *H. picroides*, *H. prenanthoides* and *H. umbellatum* and the F_1 generation of crosses between diploid *H. intybaceum* and *H. prenanthoides* (Table 1). Genome size was determined by flow cytometry using a Partec CyFlow cytometer equipped with a green (532 nm) solid-state laser. *Pisum sativum* cv. Ctirad (2C = 9.09 pg of DNA; Doležel et al., 1998) or *Bellis perennis* (2C = 3.44 pg of DNA) which was measured against *Pisum* were used as internal standards. The simplified two step-procedure described by Otto (1990) was employed for sample preparation as described in Chrtek et al. (2009). Samples were stained for 5 min at room temperature before being analyzed. As a rule, 5000 nuclei were analyzed for each sample. Nuclear genome size was

calculated as a linear relation between the ratio of 2C peaks of samples and standard using Partec FloMax version 2.4d (Partec GmbH, Münster, Germany). Each plant was measured three times on different days by the same operator to eliminate potential artefacts. In order to determine the ploidy level in populations from which fresh leaves were not available and to confirm putative within-population ploidal homogeneity (e.g., Mráz and Zdvořák, 2019), we additionaly analysed from one to four herbarized individuals. Here we used 4',6-diamino-2-phenylindole (DAPI) as a fluorescent dye and a Partec Cyflow instrument equipped with a HBO lamp as ploidy level analyzer. The results of flow cytometry measurements were calibrated by chromosome numbers gathered in this study (GISH, FISH) or previously published for our plants (Chrtek et al., 2007; Chrtek et al., 2009; Mráz and Zdvořák, 2019).

Sampling and choice of molecular markers for genotyping

One sample per population of *H. pallidiflorum* and *H. picroides* (Table 1) was genotyped with three unlinked molecular markers in order to confirm their morphologically inferred identity as allopolyploids with contribution of *H. intybaceum* and *H. prenanthoides* and to determine the direction of the cross. For comparison, diploid and polyploid accessions of the parental species were included (Table 3). For two markers, the multicopy external transcribed spacer of nuclear ribosomal DNA (ETS) and the plastid intergenic spacer *trn*T-*trn*L, sequence data of a broad range of diploid and polyploid basic species of *Hieracium* from a previous study (Fehrer et al., 2009; Table S1, see the Supplementary data with this article) were used to embed them in a phylogenetic context and as a reference database to detect potential introgression by other *Hieracium* species. As a low-copy nuclear marker, the gene for gamma-glutamylcysteine synthetase (*gsh1*) was used. Sequences of three species were obtained from Krak et al. (2012), those for other basic species were newly generated.

Due to massive ribotype, haplotype and allele sharing among *Hieracium* species (Fehrer et al., 2009; Krak et al., 2013), it is difficult to find species-specific markers for tracing hybrid origins in this genus. While *H. intybaceum* can be easily recognized due to large genetic divergence of its nuclear genome compared to all other *Hieracium* species (Fehrer et al., 2007a), *H. prenanthoides* falls into a group of other species of Western European origin and is only distinguished from them by a peculiar signature of several polymorphisms at the 3' end of ETS sequences (Fehrer et al.,

2009). ETS copies of different parental origin tend to be preserved in *Hieracium* species of hybrid origin. Therefore we expected to see additivity of substitutions diagnostic for *H. intybaceum* as well as for 'Western' species (the group to which *H. prenanthoides* belongs). In addition, the above-mentioned polymorphisms diagnostic for *H. prenanthoides* should be present.

Table 3. Assessment of *Hieracium picroides* and *H. pallidiflorum* allopolyploid origins with molecular markers.

Species, ploidy, accession	**ETS**[1] (multicopy nuclear)	***gsh1***[2] (single copy nuclear)	***trn*T-*trn*L**[3] (cpDNA)	**Genbank accession numbers**	
				gsh1	***trn*T-*trn*L**
***H. prenanthoides* (2*x*) 1252**	pre	pre (direct)	pre (ht1)	MK465644	EU867745
H. prenanthoides (3*x*) 1161/2	pre (+) × Wx	pre X1,X2,X10	pre (ht2*)	MK465645, MK465646, MK465647	EU867732
H. prenanthoides (3*x*) 1187/1	pre (+) × umb	pre X7,X10; umb X9	pre (ht1)	MK465648, MK465649, MK465650	EU867744
H. prenanthoides (3*x*) 1/4/4	pre	pre (direct)	pre (ht1)	MK465524	MK465503
H. prenanthoides (4*x*) 3/9/3	pre?	pre X4; umb X1	umb	MK465525, MK465526	MK465504
H. prenanthoides (4*x*) 1/3/2	pre?	pre X1; umb X4	pre (ht2)	MK465527, MK465528	MK465505
H. prenanthoides (4*x*) R4.3.1	pre?	pre X1; umb X3; W X6	pre (ht2)	MK465529, MK465530, MK465531	MK465506
H. picroides (3*x*) 1509/2	pre (+) × inb	pre (direct)	pre (ht1)	MK465532	MK465507
H. picroides H1619	pre (+) × inb	pre (direct)	pre (ht1)	MK465533	MK465508
H. picroides H1621	pre (+) × inb	pre (direct)	pre (ht1)	MK465534	MK465509
H. picroides (3*x*) H1613	pre (++) × inb	pre X3; inb X1,X2	pre (ht1)	MK465535, MK465536, MK465537	MK465510
H. picroides (3*x*) H1620	pre × inb (equal)	pre X4,X5	pre (ht2*)	MK465538, MK465539	MK465511
***H. intybaceum* (2*x*) inb.Kaer**	inb	inb X1,X3	inb	MK465606, MK465607	AY573323
***H. intybaceum* (2*x*) 1069/1**	inb	inb (direct)	inb	MK465605	JX129600
H. intybaceum (4*x*) INT1_PEL	inb (+) × pre	pre (direct)	inb	MK465540	MK465512
H. picroides ssp. *lutescens* (3x) H1605	pre (+) × inb × umb	pre (direct)	inb	MK465541	MK465513
H. picroides (3*x*) H1608	pre (++) × inb	pre X1,X3	inb*	MK465542, MK465543	MK465514
H. picroides (3*x*) H1615	pre (+) × inb	pre X6, X8	inb	MK465544, MK465545	MK465515
H. picroides (3*x*) 1/1/2	pre (+) × inb	pre (direct)	inb	MK465546	MK465516
H. picroides (3*x*) H1618	pre (++) × inb	pre X3,X4	inb	MK465547, MK465548	MK465517
H. picroides H1623	pre? × inb (equal)	pre (direct)	inb	MK465549	MK465518
H. cf. *picroides* H1637	pre? × inb (equal)	pre (direct)	inb	MK465550	MK465519
H. pallidiflorum (3*x*) H1609	pre × inb (equal)	pre X3; inb X5	inb	MK465551, MK465552	MK465520
H. pallidiflorum (3*x*) H1614	pre? × inb (equal)	pre X3; inb X1,X6	inb	MK465553, MK465554, MK465555	MK465521
H. pallidiflorum (3*x*) 1/4/2	pre? × inb (equal)	pre (direct)	inb	MK465556	MK465522
H. pallidiflorum (3*x*) H1616	pre × inb (+)	pre (direct)	inb	MK465557	MK465523

[1] diagnostic characters corresponding to pre: *H. prenanthoides*, inb: *H. intybaceum*, Wx: unknown Western 1, umb: *H. umbellatum* clade (for details see Fehrer et al. 2009), ×: allopolyploid origin (additivity); +, ++, equal: copies of the respective species dominating, strongly dominating, or approximately equal proportions of ETS variants, pre?: specific contribution of *H. prenanthoides* not visible (for more details, see Table S2)

[2] direct sequence or clones (X+ number) corresponding to *H. prenanthoides* (pre), *H. intybaceum* (inb), *H. umbellatum*-clade (umb) or Western clade (W); for details, see Fig. S3.

[3] ht1, ht2: different haplotypes of *H. prenanthoides*, *: single substitution or indel derived from the respective haplotype (see also Fig. 8)

With nuclear markers, *H. intybaceum* adopts a position among outgroup taxa in phylogenetic trees (Fehrer et al., 2007a, 2009; Krak et al., 2013). However, it shows plastid capture of *Hieracium* due to ancient wide hybridization (Fehrer et al., 2007a). Despite this, the plastid *trn*T-*trn*L intergenic spacer is able to distinguish *H. intybaceum* from all other *Hieracium* taxa, and the *H. prenanthoides* haplotype can be distinguished from most Western *Hieracium* taxa with this marker. This plastid region was therefore used to investigate the origins of *H. picroides* and *H. pallidiflorum* accessions and to determine the direction of the cross, i.e. their maternal origins.

Sequences of *gsh1*, which was developed as a highly variable low-copy nuclear marker for Asteraceae (Krak et al., 2012), were found to show higher species specificity in *Hieracium* in a pilot study than other molecular markers employed so far. Most importantly, three accessions of *H. prenanthoides* were clearly distinguished from all other species. The gene was therefore used as an additional nuclear marker to possibly confirm the involvement of *H. prenanthoides* in *H. pallidiflorum*, *H. picroides*, especially if this parental species could not be distinguished from other Western European species by other markers.

DNA isolation, PCR amplification and Sanger sequencing

DNA was isolated by a sorbitol extraction method (Štorchová et al., 2000) or by means of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from fresh or herbarium material. The ETS region was amplified and sequenced as described in Fehrer et al. (2009); the *trn*T-*trn*L intergenic spacer was treated as in Fehrer et al. (2007a). A part of the *gsh1* gene spanning exons 11 through 15 were amplified, sequenced and, if necessary, cloned as described in Krak et al. (2012) and Mráz et al. (2019). Sequences were submitted to the GenBank database (accession numbers MK465503–MK465681). All sequences were aligned in BioEdit (Hall 1999) and compared to datasets of other *Hieracium* species. A list of *gsh1* sequences for basic species of *Hieracium* along with their GenBank accession numbers is provided in Table S1. ETS, *trn*T-*trn*L and *gsh1* data for *H. picroides* and *H. pallidiflorum* and their parental species are summarized in Table 3.

Genotyping and phylogenetic analysis

The two parental species, *H. intybaceum* and *H. prenanthoides*, differ by 18 substitutions and a 1 bp indel in the ETS region. Polymorphic sites were identified in both reading directions and represented by the IUPAC ambiguity codes. Based on the alignment, all polymorphic positions and substitutions of *H. intybaceum*, *H. prenanthoides* and the allopolyploids *H. picroides* and *H. pallidiflorum* were summarized (Table S2). Comparison with the dataset of other *Hieracium* species revealed two samples that showed signs of introgression by a species of the *H. umbellatum* clade, therefore sequences of *H. umbellatum* (its most widespread diploid representant) were also included. In Table S2, each accession is represented twice, once showing all polymorphisms (including very small additional peaks) and once showing the major or dominant sequence. Unequal ratios may be indicative of different genome dosage in polyploids (e.g. one or two chromosome sets of particular parents in triploids) or of intragenomic processes like concerted evolution or locus loss after hybridization.

The alignment of the plastid *trn*T-*trn*L region consisted of 698 aligned characters. It contained 18 indels that were coded with FastGap (Borchsenius, 2009). A total of 51 characters was variable of which 28 were parsimony informative. The model of molecular evolution best fitting the data was determined with Modeltest (Posada and Crandall, 1998). Phylogenetic analyses were performed using Maximum likelihood (ML), Bayesian (BI) and Maximum Parsimony (MP) approaches. For ML, analyses were done with MEGA (Kumar et al., 2016) using a Jukes-Cantor model (F81+Γ) with gamma distributed rates among sites (2 discrete categories), extensive subtree-pruning-regrafting, very strong branch swap filter and 1000 bootstrap replicates. BI was done with MrBayes (Ronquist and Huelsenbeck, 2003) using one substitution rate and gamma distribution as priors along with the default settings, but sampling every 1000th tree. Convergence was reached after 1.2 million generations; 300 trees per run were discarded as burn-in and the remaining trees were summarized. MP analyses were run in PAUP (Swofford, 2002) as heuristic searches with 100 random addition sequence replicates and TBR branch swapping, saving no more than 100 trees with length greater than or equal to 1 per replicate, automatically increasing the maximum number of trees saved. Bootstrapping was done with the same settings and 1000 replicates, but without branch swapping.

The alignment of the *gsh1* dataset consisted of 931 bp plus 142 characters from gapcoding. Altogether, 335 characters were variable of which 207 were parsimony informative. The same

principal procedures and programs were used as above with the following differences: The *gsh1* alignment contained a microsatellite $(AC)_n$ and a poly-T region in introns 13 and 14; length variation in these hypervariable regions was discarded for analyses. A General Time Reversible model (GTR+Γ+I) was used to infer phylogenetic trees (6 categories) in ML analysis. For BI, six substitution rates and gamma distribution with a proportion of invariant sites were set as priors. The dataset needed 30 million generations to converge; 25% of the trees were discarded as burn-in and the rest of the trees was summarized. To visualize character conflict within the *gsh1* dataset, Neighbor Net analysis as implemented in SplitsTree (Huson and Bryant, 2006) was performed using the default settings.

In situ hybridization

In order to determine the genome dosage of the parental species in polyploid hybridogeneous species, we performed GISH with labelled total genomic DNA of *H. intybaceum* and *H. prenanthoides* on chromosomes of *H. pallidiflorum*, *H. picroides*, and an experimental diploid F_1 hybrid for comparison (Table 1). GISH analyses were also used to validate results from genotyping which suggested introgression from *H. umbellatum* in some polyploid accessions of *H. prenanthoides* and a contribution of *H. prenanthoides* to tetraploid *H. intybaceum*. In addition, as the number of loci per haploid genome differs between the parental species, FISH with a 45S probe (pTa71) for the nrDNA locus were performed.

Roots were collected from cultivated plants and pretreated in 0.1% (w/v) colchicine for 4 h at room temperature. Root tips were fixed in 3:1 (v/v) 100% ethanol:acetic acid. The fixed root meristems were thoroughly washed in water and enzyme buffer (10 mM citrate buffer at pH 4.6) and partially digested in 0.3% (w/v) cytohelicase, pectolyase and cellulase (Sigma, St. Louis, MS, USA) at 37°C for 4 h followed by washes in enzyme buffer and water (Lysak et al., 2001). The material, in a water drop, was carefully transferred onto a grease-free microscope slide, and the cells were spread according to the technique of Pijnacker and Ferwerda (1984) with modifications, as previously described (Belyayev et al., 2001).

For GISH experiments, total genomic DNA (gDNA) of *H. intybaceum*, *H. prenanthoides* and *H. umbellatum* were used, sonicated by Bioruptor® Pico (Diagenode, Liège, Belgium) (DNA fragment length 500–2000 bp) and labeled with Cy3 (Amersham, Amersham, UK) and biotin

(biotin-16-dUTP, Roche, Basel, Switzerland) according to a standard oligolabeling protocol (Schwarzacher and Heslop-Harrison, 2000). In addition, probe pTa71 labelled with Cy5 (Amersham, Amersham, UK) was used for the detection of chromosomal positions of 45S rDNA. Two differently labeled gDNA probes together with pTa71 were hybridized simultaneously on the chromosomes of *H. pallidiflorum*, *H. picroides*, F_1 *H. intybaceum* × *H. prenanthoides* hybrid, and tetraploid *H. intybaceum*. The number of 45S rDNA loci was checked on the same metaphase plate analyzed by GISH. Chromosomes of polyploid *H. prenanthoides* were simultaneously hybridized with gDNAs of diploid *H. prenanthoides* and *H. umbellatum*. *In situ* hybridization was carried out on a hot plate (StatSpin® ThermoBrite™ Slide Processing System, Abott Molecular, Chicago, IL, USA) at 63°C, overnight for GISH and 3 h for FISH. Biotin was detected with fluorescein isothiocyanate (FITC)-conjugated avidin (Termo Fisher Scientific, Carlsbad, CA, USA). Slides were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in antifade mounting medium (Vector Laboratories, Peterborough, UK), and examined and photographed on a Zeiss Axio Imager.Z2 microscope system equipped with an ApoTome.2 (Carl Zeiss, Jena, Germany).

Repeatome comparisons

The repeatomes of the parental species and *H. umbellatum* were compared utilizing genome skimming and a similarity-based read clustering approach (RepeatExplorer pipeline) (Novák et al., 2010, 2013). It has been demonstrated that this approach provides a good overview of all major repeat families which substantially contribute to the overall plant repeatome architecture (Macas et al., 2007, 2015; Novák et al., 2010) and are therefore mainly responsible for species specific differences in GISH experiments.

Genomic DNA of two plants of each *H. intybaceum* and *H. prenanthoides* as well as one plant of *H. umbellatum* (Table 1) was extracted from fresh or silica-gel dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Library preparation and Illumina next-generation sequencing were performed at GATC Biotech (Konstanz, Germany) / Eurofins Genomics (Ebersberg, Germany); 150 bp paired-end reads from libraries of insert size ~450 bp were obtained. The NGS reads were first quality-filtered using Trimmomatic (Bolger et al., 2014) and then plastid and mitochondrial reads were filtered out using the script ‘bbsplit.sh’ from BBTools v.37.44 (Bushnell, 2017) against the plastid genome of *Lactuca sativa* (NCBI

DQ383816.1) and the mitochondrial genome of *Helianthus annuus* (NCBI MG770607.2). Each set of reads was randomly sampled according to absolute genome sizes for the same coverage of 0.05x resulting in 1.230.000 and 1.236.668 reads for *H. intybaceum* (accessions: 1531/8, int_6/14/25), 1.176.668 and 1.170.000 reads for *H. prenanthoides* (pre_6/5/5, pre_6/8/5), and 1.360.000 reads for *H. umbellatum* (H1617). The samples were subjected to pairwise comparative analyses (two by two, resulting in eight runs), using the RepeatExplorer pipeline implemented in a GALAXY server environment (https://repeatexplorer-elixir.cerit-sc.cz/galaxy/), using the default settings. The pipeline, based on sequence similarity of input reads produces clusters corresponding to families of repetitive DNA sequences. The proportion of reads in each cluster is in theory directly related to the genome proportion of repetitive DNA families of the analyzed accession. In all eight runs, the pairwise differences in read abundances for all clusters above a threshold of 0.2% were obtained (Fig. S1); the threshold was adopted from a study on closely related *Taraxacum* (Ferreira de Carvalho et al., 2016). The magnitude of intra- and inter-specific differences between accessions was assessed statistically by summarizing the absolute differences in proportions of reads across clusters, which were subsequently used in a linear regression mode. To test for statistically significant differences between runs, a post-hoc analysis (Tukey HSD; Hsu, 1996) and Levene test of variance were conducted with the R package multcomp (Hothorn et al., 2008). Absolute differences were square-root transformed to ensure normalization of the residuals. The analyses were performed within the R environment (R version 3.5.1, R Core team, 2014).

Results

Multivariate morphometric analyses

The Spearman rank correlation coefficients did not exceed 0.90 for any character pair, and thus all of the measured characters were retained for further analyses. The PCA based on the complete set of data (dataset 1) showed that the parental species *H. intybaceum* and *H. prenanthoides*, were completely separated along the first component axis, PC1. *Hieracium pallidiflorum* and *H. picroides* occupied intermediate positions between the parental species and formed, with some small overlaps, well distinguished clouds in the ordination space. Experimental diploid F_1 hybrids between *H. intybaceum* and *H. prenanthoides* fell into the variation of *H. picroides* (PCA1; Fig. 3). The predicted number of significant PC axes was 1 (based on rnd-LambdaF statistic) and 3 (based on rnd-F statistic), respectively. The first axis explained 45% of the variation, with plant height (PLH) and pubescence (NCT, NGT, and LGT) being important drivers of among-species variation (Table 4). The permuation test supports the discrimination of the groups (ratio = 0.503, P = 0.001). In CDA, based on individual plants as OTU´s and species as predefined groups performed on the complete dataset (except for experimental hybrids), a fairly distinct separation of plants attributed *a priori* to *H. intybaceum*, *H. prenanthoides*, *H. pallidiflorum* and *H. picroides* was achieved (CDA1; Fig. 4A). The first canonical axis represents 41% of the variation among the species with the variables strongly contributing to the separation of species along this axis (in descending order): NCT, LGT, NGT and NFC (Fig. 4B). The second canonical axis accounted for 34% of variation among the species. The most important character that correlated (higher than 0.35) with this canonical axis was NMG. All but one (LLB) morphological characters explained a significant part of the overall variation when used as the only predictors in independent tests (i.e., marginal effect, $\alpha = 0.05$). However, only 11 characters showed significant unique contribution when included into the model during a stepwise selection of best model predictors (Table 5). Success of classification of these four groups of specimens was 97.14% in *H. intybaceum* (one plant classified as *H. pallidiflorum*), 100% in *H. pallidiflorum*, 91.43% in *H. picroides* (two plants classified as *H. pallidiflorum* and one plant as *H. prenanthoides*) and 100% in *H. prenanthoides*, respectively.

Hieracium picroides accessions with *H. intybaceum* as a maternal parent were not clearly separated from those with *H. prenanthoides* as a maternal parent in the PCA scatterplot (dataset 2,

PCA2; Fig. 5). The predicted number of significant PC axes was 2 (based on rnd-LambdaF statistic) and 3 (based on rnd-F statistic), respectively. CDA2 (Fig. S2A) showed certain morphological trends, but with considerable overlap among the two groups in congruence with rather low to moderate values of correct classification (66.7% and 82.6%, respectively).

The PCA3 revealed a morphological distinction between accessions of *H. pallidiflorum* from the Western Alps and those from the Eastern Alps (dataset 3, Fig. 6). The predicted number of significant PC axes was 2 (based on rnd-LambdaF statistic) and 2 (based on rnd-F statistic), respectively. The first axis explained 33% of the variation and was most strongly correlated with the characters SHR, PLH, LML, NSL and WML. The permutation test supported the discrimination of the groups (ratio = 0.207, P = 0.001). The histogram of CDA3 showed two clearly separated peaks (Fig. S2B); the most important characters that correlated with the canonical axis were SHR and LML (Table S3). The classificatory DA 3 showed all specimens to be correctly classified.

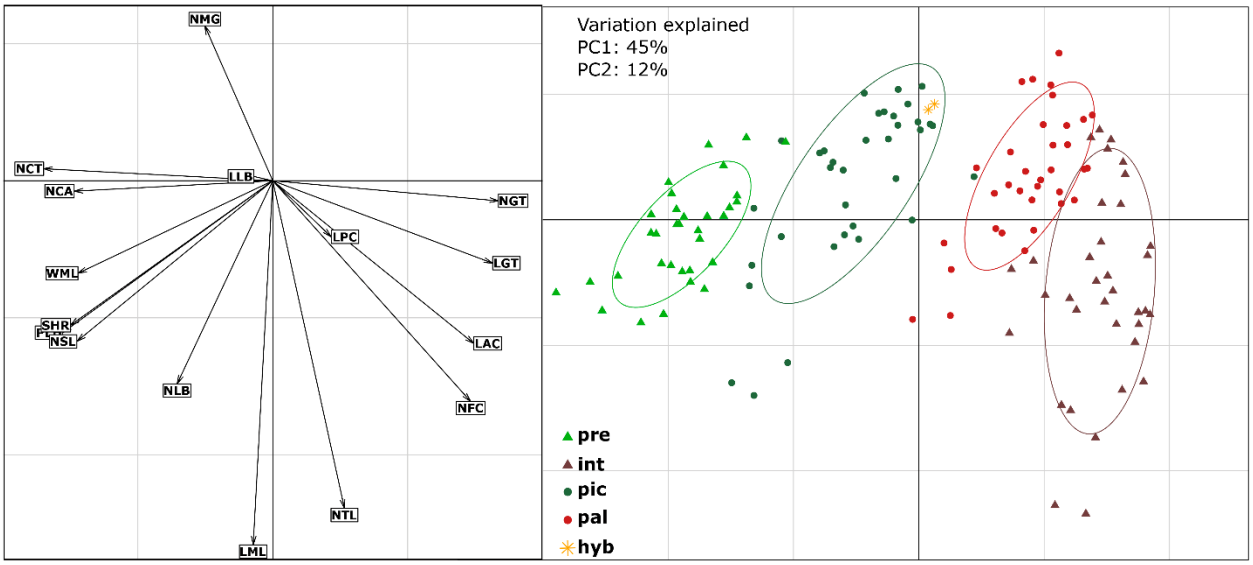

Fig. 3. Principal component analysis of individual plants of the parental species *Hieracium intybaceum* (int, 2*x*; n = 35) and *H. prenanthoides* (pre, 2*x*; n = 33), and their (i) stabilized hybridogeneous species *H. pallidiflorum* (pal, 3*x*; n = 34) and *H. picroides* (pic, 3*x*; n = 35) and (ii) experimental diploid F_1 hybrids (n = 2) between diploid cytotypes of *Hieracium intybaceum* and *H. prenanthoides* (hyb; n = 2) based on a set of 16 morphological characters (PCA1).

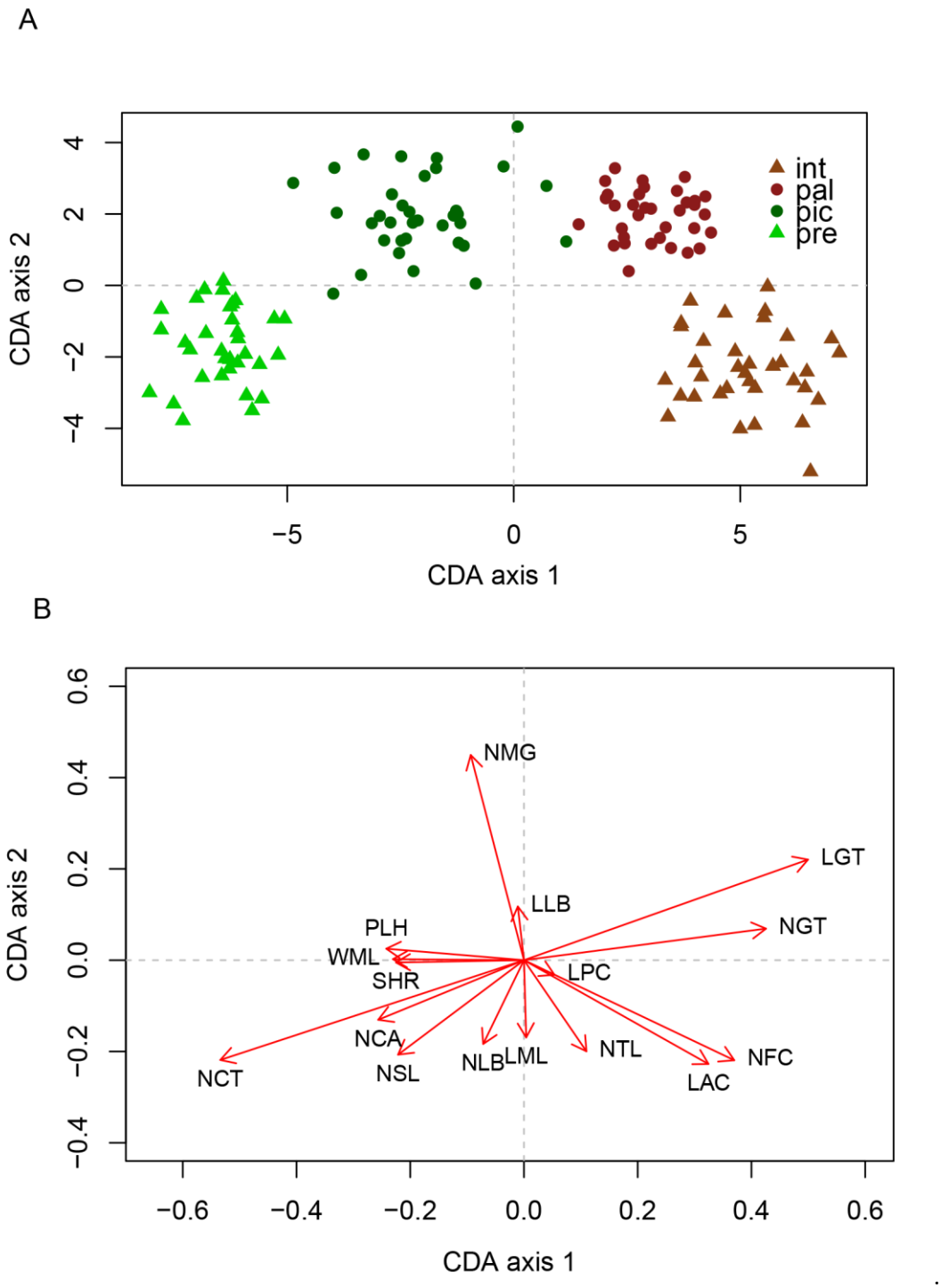

Fig. 4. Canonical discriminant analysis performed on individual plants of four predefined groups based on genome composition, genome size and ploidy: the parental species *Hieracium intybaceum* (int, 2*x*, n = 35) and *H. prenanthoides* (pre, 2*x*; n = 33) and their (i) stabilized hybridogeneous species *H. pallidiflorum* (pal, 3*x*, n = 34) and *H. picroides* (pic, 3*x*; n = 35) (CDA1, A), and 16 morphological characters (B). For total canonical structure, see Table S3.

Table 4. Results of principal component analyses of (i) the parental species *Hieracium intybaceum* and *H. prenanthoides* and their stabilized hybridogeneous species *H. pallidiflorum* and *H. picroides* and experimental F_1 hybrids (PCA1), and (iii) accessions of *H. pallidiflorum* (PCA3). PC1, PC2, eigenvector values for the first and second principal components; for an explanation of the character acronyms, see Table 2.

	PCA1 (Fig. 3)		PCA3 (Fig. 6)	
Character	PC1	PC2	PC1	PC2
PLH	0.834	0.266	-0.805	0.313
SHR	0.803	0.278	-0.891	-0.217
LAC	-0.802	0.314	-0.128	0.628
LLB	0.075	-0.094	0.118	0.900
LPC	-0.232	0.088	-0.210	0.206
LML	0.067	0.737	-0.731	0.222
WML	0.767	0.193	-0.714	-0.190
LGT	-0.873	0.130	-0.652	0.251
NCA	0.784	0.017	0.123	0.766
NFC	-0.788	0.421	-0.586	0.440
NSL	0.774	0.341	-0.728	-0.039
NLB	0.377	0.454	-0.684	-0.329
NMG	0.273	-0.386	-0.543	0.209
NTL	-0.371	0.671	-0.104	0.160
NGT	-0.892	0.027	0.429	0.224
NCT	0.913	0.015	-0.634	-0.333

Table 5. Unique contributions of the characters (i.e., the addition of each character into the model with all other characters), based on 16 morphological characters (for acronyms see Table 2) measured for the parental species *Hieracium intybaceum* and *H. prenanthoides* and their stabilized hybridogeneous species *H. pallidiflorum* and *H. picroides* and experimental F_1 hybrids (CDA 1).

Character	ChiSquare	F	Prob>F
PLH	0.031	2.569	0.035
SHR	0.020	1.631	0.125
LAC	0.054	4.439	0.003
LLB	0.029	2.333	0.055
LPC	0.002	0.140	0.985
LML	0.087	7.060	0.001
WML	0.078	6.272	0.001
LGT	0.182	14.698	0.001
NCA	0.031	2.513	0.061
NFC	0.039	3.189	0.017
NSL	0.031	2.546	0.033
NLB	0.025	2.025	0.081
NMG	0.166	13.443	0.001
NTL	0.100	8.122	0.001
NGT	0.076	6.152	0.001
NCT	0.120	9.670	0.001

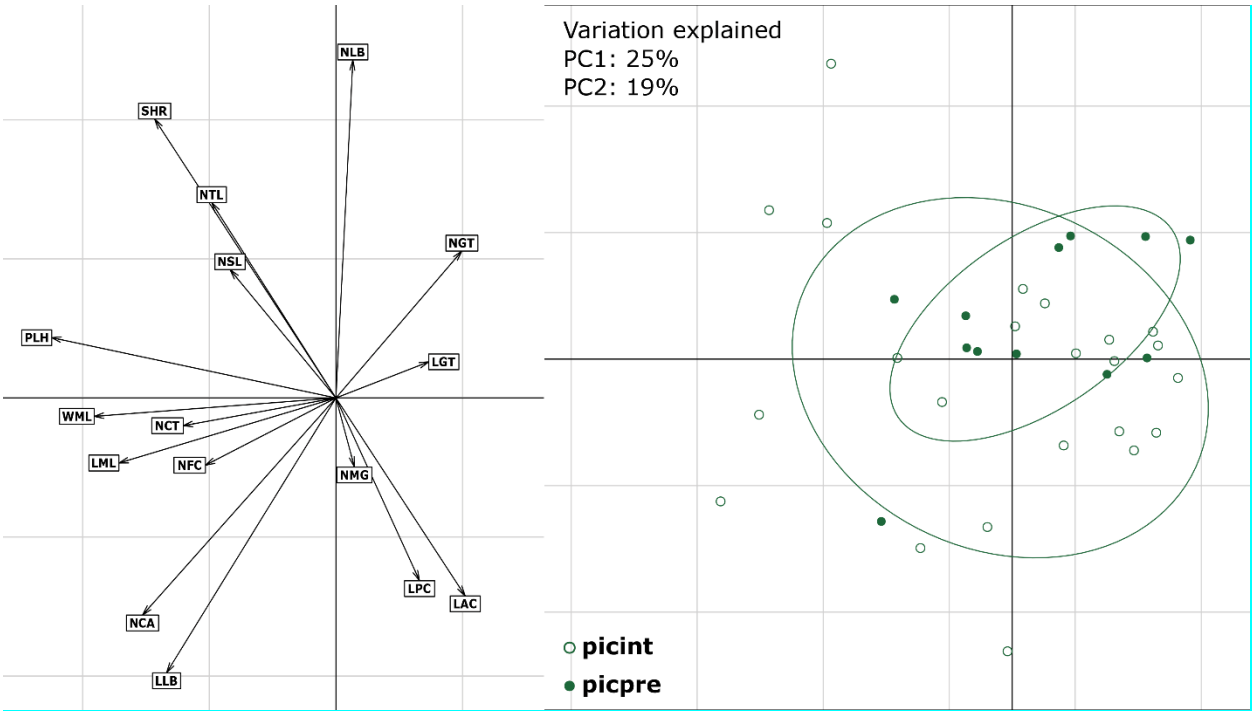

Fig. 5. Principal component analysis of the *Hieracium picroides* plants with *H. intybaceum* as the maternal parent (picint; n = 23) and with *H. prenanthoides* as the maternal parent (picpre; n = 12; according to ptDNA, see Table 3) based on 16 morphological characters (PCA2).

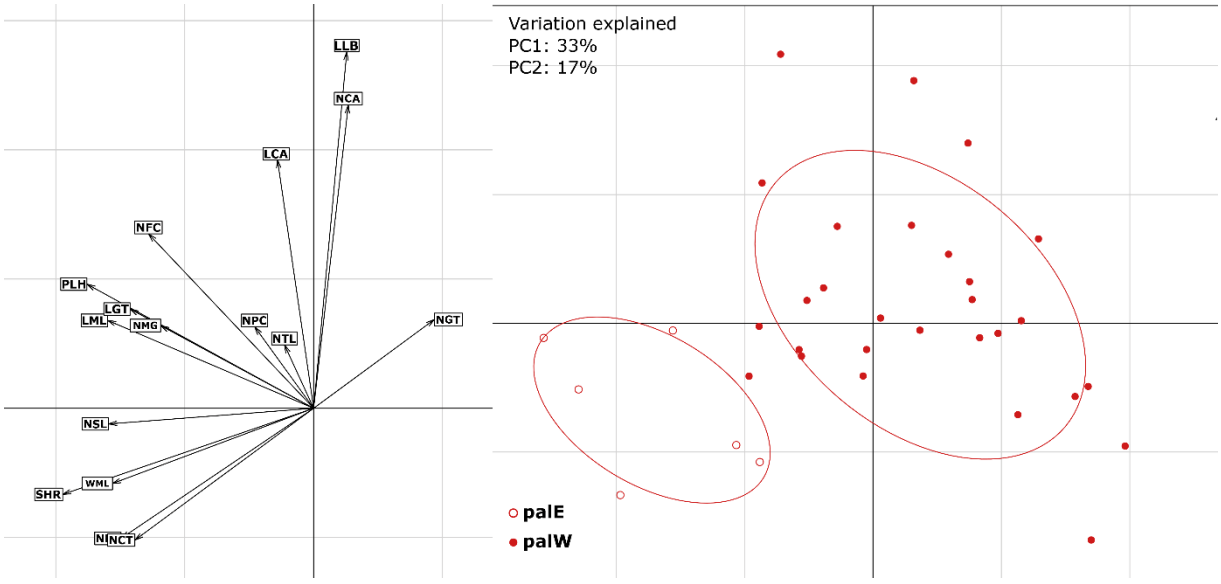

Fig. 6. Principal component analysis of *Hieracium pallidiflorum* plants from the Western (palW; n = 28) and Eastern (palE; n = 6) Alps, based on 16 morphological characters (PCA3).

Pollen size variation

All diploid and polyploid accessions produced pollen except for the accessions of tetraploid *H. intybaceum* which were completely pollen sterile – its dissected anthers did not contain any pollen grains. Polyploid species showed generally larger pollen than diploid accessions, with the exception of diploid *H. intybaceum*, where pollen equatorial size was very similar to that of most of polyploids (Fig. 7A, Table 6). We found a very obvious trend in the level of homogeneity of pollen size with polyploids producing pollen of more variable size than diploids (Fig. 7B); the coefficients of variation of the former usually exceeded 10% while these values were lower in diploids (Fig. 7B, Table 6).

Genome size variation

Flow cytometric analyses yielded high-resolution histograms with CVs of G_0/G_1 peaks for 21 samples ranging from 1.49 to 3.83 % (mean 2.72%), the values for the internal reference standard were 1.76 to 5.72 % (mean 3.62%). The genome size of *H. pallidiflorum* was higher (mean ± SD: 11.373 ± 0.194 pg, range: 11.202 – 11.584 pg) in comparison with *H. picroides* (mean ± SD: 11.069 ± 0.224 pg, range: 10.659 – 11.412 pg). Genome sizes (2C values) of *H. intybaceum* (2*x* and 4*x*), *H. prenanthoides* (2*x*, 4*x*), *H. umbellatum* (2*x*) and an experimental F_1 hybrid between *H. intybaceum* and *H. prenanthoides* are given in Table 7.

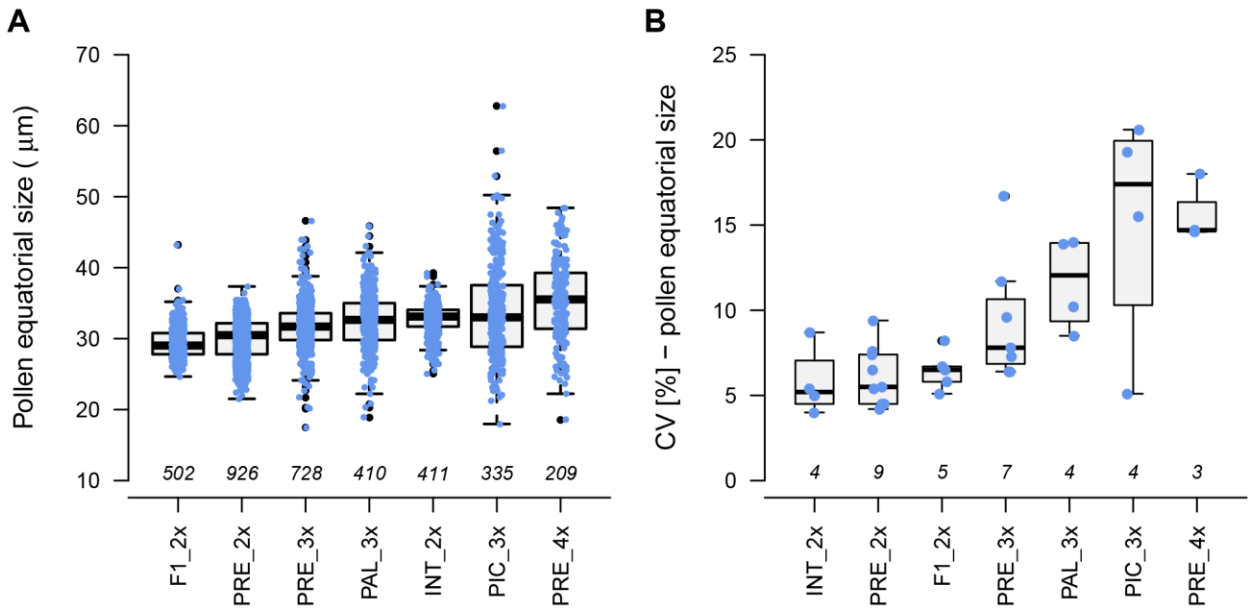

Fig. 7. Variation in pollen equatorial size (µm) **(A)** and variance in this parameter expressed as coefficient of variation (CV) **(B)** in diploid (2*x*) and polyploid (3*x* or 4*x*) *Hieracium* taxa included in this study. Boxplots in both cases were ordered according to increasing mean values of particular group (species by ploidy combination). Above the x axis is the total number of pollen grains (A), and the total number of plants (B) measured per group. Abbreviations: (F_1) diploid experimental hybrids between diploid *H. intybaceum* and diploid *H. prenanthoides*, (INT) *H. intybaceum*, (PAL) *H. pallidiflorum*, (PIC) *H. picroides*, (PRE) *H. prenanthoides*.

Table 6. Summary statistics of pollen equatorial size variation in diploid, triploid and tetraploid taxa of *Hieracium* s.str. and their F_1 hybrid. Abbreviations: (int) *Hieracium intybaceum*, (pre) *H. prenanthoides*, (preint) F_1 hybrid between *H. prenanthoides* and *H. intybaceum*, (pal) *H. pallidiflorum*, (pic) *H. picroides*, (P) parental sexual species, (F_1) F_1 sexual hybrid, (A) apomictic polyploid, (Nplant) number of accessions analysed per species by ploidy combination, (Npollen) total number of pollen measured per species by ploidy combination, Cv) coefficient of variation.

Species	Ploidy	Origin	Nplant	Npollen	Mean [µm]	SD	Max [µm]	Min [µm]	Cv [%]
int	2*x*	P	4	411	32.7	2.2	39.3	25.1	6.6
pre	2*x*	P	9	926	29.9	2.9	37.4	21.5	9.7
preint	2*x*	F_1	5	502	29.3	2.2	43.2	24.7	7.4
pre	3*x*	A	7	728	31.7	3.2	46.6	17.5	10.1
pal	3*x*	A	4	410	32.5	3.9	45.9	18.9	12.0
pic	3*x*	A	4	335	33.9	6.6	62.8	18.0	19.5
pre	4*x*	A	3	209	35.4	5.8	48.4	18.6	16.2

Table 7**.** Genome size in *H. intybaceum* (2*x*, 4*x*), *H. prenanthoides* (2*x*, 3*x*, 4*x*), *H. pallidiflorum* (3*x*), *H. picroides* (3*x*), an experimental hybrid between *H. intybaceum* and *H. prenanthoides* (2*x*) and *H. umbellatum* (2*x*). B = *Bellis perennis*, P = *Pisum sativum* cv. Ctirad.

Species / ploidy / accession	**Locality**	**Absolute genome size, 2C (pg), standard**
Hieracium intybaceum* 2*x		
1531/8	A: Arlberg	7.540 (P)
int_6/14/25	F: Col du Petit Saint-Bernard	7.590 (P)
Hieracium intybaceum* 4*x		
INT1_PEL	F: massif des Écrins	14.890 (P)
16439/5	F: massif des Écrins	14.737 (B)
Hieracium prenanthoides* 2*x		
pre_6/5/5	I: Claviere	7.220 (P)
pre6	I: Claviere	7.089 (B)
Hieracium prenanthoides* 4*x		
R4.3.1	CZ: Rýchory	15.064 (B)
rap_3_8_6_5	F: Ailefroide	14.961 (B)
Hieracium pallidiflorum* 3*x		
H1609	A: Muhr	11.334 (B)
H1508	F: Bonneval sur Arc	11.202 (B)
H1614	F: Col du Petit Saint-Bernard	11.584 (B)
Hieracium picroides* 3*x		
H1608	A: Muhr	11.060 (B)
H1605	A: Wirl I	11.071 (B)
H1603	A: Wirl II	11.181 (B)
H1604	A: Wirl III	11.087 (B)
H1613	F: Col du Lautaret	11.412 (B)
H1615	F: Col du Petit Saint-Bernard	10.659 (B)
pic_1/1/4	F: Col du Petit Saint-Bernard	11.015 (B)
Primary hybrid 2*x*		
17038-4		7.341 (B)
Hieracium umbellatum		
H1617	CZ: Praha	8.336 (B)
umbellatum 8/9/2	SK: Prakovce	8.400 (P)

Molecular identification of allopolyploids

Direct sequencing of the ETS region showed that all *H. picroides* and *H. pallidiflorum* accessions are indeed composed of ETS copies of *H. intybaceum* and a *Hieracium* of Western European origin (Table S2), i.e. their allopolyploid origin was confirmed. Most of them also showed the signature of *H. prenanthoides* specific polymorphisms indicating that this species was involved in their origin. However, the *H. prenanthoides* specific polymorphisms were missing in three tetraploid *H. prenanthoides* accessions (3/9/3, 1/3/2, R.4.3.1), in two *H. pallidiflorum* (1/4/2, H1614) and two *H. picroides* accessions (H1623, H1637), therefore, additional markers were needed to unequivocally demonstrate the involvement of *H. prenanthoides* at the molecular level. One triploid *H. prenanthoides* (1187/1) and one triploid *H. picroides* ssp. *lutescens* (Zahn) Greuter (H1605) showed introgression by a species of the *H. umbellatum* clade in ETS direct sequences (Table S2). In eight accessions of *H. picroides*, the dominant ETS variant was that of *H. prenanthoides*. Three accessions of each *H. picroides* and *H. pallidiflorum* showed approximately equal composition of parental ETS copies; a single *H. pallidiflorum* (H1616) had predominantly *H. intybaceum* ETS sequences. Surprisingly and not evident from morphology, the tetraploid accession of *H. intybaceum* also showed an introgression from *H. prenanthoides* including the diagnostic polymorphisms, but ETS copies of *H. intybaceum* were strongly dominating (see also Table 3).

All *H. pallidiflorum* and more than half of the *H. picroides* accessions investigated had *H. intybaceum* as their maternal parent (Fig. 8, Table 3), one sample of *H. picroides* (H1608) differed by one substitution from the main haplotype. Two haplotype groups of *H. prenanthoides* were found that differed by a 7 bp indel. One comprised diploid and triploid accessions of *H. prenanthoides* and most *H. picroides* samples and was also shared by *H. lucidum* Guss., a Sicilian endemic with strongly different morphology (ht1). The second haplotype group (with indel variations in two samples) occurred in triploid and tetraploid *H. prenanthoides*, one *H. picroides* and another species of Western origin, *H. tomentosum* (ht2). One of the tetraploid *H. prenanthoides* (3/9/3) accessions showed a haplotype with identical sequence to most species of the *H. umbellatum* clade indicating introgression which was not detected with ETS.

With the *gsh1* marker, all allopolyploids showed *H. prenanthoides* specific alleles (Table 3, Fig. 9), even the tetraploid *H. intybaceum* which was introgressed by *H. prenanthoides* according to ETS. In ten allopolyploid accessions, only a single *gsh1* allele of *H. prenanthoides*

was found, four accessions showed two *H. prenanthoides* alleles each (Table 3), but no *H. intybaceum* allele occurred in any of them. Additional alleles of *H. intybaceum* were found in only three accessions although the contribution of this species according to ETS copies, morphology and, for most of them, also *trn*T-*trn*L haplotypes, was unequivocal. Two *H. intybaceum* alleles were present in *H. picroides* H1613 and *H. pallidiflorum* H1614 in addition to one *H. prenanthoides* allele. All polyploid *H. prenanthoides* samples except one (1/4/4) showed two or three different alleles; five out of six investigated accessions were introgressed, i.e. they represented allopolyploids as well, like tetraploid *H. intybaceum*. Apart from a large group comprising the majority of *H. prenanthoides* alleles (Fig. 9), a second clade comprised one allele of a triploid *H. prenanthoides* (1161/2) previously attributed to an extinct or unknown lineage (Wx, Fehrer et al., 2009) along with one allele of each *H. prenanthoides* 3/9/3 and *H. picroides* H1618. Some polyploid *H. prenanthoides* accessions showed additional alleles in other species groups that were apparently the result of introgression. In the case of tetraploid *H. prenanthoides* R4.3.1, the donor was most likely triploid *H. lachenalii* whose origin involved a Western species and a taxon from the *H. umbellatum* clade (Table S1). The *gsh1* tree was characterized by a general lack of resolution of the backbone (see Fig. S3 for details) due to strong character conflict apparent in a network representation (Fig. S4). The network also showed *H.* cf. *picroides* H1637 as belonging to the main group of *H. prenanthoides* alleles. Also, several species groups identified by other markers that formed different unsupported branches in the tree grouped together indicating that the network is a more adequate representation of the data. Given that the number of different alleles never exceeded the ploidy of the sample (Table 3, Table S1), *gsh1* can be considered as a single copy gene in *Hieracium*. For other basic species of *Hieracium* s.str., its alleles generally showed the same species origins inferred previously, but also higher species specificity compared to other markers (Table S1).

Comparison of the results obtained by three molecular markers (Table 3) showed that all *H. picroides* and *H. pallidiflorum* populations have different origins except for three accessions of *H. picroides* (1509/2, H1619, H1621) from two populations that cannot be distinguished and even have identical *gsh1* alleles (Fig. S3).

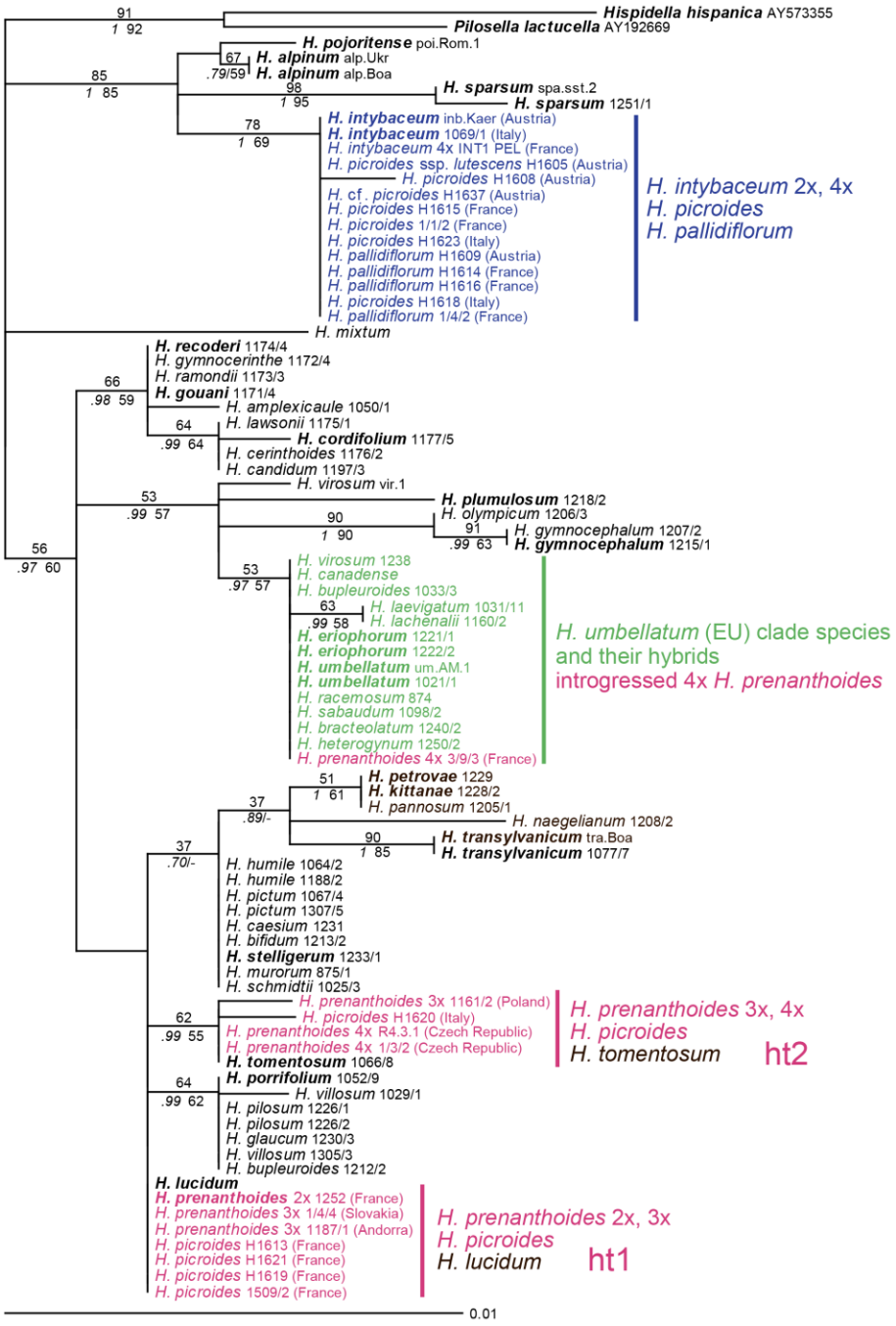

Fig. 8. Maternal origin of *H. picroides* and *H. pallidiflorum* based on *trn*T-*trn*L. A ML tree (log likelihood -1444.24) is shown with bootstrap support (BS) above branches. Posterior probabilities of Bayesian analysis and BS of MP analysis are given below branches. Two main haplotypes (ht1, ht2) are found in *H. prenanthoides*; both are shared with one additional species each. One tetraploid accession of *H. prenanthoides*, introgressed by a species of the *H. umbellatum* clade, shares the plastid haplotype of that group. Diploid accessions are given in bold.

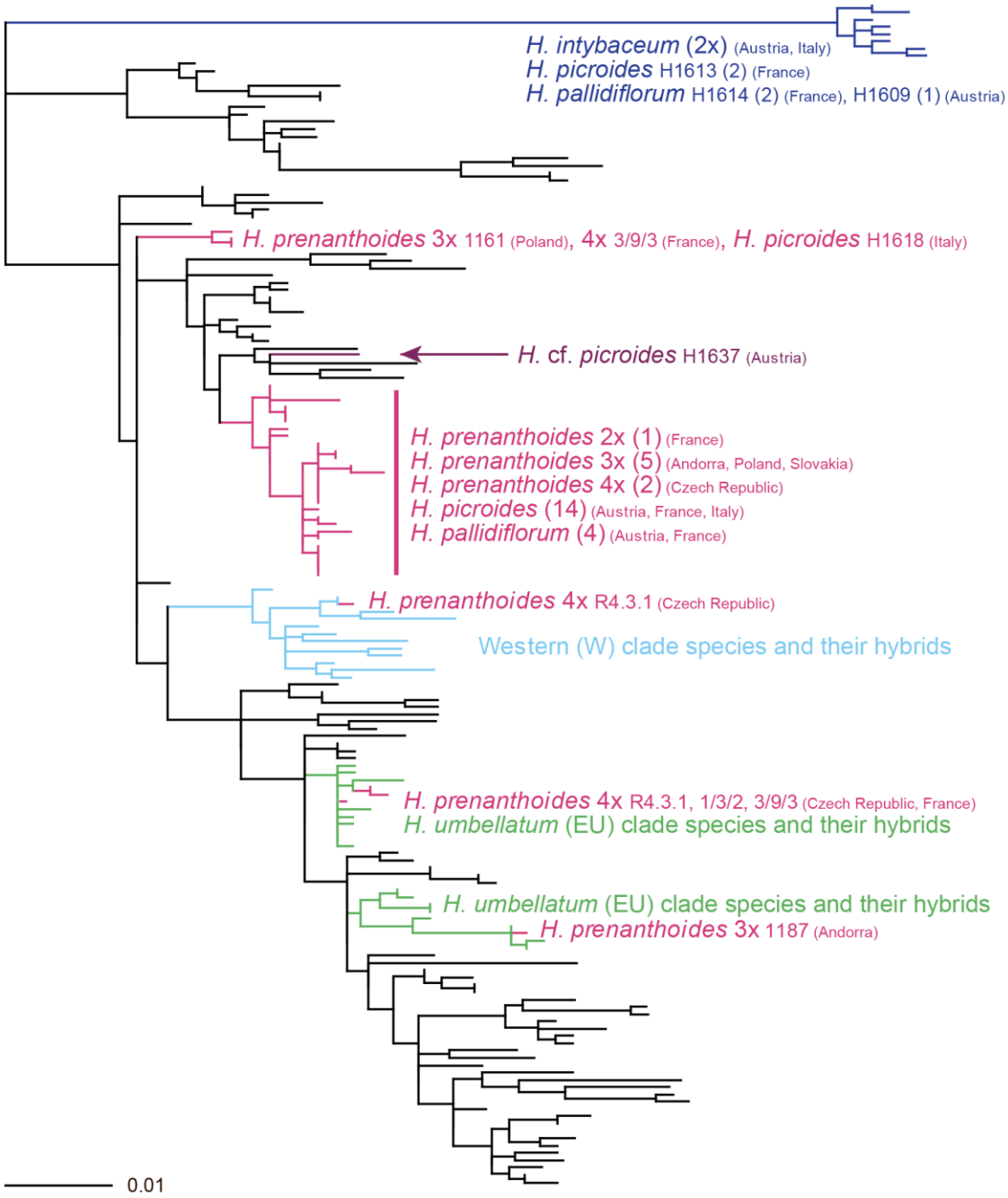

Fig. 9. Placement of *H. intybaceum* and *H. prenanthoides* alleles in the *gsh1* tree. A simplified tree shows alleles of the parental species and their allopolyploid intermediates *H. picroides* and *H. pallidiflorum*. Alleles of *H. intybaceum* are in dark blue, alleles of *H. prenanthoides* in pink. Alleles of intermediate species are shown in the color of the respective parental cluster of alleles. Additional species clades are indicated for introgressed accessions of polyploid *H. prenanthoides* (Western species, *H. umbellatum* group). The ploidy of the parental taxa is indicated along with the number of plants; for individual accessions, the number of alleles in the respective clade is shown. For tree and sample details, see Fig. S3.

Genomic composition in allopolyploids

The genome of *H. pallidiflorum* was composed of nine chromosomes of *H. prenanthoides* and 18 chromosomes of *H. intybaceum* (Fig. 10A) while the genome of *H. picroides* is composed of nine chromosomes of *H. intybaceum* and 18 chromosomes of *H. prenanthoides* (Fig. 10C). However, based on the intensity of red-orange fluorescence, the differentiation of parental haplomes in allopolyploids was rather poor. In all accessions of *H. pallidiflorum*, we observed seven 45S rDNA clusters, four of which were located on the *H. intybaceum* haplome and three on the *H. prenanthoides* haplome (Fig. 10 A, B). Seven clusters were also observed in two samples of *H. picroides* (H1613-1, H1615-1), two of which were detected on the *H. intybaceum* haplome and five on the *H. prenanthoides* haplome. In another two accessions of this species (H1608 and Pic 1/2/1) eight clusters were found; two on the *H. intybaceum* haplome and six on the *H. prenanthoides* haplome). GISH analysis of the diploid F_1 artificial hybrid between *H. intybaceum* and *H. prenanthoides* (92preint_1) revealed nine chromosomes of each parental species (Fig. 10E). GISH was also used to confirm introgression of other *Hieracium* species detected by genotyping in polyploid accessions of parental species and to determine the genome dosage. The presence of nine chromosomes of *H. prenanthoides* was revealed in a tetraploid plant of *H. intybaceum* (INT1_PEL; Fig. 10F). Probing chromosomes of triploid and tetraploid accessions of *H. prenanthoides* (1187/1, 3/8/7, 1/3/2, R4.3.1) with total DNA of diploid *H. umbellatum* revealed contribution of this species to the genome of *H. prenanthoides* (in each case nine chromosomes of *H. umbellatum*, 18 or 27 chromosomes of *H. prenanthoides*, depending on the ploidy; Fig. 10G, and data not shown).

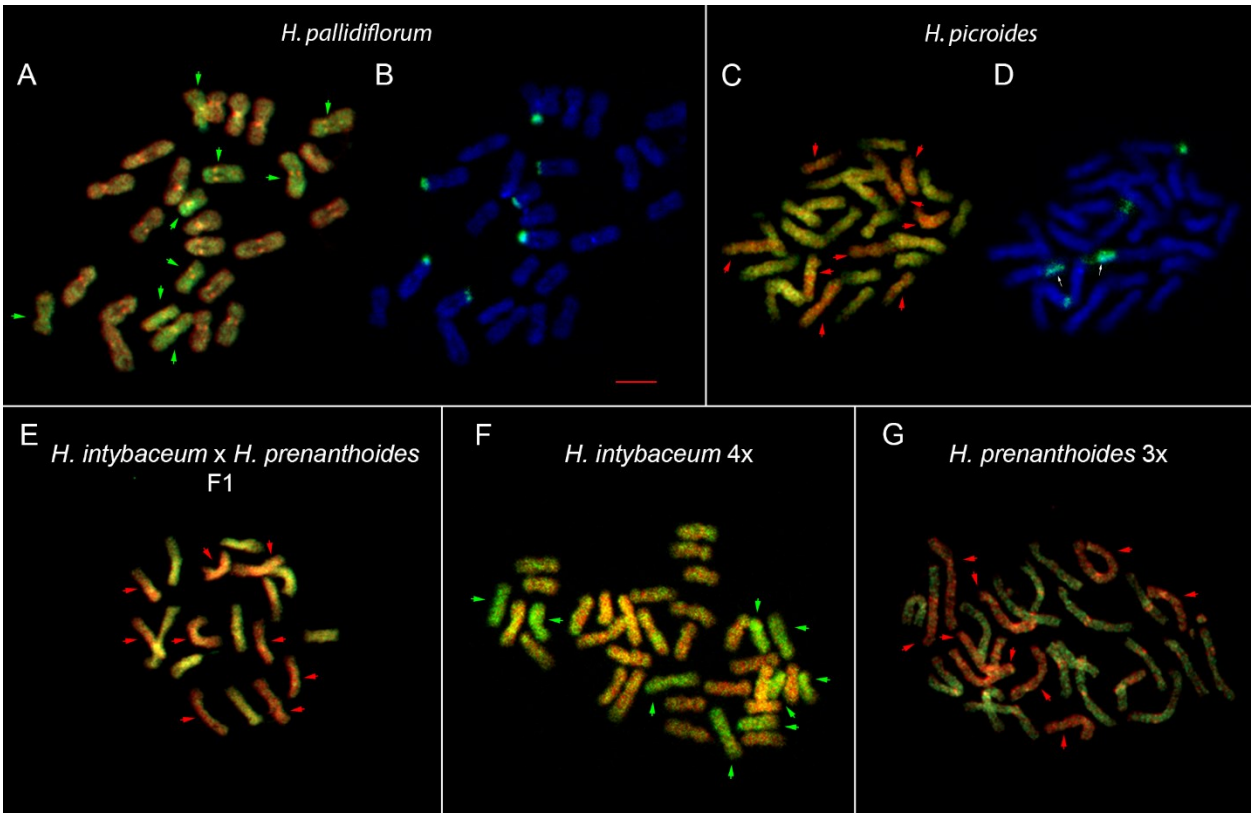

Fig. 10. Genomic and fluorescent *in situ* hybridization on metaphase chromosomes of triploid apomictic *H. pallidiflorum* and *H. picroides*, an experimental F_1 diploid hybrid between *H. intybaceum* and *H. prenanthoides*, and polyploids of *H. intybaceum* and *H. prenanthoides*. **(A)** Chromosomes of *H. pallidiflorum* (pal_1/4/5) probed with total DNA of *H. intybaceum* (red signal) and *H. prenanthoides* (green signal). *H. intybaceum* DNA hybridized preferentially on 18 chromosomes and *H. prenanthoides* on 9 chromosomes (shown by green arrows) of *H. pallidiflorum.* **(B)** The same metaphase plate as in (A) with positions of 45S rDNA (green signal), chromosomes counterstained by DAPI. **(C)** Chromosomes of *H. picroides* (H1613/1) probed with total DNA of *H. intybaceum* (red signal, shown by red arrows) and *H. prenanthoides* (green signal). **(D)** The same metaphase plate as in (C) with positions of 45S rDNA (green signal), chromosomes counterstained by DAPI. Fused 45S rDNA blocks from two chromosomes on this image are shown by white arrows. **(E)** Chromosomes of a F_1 diploid hybrid between *H. intybaceum* and *H. prenanthoides* (92preint_1) probed with total DNA of *H. intybaceum* (red signal, shown by arrows) and *H. prenanthoides* (green signal). **(F)** Chromosomes of tetraploid *H. intybaceum* (INT1_Pel) probed with total DNA of diploid *H. intybaceum* (red signal) and *H. prenanthoides* (green signal, shown by green arrows). **(G)** Chromosomes of triploid *H. prenanthoides* (1187/1) probed with total DNA of diploid *H. prenanthoides* (green signal) and *H. umbellatum* (red signal, shown by red arrows). Bar represents 5 µm.

Repeatome comparison

In order to compare the repeatomes of *H. prenanthoides*, *H. intybaceum* and *H. umbellatum*, comparative clustering analyses of genome skimming data were performed (Fig. S1). The analyses did not reveal any species-specific cluster; all three species differed only in their relative abundance of repeats. The statistical analysis of between-species differences in proportion of reads in clusters above the threshold of 0.2% corresponding to the approximately 100 most abundant clusters (Fig. 11) confirmed that 1) interspecific differences exceeded intraspecific ones (Tukey test: $p < 0.001$), and 2) the repeatome of *H. umbellatum* was significantly more different from *H. prenanthoides* than *H. prenanthoides* (Tukey test: $p < 0.001$) and *H. intybaceum* differed from each other. Also, the variance in the interspecific comparison of *H. umbellatum* and *H. prenanthoides* was significantly higher than in comparisons between *H. intybaceum* and *H. prenanthoides* (Levene test of variance $F = 32.274$, $p<0.001$).

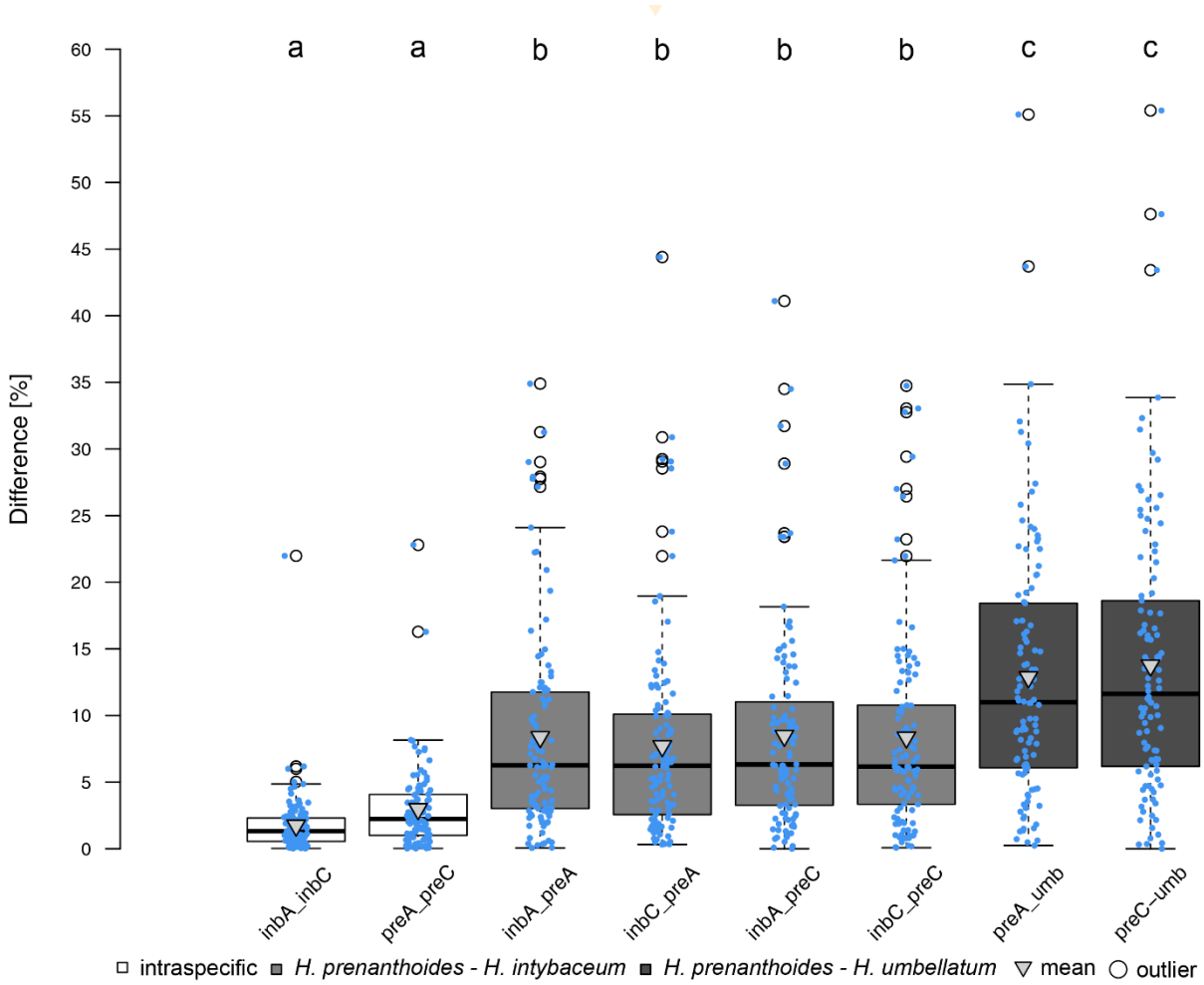

Fig. 11. Intra- and interspecific differences in parental repeatomes. Summarized are differences in the genome proportions of clusters of repetitive elements. Results of the post-hoc Tukey pairwise comparisons of means are included; differences in letters indicate significant differences between the pairs tested. (inbA, inbC) *H. intybaceum* accessions 1531/8, int_6/14/25; (preA, preC) *H. prenanthoides* accessions pre_6/5/5, pre_6/8/5; (umb) *H. umbellatum* accession H1617; all plants are diploid.

Discussion

Although the majority of polyploid apomictic *Hieracium* species (so called 'intermediate' species) is supposed to be of hybrid origin, there is only little evidence for this evolutionary history, except for their morphologically intermediate position. Here we demonstrate the hybrid origins of two intermediate species, *H. pallidiflorum* and *H. picroides*, with the same parental combination of *H. intybaceum* and *H. prenanthoides* using multivariate morphometric analysis (incl. pollen size), sequencing of nuclear and plastid molecular markers, genomic *in situ* hybridization and flow cytometric genome size estimation. The two intermediate species clearly differed from each other in morphology and genome composition. However, comparative analysis of molecular markers and genome size estimation did not show a clear borderline between the taxa, but only some tendencies for their delineation. Generally, a much larger intraspecific variation was detected in *H. picroides* while *H. pallidiflorum* accessions were more uniform in all approaches.

Allopolyploid origins and delimitation of the morphologically intermediate species *H. pallidiflorum* and *H. picroides*

The pattern of two (or sometimes up to four) morphologically well defined intermediate species between two parents seems to be common in *Hieracium* (Zahn, 1921–1923), although the processes leading to this pattern are still unexplored.

Our multivariate morphometric analyses revealed two clusters between *H. intybaceum* and *H. prenanthoides*, corresponding to plants traditionally referred to as *H. pallidiflorum* and *H. picroides*, respectively, the former morphologically closer to *H. intybaceum*, the latter closer to *H. prenanthoides* (Fig. 3).

The genome composition of *H. pallidiflorum* (2int + 1pre) and *H. picroides* (1int + 2pre) is generally consistent with morphology. The number and sites of 45S rDNA clusters in *H. pallidiflorum* (1/4/5, Fig. 10B) matches well the expectations given that diploid *H. intybaceum* has two loci per haploid genome like the majority of *Hieracium* species as yet investigated whereas diploid *H. prenanthoides* has three loci per haploid genome (Belyayev et al., 2018). Intraspecific variation in the number of 45S rDNA loci was observed in *H. picroides* (7–8 loci of which 5–6 were located on the *H. prenanthoides* haplome, Fig. 10D and data not shown). This variation can

be explained by the loss of one 45S rDNA block during speciation, as reported in other plant groups (Raskina et al., 2004) or by potential (so far undetected) intraspecific variation in the number of clusters in *H. prenanthoides*.

Similarly to cytogenetic markers, genome size data for the two intermediate species generally corresponded with our expectations. We confirmed small, but stable differences in genome size between diploid cytotypes of the parental species *H. intybaceum* and *H. prenanthoides* (higher values of absolute genome size in the former), previously reported by Chrtek et al. (2009). Assuming the additivity of genome sizes, higher values in *H. pallidiflorum* likely reflect two haplomes of *H. intybaceum*, whilst lower values in *H. picroides* reflect two haplomes of *H. prenanthoides* (Table 5). However, the mean observed genome size in both species is slightly higher compared to the genome size calculated according to this scenario (2int + 1pre, observed 11.373 pg, expected 11.142 pg in *H. pallidiflorum*, and 2pre + 1int, observed 11.065 pg, expected 10.937 pg in *H. picroides*) which contradicts the often claimed genome downsizing in polyploids caused by e.g. non-random deletion of coding and non-coding sequences, changes in retroelements, and chromosome reorganization (Leitch and Leitch, 2013). However, all mechanisms of downsizing have been proposed for sexually reproducing polyploids and thus cannot be fully applied to our plants. Theoretically, one might expect if a genome is frozen by apomixis, genome size of the polyploids would be additive with respect to their parents' and may not undergo downsizing. The observed genome increases do not fit either interpretation and should be investigated systematically in other apomictic allopolyploids in order to assess if a general trend can be observed. Moreover, genome size data seem to have a good predictive value for inferring evolutionary relationships and genome constitution (i.e. putative parental combinations) in hybridogenous *Hieracium* species, as previously reported in the closely related genus *Pilosella* (Suda et al., 2007) and other genera of vascular plants (e.g., Chumová et al., 2015) and can help to delineate taxonomically problematic and morphologically poorly separated taxa.

In contrast, the concept of two well separated intermediate species is only partly supported by molecular markers: In the majority of accessions of *H. picroides*, the dominant ETS variant was that of *H. prenanthoides*, in the rest of samples approximately equal compositions were found like in most accessions of *H. pallidiflorum* (Table 3). Likewise, ptDNA did not separate the two taxa. Here again, *H. pallidiflorum* was more uniform showing exclusively a maternal origin from *H. intybaceum* whereas *H. picroides* accessions originated from the one or other maternal parent.

Gsh1 alleles of both intermediate species were predominantly those of *H. prenanthoides*; the reason for the preferential loss of *H. intybaceum* alleles is unknown. Thus, while we were able with three unlinked molecular markers to prove the origins of the intermediate species from the same parents, no separation into different taxa was possible. This finding most likely reflects the complexity of molecular marker dynamics in the allopolyploids.

Polytopic origins and intraspecific diversity of hybridogenous species

Besides clear-cut evidence of allopolyploid origins of all accessions of both *H. pallidiflorum* and *H. picroides*, we detected considerable variation within both species indicating their multiple polytopic origins. A combination of three molecular markers indicated that each plant analyzed differed from all others with the exception of three accessions of *H. picroides* (1509/2, H1619, H1621) from two nearby populations. These could not be distinguished and may belong to the same apomictic clone. These results paint a very contrasting picture to the sister genus *Pilosella* where individual clones of several apomictic species were found to occupy large geographic ranges; up to hundreds or thousands of kilometers in their European native range (Fehrer et al., 2005; Krahulec et al., 2008) and even as invasives in North America (Wilson et al., 2006). More surprisingly even, apomicts of genus *Pilosella* (sometimes treated as a subgenus of *Hieracium*) show relatively high residual sexuality (facultative apomixis) and a tendency to form hybrid swarms (Fehrer et al., 2007b) whereas recent natural hybridization in *Hieracium* s.str. is very rare (Mráz et al., 2005, 2011).

Morphological differences also corroborate independent origins, which is already reflected in taxonomic treatments (e.g., Zahn, 1921–1923), that recognize only two subspecies in *H. pallidiflorum* and eight subspecies in *H. picroides* in the Alps. Our study was not aimed at a detailed taxonomic analysis of intermediate species, therefore the affiliation of our plants to previously delimited subspecies shall not be discussed here, but it certainly deserves further attention. Recently, ample data have shown considerable genetic variation in morphologically almost invariant and presumably obligately apomictic polyploid (micro)species (usually more narrowly defined than our intermediate species) of *Hieracium* (Shi et al., 1996; Stace et al., 1997; Štorchová et al., 2002; Rich et al., 2008; Reisch et al., 2014), which is consistent with our results. In a simplified view, this can be caused by either independent polytopic hybridizations involving

different genotypes of parental species or by residual sexuality acting in allopolyploid apomicts. In the related genus *Taraxacum* (Asteraceae), apomictic microspecies (more narrowly defined than our intermediate species and usually with unclear evolutionary history) consist, as a rule, of one, more or less dominant, clone and several minority clones, each usually confined to a single plant. This pattern corresponds well to the mutational clone cluster hypothesis and can be explained by somatic mutations, as a consequence of repeated origins from different lineages or recent hybridization or both (Reisch, 2004; Majeský et al., 2015; Kirschner et al., 2016). In at least some groups of *Rubus* (Rosaceae), hybridogeneous apomictic microspecies have considerably low genetic variation and are as a rule derived from a single distinct genotype differing from the parental taxa, suggesting stabilized clonal reproduction (Šarhanová et al., 2017).

Hieracium pallidiflorum and *H. picroides* are very similar in pollen size, but size variation is higher in the latter. Generally, a lower amount of heterogeneously-sized pollen or even male sterility have been observed in many polyploid apomicts (Asker and Jerling, 1992), and our results confirm this trend. Because the successful production of seeds in autonomous apomicts is completely independent of pollination and fertilization, the retention of the male function is explained by either their phylogenetically too recent origin to have accumulated enough mutations for male sterility (Maynard Smith, 1978), or by the advantage to mate with co-occurring diploids (Mogie, 1992).

Diversity of absolute genome sizes within *H. pallidiflorum* and *H. picroides* and their higher variance in the latter confirm the results from genotyping. Worth mentioning are two outliers in *H. picroides* – H1613 with the highest genome size and H1615 with the lowest (both from the French Alps). In both cases, the values do not correspond with any differences in either morphometric or genetic analyses, and also genome dosage is the same; therefore the interpretation is not straightforward. Because nuclear genome size may affect several phenotypic and developmental characteristics irrespective of the information coded in the DNA, the variation we found might represent adaptation to different environmental conditions. However, it can be argued that all accessions originated from (at least recently) similar habitats (subalpine and alpine grasslands) and that they are recent apomicts, which strongly influences their adaptive potential (Hartmann et al., 2018). Aneuploidy cannot be fully excluded, as we have chromosome counts only for those plants analyzed using *in situ* hybridization. However, aneuploidy is very rare in stabilized *Hieracium* species and its occurrence in our system seems to be unlikely.

Worth mentioning is also the difference between *H. pallidiflorum* and *H. picroides* with respect to their maternal origins. Also in this respect, *H. picroides* shows more diversity. While *H. prenanthoides* as the maternal parent was found in accessions collected nearly exclusively (except for H1620, Col du Grand-Saint-Bernard) within the range of the diploid sexual cytotype of *H. prenanthoides* in the Western Alps (Mráz and Chrtek, unpubl.), *H. intybaceum* was the mother in areas recently occupied by exclusively triploid and tetraploid cytotypes of *H. prenanthoides*. In contrast, all accessions of *H. pallidiflorum* shared *H. intybaceum* as their maternal parent.

The higher intraspecific diversity of *H. picroides* may have various reasons. (i) More accessions of this taxon were investigated (it is more widespread than *H. pallidiflorum*) and therefore this may be a result of sampling bias. However, more or less the same area was covered so that the lower diversity among accessions of *H. pallidiflorum* is not due to geographical distance. (ii) Genomes of all *H. picroides* accessions analyzed cytogenetically show two haplomes of *H. prenanthoides*. *Hieracium intybaceum* has been shown to be genetically rather uniform across the entire Alps (Zahradníček and Chrtek, 2015) whereas *H. prenanthoides* may be genetically more variable. The latter species has not yet been investigated at the population level, however, the high diversity of *gsh1* alleles derived from *H. prenanthoides* especially in *H. picroides* (Fig. S3) is suggestive of a larger intraspecific diversity of the parent that contributed two haplomes to the triploid intermediate. It also cannot be excluded that polyploid accessions of *H. prenanthoides* may have been involved in the formation of *H. picroides* as suggested by a *gsh1* allele of H1618 identical to or highly similar to alleles of a triploid and a tetraploid accession of *H. prenanthoides*. Polyploids of that parent are most often introgressed by other species which may in turn contribute to the higher intraspecific variation of the intermediate species.

Allopolyploid origins of most polyploid accessions of *H. intybaceum* and *H. prenanthoides*

Hieracium intybaceum and *H. prenanthoides* differ in many aspects regarding morphological, cytological and genetic variation. In *H. intybaceum*, sexual diploid and apomictic tetraploid cytotypes are fairly well separated morphologically (Zahradníček and Chrtek, unpubl.), and thus the evidence of an allopolyploid origin of the tetraploid cytotype (based on molecular markers and GISH) is not much surprising. However, an introgression from *H. prenanthoides* into tetraploid *H. intybaceum* was totally unexpected based on morphology as the tetraploid shows no morphological

characters typical for *H. prenanthoides*. Furthermore, Zahradníček and Chrtek (2015) detected only a low level of genetic variation across the total distribution range of *H. intybaceum* (the Alps and the Vosges Mts) and across cytotypes using AFLP analysis. They identified four clusters/genetic groups, which were partly congruent with the ploidal pattern – the first two groups consisting exclusively of diploids, the third genetic group included both diploid and tetraploid plants, and the last group comprised exclusively tetraploid plants. However, the separation of tetraploids was not as straightforward as might be expected from the genome composition (three haplomes of *H. intybaceum* and one haplome of *H. prenanthoides*) revealed in this study. More plants from different populations would have to be investigated to see if this particular introgression holds generally for the tetraploid cytotype or not. Additional support of the allopolyploid origin of tetraploid *H. intybaceum* comes from genome size estimations. The sum of three 1Cx-values of *H. intybaceum* (3 * 3.782 = 11.349 pg) and one 1Cx value of *H. prenanthoides* (3.577 pg, altogether 14.926 pg) corresponds better to the holoploid genome size of 4*x H. intybaceum* (14.814 pg/2C) than the sum of two holoploid values of 2*x H. intybaceum* (2 * 7.565 = 15.130 pg) under the alternative autopolyploid scenario.

In contrast, *H. prenanthoides* consists of a morphologically rather invariant diploid cytotype, but morphologically extraordinarily variable triploids and tetraploids, most of which were proved to be of allopolyploid origin (Fehrer et al., 2009; this study). Only a single triploid accession (pre_1/4/4) from Slovakia was found so far which may be of autopolyploid origin; the most divergent molecular marker *gsh1* showed identical sequence with a diploid plant of *H. prenanthoides* (1252) and with alleles of a triploid accession from Poland (1161/1) as well as with two *H. picroides* plants (H1605, H1608) from Austria. The geographic location of these particular alleles may indicate a much wider past distribution of the diploid cytotype of *H. prenanthoides* which is nowadays confined to the southwestern Alps, a known glacial refuge area (Chrtek et al., 2007). All other polyploid accessions of *H. prenanthoides* were allopolyploid. Most often, these plants showed, based on one or more markers used here for genotyping, the genetic contributions of the '*H. umbellatum*' lineage, which consists of the widespread diploid *H. umbellatum* and several related taxa (Fehrer et al., 2009; this study). Four such accessions (from Andorra, Czechia and France) displayed one haplome (nine chromosomes) of *H. umbellatum*, which is consistent with allopolyploid origins. Multiple independent introgressions that involve various different species and lineages raise questions related to the taxonomic delineation of *H. prenanthoides*. Among our

accessions, a population from France possessed some morphological characters reflecting introgression from *H. umbellatum* and might be determined as *H. rapunculoides*, a hybridogeneous species between *H. prenanthoides* and *H. umbellatum* (J-M. Tison, pers. com.). On the other hand, plants from Andorra and Czechia have morphologically no signs of introgression, and, taxonomically, there is no reason to exclude them from *H. prenanthoides* despite their unquestionable hybrid origins. Similar discrepancies arise also in another polyploid apomictic complexes and make their classification extremely complicated. Furthermore, monoploid Cx values of diploid *H. prenanthoides* accessions are lower compared to triploid and tetraploid accessions which provides support for inferring an allopolyploid origin of some polyploids by introgression of *H. umbellatum* or other species with significantly higher genome size than *H. prenanthoides* (Chrtek et al., 2009; and present data).

Discrepancies between repetitive DNA and phylogenetic evidence

The two major factors influencing the performance of GISH are the genetic similarity of the parental taxa and the evolutionary age of the hybrids (Chester et al., 2010). As the repetitive DNA often represents the largest proportion of plant genomes and is often positively correlated with genome size (Kejnovsky et al., 2012), the differences in repetitive DNA content (that is, types of repeats as well as their genomic abundances) between two parental taxa significantly contribute to the success of GISH.

Based on in-depth phylogenetic studies of genus *Hieracium* (Fehrer et al., 2007a, 2009; Krak et al., 2013; Fig. 9 and S3) the phylogenetic distance between *H. intybaceum* and *H. prenanthoides* should be sufficiently large to assure successful application of GISH to their natural and experimental hybrids. However, contrary to our expectations, GISH revealed a surprisingly high similarity between the two parental genomes. Chromosomes of introgressed polyploid *H. prenanthoides* stained with genomic DNA of *H. umbellatum* were similarly or even better distinguished from *H. prenanthoides* chromosomes than the much more distantly related *H. intybaceum*. Repeatome comparisons of the most abundant repetitive clusters that substantially contribute to the genomes of the analyzed species support the GISH results: the repeat profile of *H. prenanthoides* was significantly more similar to that of *H. intybaceum* than to *H. umbellatum.*

It has been shown recently that the genomic abundance of genome-wide repeats obtained by comparative analysis of repeatomes can be used in phylogenetic studies (Dodsworth et al., 2015a, 2015b, 2017). Contrasting with these findings, the present study reports a case where the information contained in genome-wide patterns of repetitive DNA does not reflect the phylogenetic structure obtained from several unlinked molecular markers that display a level of interspecific genetic variation suitable to assess evolutionary relationships at the genus and species level.

Ploidy variation in the *Hieracium* intermediate species

All analyzed accessions of our morphologically intermediate species were found to be triploid (2n = 3*x* = 27), which is not in line with the general pattern of tetraploids´ prevalence among morphologically intermediate *Hieracium* species reported by Tyler and Jönsson (2009). These authors hypothesized that tetraploids may have originated through recent hybridization between triploids (although the mechanisms for such a process remain to be elucidated), also in formerly glaciated areas recently lacking the parental species. A contrasting hypothesis was formulated by Fehrer et al. (2009) who concluded that there is ample evidence for ancient hybridization in the genus, but no indications of recently ongoing hybridization among the extant, likely obligately apomictic species (Mráz and Zdvořák, 2019); their role as pollen parents remains to be elucidated. While traces of a sexual pathway could be found in embryological investigations of *Hieracium*, there is so far very liitle evidence that these embryos actually develop into mature plants (Hand et al., 2015; Mráz and Zdvořák, 2019). In our group, both intermediate species co-occur (at least at a wider scale, not only at the same locality) with their parental species which can theoretically allow repeated hybridization events. Besides, the similarity of *gsh1* alleles of diploid *H. prenanthoides* in areas far away from the parental species' current distribution are in keeping with a rather ancient hybridogenous origin of the apomicts.

Conclusions

In one of the first detailed multi-approach studies of apomictic allopolyploids we proved multiple origins of two hybridogeneous species, *Hieracium pallidiflorum* and *H. picroides* from the same diploid/polyploid parental species *H. intybaceum* and *H. prenanthoides*. We also detected introgression in polyploid accessions of the morphologically defined parental species. This incongruence between the phenotype, genomic constitution and genetic complexity opens new insight into the taxonomic delineation of the species, which remains a matter of debate not only in the genus *Hieracium*. Moreover, the diversification of parental repeatomes contrasts with the phylogenetic patterns and the evolutionary history of the genus. Once again, *Hieracium* behaves as puzzling as it once did in Mendel's experience (Bicknell et al., 2016).

Acknowledgments

We are grateful to Z. Dočkalová, J. Kocián, G. Mateo Sanz, M. Štefánek, J. Zahradníček and P. Zdvořák for their collaboration with field sampling and Y.J.K. Bertrand for valuable comments to the manuscript. This work was supported by the Czech Science Foundation (17-14620S to J.F. and P.M.) and the long-term research development project No. RVO 67985939 of the Czech Academy of Sciences. Access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum provided under the programme "Projects of Large Research, Development, and Innovations Infrastructures" (CESNET LM2015042), is greatly appreciated. Computational resources were also provided by the ELIXIR-CZ project (LM2015047), part of the international ELIXIR infrastructure. We thank the anonymous reviewers who provided constructive feedback that resulted in a greatly improved manuscript.

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Data availability:

All sequences generated in this study are deposited in GenBank (accession numbers MK465503–MK465681). The alignments used in this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.15dv41nsp (Chrtek et al., 2019).

Supplementary information

Appendix 1. List of localities.

***Hieracium intybaceum* (2*x*): int_1709:** Austria, Kärnten: Kötschach, 6.7 km SW of the town, 1598 m alt., leg. J. Zahradníček, 28 August 2009, 46°37'55.758"N, 12°56'7.005"E, herb. PRC; **inb.Kaer:** Austria, Kärnten: Turracher Höhe, leg. S. Jagalski, s. d., 46°55'13"N, 13°52'18"E, herb. M; **H1622:** Austria, Tirol: Sölden, Mautstelle 2.2 km WSW of the village (church), 2052 m alt., leg. J. Chrtek and Z. Dočkalová, 26 August 2009, 46°57'47.069"N, 10°58'42.538"E, herb. PRA; **1531/8:** Austria, Tirol, Arlberg Massif: St. Anton am Arlberg, Arlbergpass, 1780 m alt., leg. P. Mráz, 15 August 2015, 47°7'28.880"N, 10°12'59.094"E, herb PRC; **Arlbergpass:** Austria, Tirol, Arlberg Massif: St. Anton am Arlberg, Arlbergpass, 1780 m alt., leg. J. Chrtek, 20 August 2008, 47°7'28.880"N, 10°12'59.094"E, herb PRA; **int_1809:** Austria, Tirol: Obergaimberg near Lienz, Mt. Goiselemandl, S slopes, 3.9 km NNW of the village, 2114 m alt., leg. J. Zahradníček, 22 August 2009, 46°52'50.695"N, 12°46'34.381"E, herb. PRC; **H1626:** Austria, Tirol: Obergurgl, along the Gaisbergbach stream SW of the village, 1962 m alt., leg. J. Chrtek and Z. Dočkalová, 25 August 2009, 46°51'43.913"N, 11°1'9.138"E, herb, PRA; **H1321:** Austria, Tirol: Untergurgl (Poschach), ca 300 m SW of the village, 1820 m alt., leg. J. Chrtek and Z. Dočkalová, 25 August 2009, 46°53'15.486"N, 11°2'14.155"E, herb PRA; **Petit Bernard, int_6/9/4, int_6/14/25:** France, dépt. Savoie: La Rosière, near the road to Col du Petit Saint-Bernard, 3.6 km NNE of the village,

2060 m alt., leg. P. Mráz, 28 August 2015, 45°39'29.07"N, 6°51'49.80"E, herb PRC; **Timmelsjoch:** Italy, Trentino-Alto Adige: Rabenstein / Corvara in Passiria, along the road to the Timmelsjoch saddle, 3.3 km NW of the village, 2190 m alt., leg. J. Chrtek and Z. Dočkalová, 27 August 2009, 46°53'29.447"N, 11°7'7.350"E, herb. PRA; **int_1610:** Switzerland, Ticino: Airolo, near the road 3.5 km WNW of the village, 1968 m alt., leg. J. Zahradníček, 15 August 2010, 46°32'11.101"N, 8°34'4.643"E, herb. PRC; **int_3/2/7, int_3/2/8:** Switzerland, Wallis: Furkapass, 2200 m alt., leg. P. Mráz, 30 August 2015, 46°34'23.44"N, 8°23'34.64"E, herb. PRC; **int_1910:** Switzerland, Wallis: Oberwald, 1.5 km W of the Furka saddle, 2128 m alt., leg. J. Zahradníček, 20 August 2010, 46°34'22.641"N, 8°23'43.772"E, herb. PRC.

***Hieracium intybaceum* (4*x*): INT1_PEL, 16439/5:** France, dépt. Hautes-Alpes, Vallouise-Pelvoux, massif des Écrins: Ailefroide, along tourist path 940 m NW of Refuge Cézanne, 2105 m alt., leg. P. Mráz and J. Chrtek, 30 August 2017, 44°55'30.50"N, 6°24'34.95"E, herb. PRC.

***Hieracium pallidiflorum*: H1609:** Austria, Bundesland Salzburg, distr. Tamsweg: Muhr, near a path from the lake Unterer Rotgüldensee to Schrovinschartl, 0.6 km NNW of the Rotgüldenseehütte mountain chalet, 1980 m alt., leg. J. Chrtek and S. Chrtková, 9 August 2017, 47°6'22.191"N, 13°24'36.988"E, herb. PRA; **H1508:** France, dépt. Savoie: Bonneval-sur-Arc, near the road to Col de l´Iseran, 2.7 km NNW of the village, 2280 m alt., leg. J. Chrtek, M. Hartmann and M. Štefánek, 25 August 2014, 45°23'45.423"N, 7°2'23.998"E, herb. PRA; **H1616:** France, dépt. Savoie: Bonneval-sur-Arc, near the road to Col de l´Iseran, 2.7 km NNW of the village, 2280 m alt., leg. J. Chrtek and P. Mráz, 31 August 2017, 45°23'45.423"N, 7°2'23.998"E, herb. PRA; **H1614:** France, dépt. Savoie: La Rosière, near the road to Col du Petit Saint-Bernard, 3.6 km NNE of the village, 2050 m alt., leg. J. Chrtek and P. Mráz, 1 September 2017, 45°39'29.07"N, 6°51'49.80"E, herb. PRA; **pal_1, pal_1/4/2, pal_1/4/5:** France, dépt. Savoie: La Rosière, near the road to Col du Petit Saint-Bernard, 3.6 km NNE of the village, 2050 m alt., leg. P. Mráz, 28 August 2015, 45°39'29.07"N, 6°51'49.80"E, herb. PRC; **Col Iseran:** France, dépt. Savoie: Val-d´Isère, near the road to the saddle, 2.4 km SE of the village, 2443 m alt., leg. P. Mráz, 28 August 2015, 45°25'58.8"N, 7°0'10.8"E, herb. PRC.

***Hieracium picroides*: H1608:** Austria, Bundesland Salzburg, distr. Tamsweg: Muhr, near a path from the lake Unterer Rotgüldensee to Schrovinschartl, 0.5 km NNW of the Rotgüldenseehütte mountain chalet, 1920 m alt., leg. J. Chrtek and S. Chrtková, 9 August 2017, 47°6'19.062"N, 13°24'36.698"E, herb. PRA; **H1637:** Austria, Bundesland Tirol, distr. Imst: Sölden, Gaislach, meadow margin near the road, 1815 m alt., leg. J. Chrtek and Z. Dočkalová, 26 August 2009, 46°55'48.952"N, 10°59'37.135"E, herb. PRA; **H1603:** Austria, Bundesland Tirol, distr. Landeck: Wirl, near the street (Alte Kopsstrasse) to Zeinisjoch, 2.2 km NW of the chapel in the village, 1840 m alt., leg. J. Chrtek and S. Chrtková, 7 August 2017, 46°58'35.595"N, 10°8'31.456"E, herb. PRA; **H1604:** Austria, Bundesland Tirol, distr. Landeck: Wirl, near the street (Alte Kopsstrasse) to Zeinisjoch, 1.93 km NW of the chapel in the village, 1822 m alt., leg. J. Chrtek and S. Chrtková, 7 August 2017, 46°58'34.218"N, 10°8'37.984"E, herb. PRA; **H1605:** Austria, Bundesland Vorarlberg, distr. Bludenz: Partenen, near the road (Kopsstrasse) from the Zeinissee to Wirl, 1.08 km SEE of the hotel, 1845 m alt., leg. J. Chrtek and S. Chrtková, 7 August 2017, 46°58'37.967"N, 10°8'24.504"E, herb. PRA; **H1613:** France, dépt. Hautes-Alpes: Col du Lautaret, along the road to Col du Galibier, ca 200 of Col du Lautaret (parking place), 2070 m alt., leg. J. Chrtek and P. Mráz, 31 August 2017, 45°2'13.284"N, 6°24'12.447"E, herb. PRC; **H1621:** France, dépt. Savoie: Bonneval-sur-Arc, along the road 1.8 km NW of the village, 2190 m alt., leg. J. Chrtek and P. Mráz, 31 August 2017, 45°23'20.227"N, 7°2'33.552"E, herb. PRA; **1509/2:** France, dépt. Savoie: Bonneval-sur-Arc, L´Écot, along a path to refuge des Évettes, 2.7 km ENE of the village, 2090 m alt., leg. J. Chrtek, M. Hartmann and M. Štefánek, 25 August 2014, 45°22'32.590"N, 7°5'16.827"E, herb. PRA; **H1619:** France, dépt. Savoie: Bonneval-sur-Arc, L´Écot, along a path to refuge des Évettes, 2.7 km ENE of the village, 2090 m alt., leg. J. Chrtek and P. Mráz, 31 August 2017, 45°22'32.590"N, 7°5'16.827"E, herb. PRA; **H1615:** France, dépt. Savoie: La Rosière, near the road to Col du Petit Saint-Bernard, 3.6 km NNE of the village, 2050 m alt., leg. J. Chrtek and P. Mráz, 1 September 2017, 45°39'29.07"N, 6°51'49.80"E, herb. PRA; **pic_1/1/2, pic_1/1/3, pic_1/1/4, pic_1/2/1:** France, dépt. Savoie: La Rosière, near the road to Col du Petit Saint-Bernard, 3.6 km NNE of the village, 2050 m alt., leg. P. Mráz, 28 August 2015, 45°39'29.07"N, 6°51'49.80"E, herb. PRC; **H1620:** Italy, Aosta: Gran San Bernardo, near the road on the western bank of the Lago del Gran San Bernardo, 2450 m alt., leg. J. Zahradníček and J. Chrtek, 3 September 2010, 45°52'8.27"N, 7°9'55.21"E, herb. PRA; **H1623:** Italy, reg. Piedmont: Limone Piemonte, the saddle

of Col di Tende, near the parking place, 1880 m alt., leg. J. Chrtek and P. Mráz, 31 August 2005, 44°8'58.10"N, 7°33'37.57"E, herb. PRA; **H1618:** Italy, reg. Trentino-Alto Adige: Passo del Tonale, along a tourist path 1.7 km ESE of the church, 2047 m alt., leg. J. Chrtek and P. Mráz, 29 August 2005, 46°15'02.08"N, 10°36'19.91"E, herb. PRA.

Hieracium prenanthoides **(2*x*): H1632:** France, dépt. Hautes-Alpes, Vallouise-Pelvoux: Ailefroide, along the road to Refuge Cézanne, 1690 m alt., leg. P. Mráz and J. Chrtek, 30 August 2017, 44°54'25.2"N, 6°26'16.8"E, herb PRC; **1252:** France, dépt. Hautes Alpes: La Grave, below the village, ca 1500 m, leg. P. Mráz, 23 June 2003, leg. P. Mráz, 45°2'37"N, 6°18'21"E, herb PRC; **H1634:** France, dépt. Savoie, reg. Auvergne-Rhône-Alpes: Avrieux, 0.8 km SW of the village, 1200 m alt., 24 August 2014, leg. J. Chrtek, M. Hartmann and M. Štefánek, 45°12'30.527"N, 6°42'51.072"E, herb. PRA; **H1631:** Italy, reg. Piedmont: Claviere, 1.7 km NE of the village (church), 1570 m alt., leg. P. Mráz and J. Chrtek, 30 August 2017, 44°56'48.238"N, 6°46'11.985"E, herb PRC; **pre_6, pre_6/5/5, pre_6/8/5:** Italy, reg. Piedmont: Claviere, 1.7 km NE of the village (church), 1570 m alt., leg. P. Mráz, 27 August 2015, 44°56'48.238"N, 6°46'11.985"E, herb PRC.

Hieracium prenanthoides **(3*x*): 1187/1:** Andorra, Pirineus Mts: Canillo, SE margin of the village, 1530 m alt., leg. J. Chrtek, G. Mateo and J. A. Rosselló, 22 July 2006, 42°33'56"N, 1°36'11"E, herb. PRA; **1161/2:** Poland, Województwo dolnośląskie, Karkonosze Mts: Jagniątków, Mały Kocioł Śnieżny glacial cirque, along the path, 1250 m alt., leg. J. Zahradníček, 28 July 2006, 50°47'0.45"N, 15°33'27.59"E, herb. PRA; **PM2050, PM2051, PM2052:** Slovakia, Banskobystrický kraj, Muránska planina Mts: Nižná Kľaková, 1170 m alt., 48°46'0.7"N, 19°57'47.3"E, July 2017, leg. P. Mráz, herb. PRC; **pre_1/4/4, pre_sl_1/4/4, pre_sl_1/4/5, pre_sl_1/3/1, pre_sl_1/3/9:** Slovakia, Prešovský kraj, Belianske Tatry Mts: Ždiar, ca 3 km SW of the village, 1280 m alt., leg. P. Zdvořák, 30 July 2015, 49°14'53.16"N, 20°13'32.88"E, herb. PRC.

Hieracium prenanthoides **(4*x*): pre_1/3/2, R1/8/1:** Czechia, Královéhradecký kraj, distr. Trutnov, Krkonoše Mts: Horní Maršov, mountain massif of Rýchory, 50 m N of the Rýchorská Bouda chalet, 1000 m alt., leg. J. Kocián, 16 August 2010, 50°39'38.6"N, 15°50'59.0"E, herb.

PRA; **R4.3.1:** Czech Republic, Královéhradecký kraj, distr. Trutnov, Krkonoše Mts: Horní Maršov, mountain massif of Rýchory, 640 m N of the Rýchorská Bouda chalet, 980 m alt., leg. J. Kocián, 16 August 2010, 50°39'39.7"N, 15°51'32.0"E, herb. PRA; **pre_3/9/3, rap_3/8/6/5, rap_3/8/7:** France, dépt. Hautes-Alpes, Vallouise-Pelvoux: Ailefroide, along the road to Refuge Cézanne, 1690 m alt., leg. P. Mráz, 27 August 2015, 44°54'25.2"N, 6°26'16.8"E, herb. PRC.

Hieracium umbellatum **(2*x*): H1617:** Czechia, Praha: Troja, near the settlement Pod Havránkou, 235 m alt., leg. J. Chrtek, 21 September 2017, 50°7'10.23"N, 14°25'15.00"E, herb. PRA; **umbellatum 8/9/2:** Slovakia, Košický kraj: Gelnica, Prakovce, 370 m alt., leg. P. Mráz, 31 October 2015, 48°48'54"N, 20°54'43.20"E, herb. PRC.

Table S1. Species/accessions, lineage inferred from ETS, ptDNA, *sqs* and *gsh1* clones.

Species	Accession	Ploidy	Inferred origin / clade				variants[4]	GenBank accession numbers
			ETS[1]	*trn*T-*trn*L[2]	*sqs*[3]	*gsh1*	*gsh1*	*gsh1*
Hieracium alpinum	alp.Ukr	2x	EA	EA	EA	EA	X3, X4	MK465558, MK465559
	alp.Boa.2	2x	EA	EA	EA	EA	X10, 2nd[5]	MK465560, MK465561
H. amplexicaule	1050/1	3x	WP-E	dWP1	WP-E	WP	dir	MK465562
H. bifidum	1213/2	3x	W	W	W	W	X1, X2	MK465563, MK465564
H. bracteolatum	1240/2	3x	Wx-EU	EU	Wx-EU	EU	X2, X3	MK465565, MK465566
H. bupleuroides	1212/2	3x	Epo	Epo	Epo	Epo	X3, X8, X13	MK465567, MK465568, MK465569
	1033/3	3x	EU-Epo	EU	EU-Epo	EU-Epo	X1, X8, X9	MK465570, MK465571, MK465572
H. caesium	1231	4x	W-EU	W	W-EU	W-EU	X2, X12, X17	MK465573, MK465574, MK465575
H. canadense	canad	3x	EU	EU	EU	EU	X1, X5	MK465576, MK465577
H. candidum	1197/3	3x	WP-W	WP2	WP-W	WP	X2, X5	MK465578, MK465579
H. cerinthoides	1176/2	3x	WP-W	WP2	WP-W	WP-W	X1, X4, X5	MK465580, MK465581, MK465582
H. cordifolium	1177/5	2x	WP-W	dWP2	WP	WP	X7, X8	MK465583, MK465584
H. eriophorum	1221/1	2x	EU	EU	EU	EU	dir	MK465585
	1222/2	2x	EU	EU	EU	EU	dir	MK465586
H. glaucum	1230/3	3x	W-Epo	Epo	W-Epo	Epo	X2, X8, X9	MK465587, MK465588, MK465589
H. gouani	1171/4	2x	WP-E	WP1	WP	WP	dir	MK465590
H. gymnocephalum	1215/1	2x	Wy-Ex	unique	?	unique	X3, X5	MK465591, MK465592
	1207/2	3x	Wy-Ex	unique	?	unique	X1, X4, X6	MK465593, MK465594, MK465595
H. gymnocerinthe	1172/4	3x	WP-W	WP1	WP	WP	X2, X3	MK465596, MK465597
H. heterogynum	1250/2	3x	W-Wy-Ex-EU	EU	W-?-Epo	?	X2, X5	MK465598, MK465599
H. humile	1064/2	4x	W	W	W	unique	X5, X6	MK465600, MK465601
	1188/2	3x	W	W	W	unique	X3, X5, X12	MK465602, MK465603, MK465604
H. intybaceum	1069/2	2x	inb	dEA	inb	inb	dir	MK465605
	inb.Kaer	2x	inb	dEA	inb	inb	X1, X3	MK465606, MK465607
H. kittanae	1228/2	2x	EB	EB	EB	?	X6, X9	MK465608, MK465609
H. lachenalii	1160/2	3x	W	EU	W-?	W-EU	X1, X13, X18	MK465610, MK465611, MK465612
H. laevigatum	1031/11	3x	W-EU	EU	W-EU	EU	X3, X7	MK465613, MK465614
H. lawsonii	1175/1	3x	WP	WP2	WP	WP	X2, X3, X9	MK465615, MK465616, MK465617
H. lucidum	H. lucidum	2x	W-Wx	W	Wx-?	Wx	dir	MK465618
H. mixtum	H. mixtum	3x	W-E	unique	WP-unique	WP	dir	MK465619
H. murorum	875/1	3x	W	W	W	W	X8, X9	MK465620, MK465621
H. naegelianum	1208/2	3x	EB	unique	EB	?	X3, X8, X14	MK465622, MK465623, MK465624
H. olympicum	1206/3	3x	Wx-EB	unique	Wx-EB	Wx-unique	X4, X5, X9	MK465625, MK465626, MK465627
H. pannosum	1205/1	3x	EB	EB	EB-?	EB	X1, X3, X9	MK465628, MK465629, MK465630
H. petrovae	1229	2x	EB	EB	EB	EB-?	X1, X6	MK465631, MK465632
H. pictum	1067/4	3x	W	W	W-?	W	X5, Y1, Y3	MK465633, MK465634, MK465635
	1307/5	3x	W	W	n.d.	n.d.	-	-
H. pilosum	1226/1	3x	Epo	Epo	Epo	Epo	X3, X5	MK465636, MK465637
	1226/2	3x	Wy-Epo	Epo	n.d.	Epo	X2, X4	MK465638, MK465639
H. plumulosum	1218/2	2x	W-Wy-Ex-E	unique	W	unique	X4, X6	MK465640, MK465641
H. pojoritense	poi.Rom.1	2x	EA-EU	EA	EU-W	unique	X2, X4	MK465642, MK465643
H. porrifolium	1052/9	2x	Epo	Epo	Epo	Epo	Y3, Y9	HQ131786, HQ131785
H. prenanthoides	1252	2x	W-E	W	?/pren	unique/pren	dir	MK465644
	1161/2	3x	W-E-Wx	dW	?/pren	pren	X1, X2, X10	MK465645, MK465646, MK465647
	1187/1	3x	W-E-EU	W	?/pren-EU	pren-EU	X7, X9, X10	MK465648, MK465649, MK465650
H. racemosum	874	3x	Wx-EU	EU	EU	Wx-EU	X6, X12, X15	MK465651, MK465652, MK465653
H. ramondii	1173/3	3x	WP	WP1	WP	WP	X1, X9	MK465654, MK465655
H. recoderi	1174/4	2x	WP	WP1	WP	WP	dir	MK465656
H. sabaudum	1098/2	3x	Wx-EU	EU	Wx-EU	EU	X4, X8	MK465657, MK465658
H. schmidtii	1025/3	3x	W	W	W	W	dir	MK465659
H. sparsum	1251/1	2x	EB	dEA	E	?	X7, X9	MK465660, MK465661
	spa.sst.2	2x	EB	dEA	E	?	X1, X2	MK465662, MK465663
H. stelligerum	1233/1	2x	W	W	W	W	dir	MK465664
H. tomentosum	1066/8	2x	W	W	W	W	dir	MK465665
H. transylvanicum	tra.Boa	2x	W	unique	E	unique	X1, X8	MK465666, MK465667
	1077/7	2x	W	unique	E	unique	dir	MK465668
H. umbellatum	1021/1	2x	EU	EU	EU	EU	Y3, Y10	HQ131788, HQ131787
	um.AM.1	2x	EU	EU	EU	EU	a1, a2	MK465669, MK465670
H. villosum	1029/1	4x	Epo	dEpo	E-Epo	Epo	X3, X4, X14	MK465671, MK465672, MK465673
	1305/3	3x	Wy-Epo	Epo	Epo	Epo	X1, X2	MK465674, MK465675
H. virosum	1238/1	3x	EU	EU	EU	EU-unique	X3, X4, X5	MK465676, MK465677, MK465678
	vir.1	3x	EU	unique	EU	EU-unique	X1, X3, X10	MK465679, MK465680, MK465681

[1] Apart from Eastern (E) and Western (W) ribotypes that could not be further differentiated, the following subgroups are indicated: Pyrenean (WP), unknown Western 1 (Wx), unknown Western 2 (Wy), *H. alpinum* (EA), *H. umbellatum* group (EU), *H. porrifolium* group (Epo), Balkan species (EB), unknown Eastern (Ex). Data from Fehrer et al. (2009).

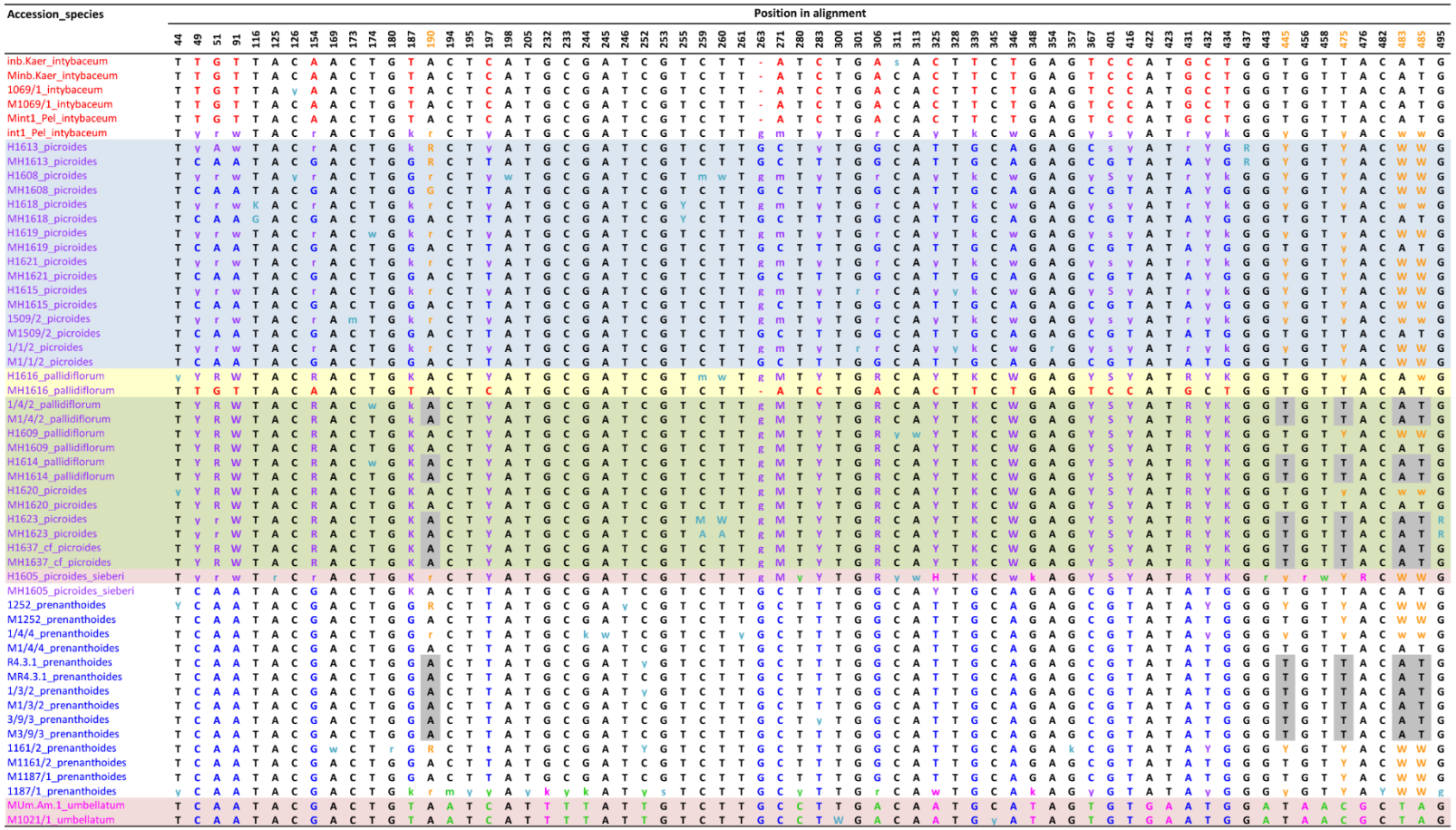
[2] WP1 and WP2 are different plastid haplotypes of Pyrenean species. Haplotypes derived from other haplotypes / haplotype groups are given as, e.g., dWP1.

[3] Question marks refer to clades composed of alleles of different species without correspondence to identifyable species groups. Data from Krak et al. (2013), simplified.

[4] Direct sequences (dir), cloned sequences (X or Y + number) or alleles (a1, a2) differing only by a single substitution are shown. The number of alleles is always equal or lower than the ploidy of the sample indicating single copy status (e.g. diploids are homozygous or heterozygous, triploids are composed of maximal three different alleles etc.). If the placement in the phylogenetic tree was equivocal, only identifyable groups are indicated.

[5] Only identical cloned sequences were found for *H. alpinum* alp.Boa.2; the second allele was inferred by peak subtraction from the electropherogram of the direct sequence.

Table S2. Diagnostic positions and character additivity in ETS sequences: Variable positions of the alignment are shown; different species, substitutional patterns and diagnostic polymorphisms are indicated in different colors (see detailed Legend below). Polymorphisms are indicated in the IUPAC nomenclature code (Y=C+T, R=A+G, S=C+G, M=C+A, K=T+G, W=A+T, H=A+T+C).



Legend

Lower case letters indicate small additive peaks or optional indels

M before accession names reflect higher peaks in direct sequences; polymorphisms in approx. equal amounts are retained

H. picroides with predominantly Western ETS copies

H. pallidiflorum with predominantly *H. intybaceum* ETS copies

H. pallidiflorum and *H. picroides* with approx. equal amounts of *H. intybaceum* and Western copies

H. picroides ssp. *sieberi* with several Eastern and *H. umbellatum* clade-specific polymorphisms

H. picroides , *H. pallidiflorum* and polyploid *H. prenanthoides* accessions lacking all *H. prenanthoides* -specific polymorphisms

red: *H. intybaceum* and species-specific substitutions / indel

blue: *H. prenanthoides* , Western clade and general substitutions differing from *H. intybaceum*

violet: *H. picroides* , *H. pallidiflorum* and additive polymorphisms between *H. intybaceum* and *H. prenanthoides* / Western clade / general *Hie*

orange: *H. prenanthoides* -specific polymorphisms / positions

green: Eastern clade-specific substitutions (some shared with *H. intybaceum*)

pink: *H. umbellatum* clade-specific substitutions

turquoise: Accession-specific polymorphisms

Table S3. Results of canonical discriminant analyses (CDA1, CDA2, CDA3) showing total canonical structure values that express correlations of morphological characters with canonical axes. Character acronyms are explained in Table 2

	CDA 1		CDA 2	CDA 3
Character	Can 1	Can 2	Can 1	Can 1
PLH	-0,242	0,025	-0,050	0,228
SHR	-0,225	-0,005	-0,108	0,460
LAC	0,324	-0,227	-0,104	-0,142
LLB	-0,011	0,118	-0,023	-0,191
LPC	0,054	-0,032	0,055	0,007
LML	0,004	-0,169	-0,005	0,306
WML	-0,230	0,002	0,024	0,155
LGT	0,499	0,220	-0,220	0,080
NCA	-0,256	-0,131	0,044	-0,072
NFC	0,370	-0,219	-0,070	0,097
NSL	-0,221	-0,207	-0,066	0,103
NLB	-0,072	-0,184	-0,237	0,134
NMG	-0,094	0,449	0,040	0,051
NTL	0,110	-0,200	0,027	0,038
NGT	0,426	0,069	-0,520	-0,125
NCT	-0,534	-0,219	0,503	0,181

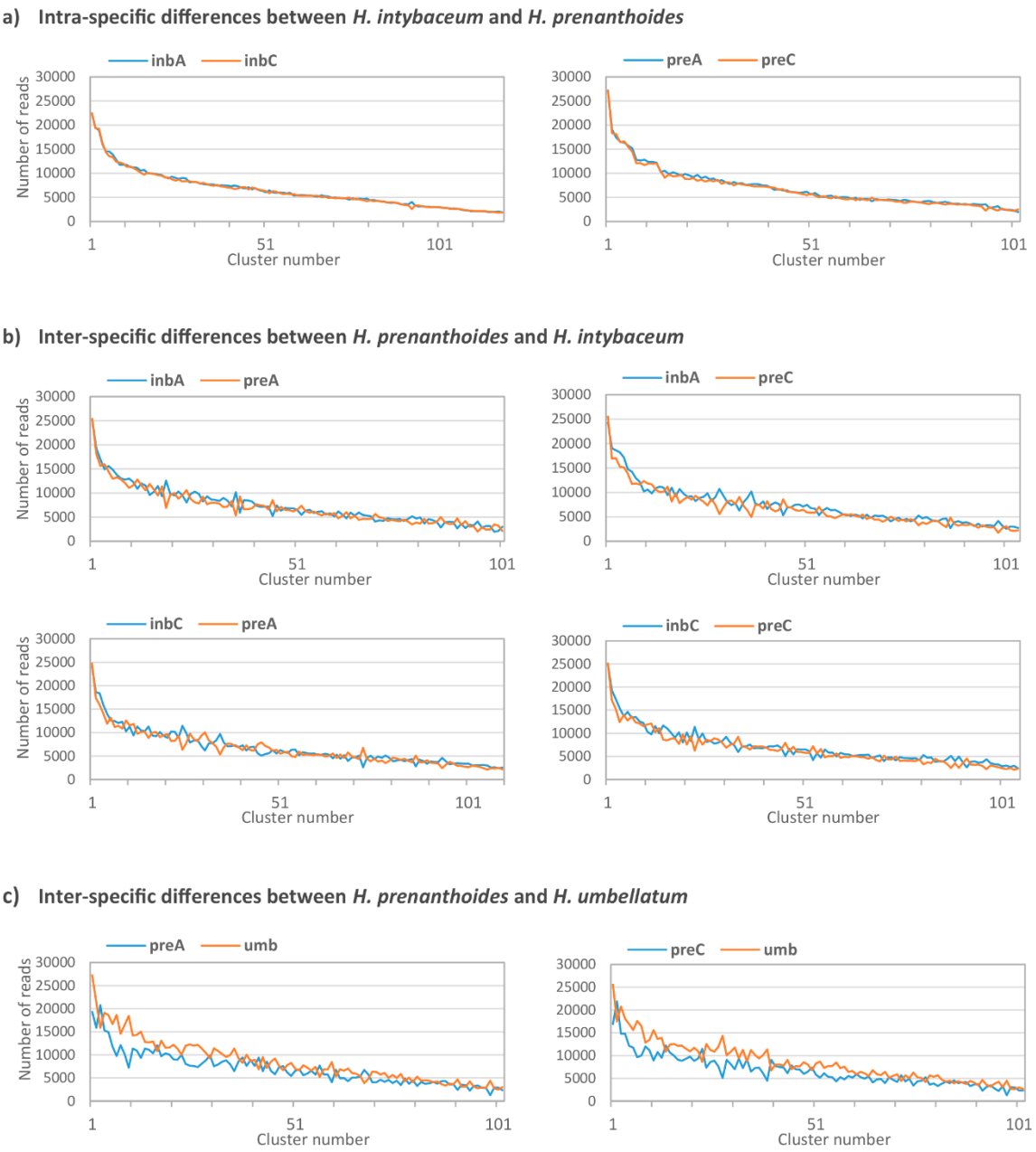


Fig. S1. Differences in the genome proportions of the most abundant clusters of repetitive elements: Differences in the number of reads per tested pair of samples, for clusters with the genome proportion of at least 0.2%. Labelling: (inbA, inbC) *H. intybaceum* accessions 1531/8, 6/14/25; (preA, preC) *H. prenanthoides* accessions 6/5/5, 6/8/5; (umb) *H. umbellatum* accession H1617.

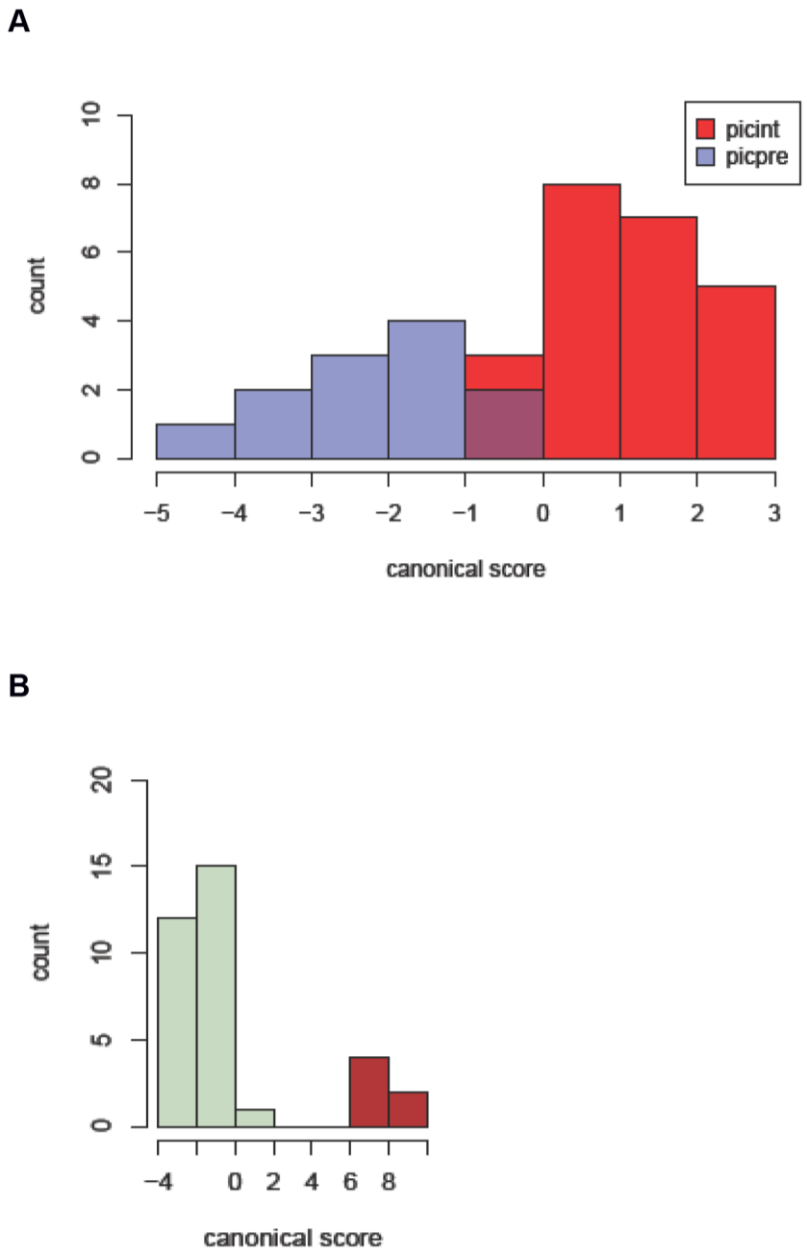


Fig. S2. Canonical discriminant analysis of plants of *Hieracium picroides* (A) and *H. pallidiflorum* (B) based on 16 morphological characters with groups defined as follows: A: (i) a group of accessions with *H. intybaceum* as the maternal parent (red, n = 23), and (ii) a group of accessions with *H. prenanthoides* as the maternal parent (blue, n = 12) (CDA2, based on the cpDNA analysis, see Table 3). B: (i) a group of accessions from the Western Alps (greenish, n = 28), and (ii) a group of accessions from the Eastern Alps (red, n = 6) (CDA3). Numbers on the x axis represent values of the discriminant function.

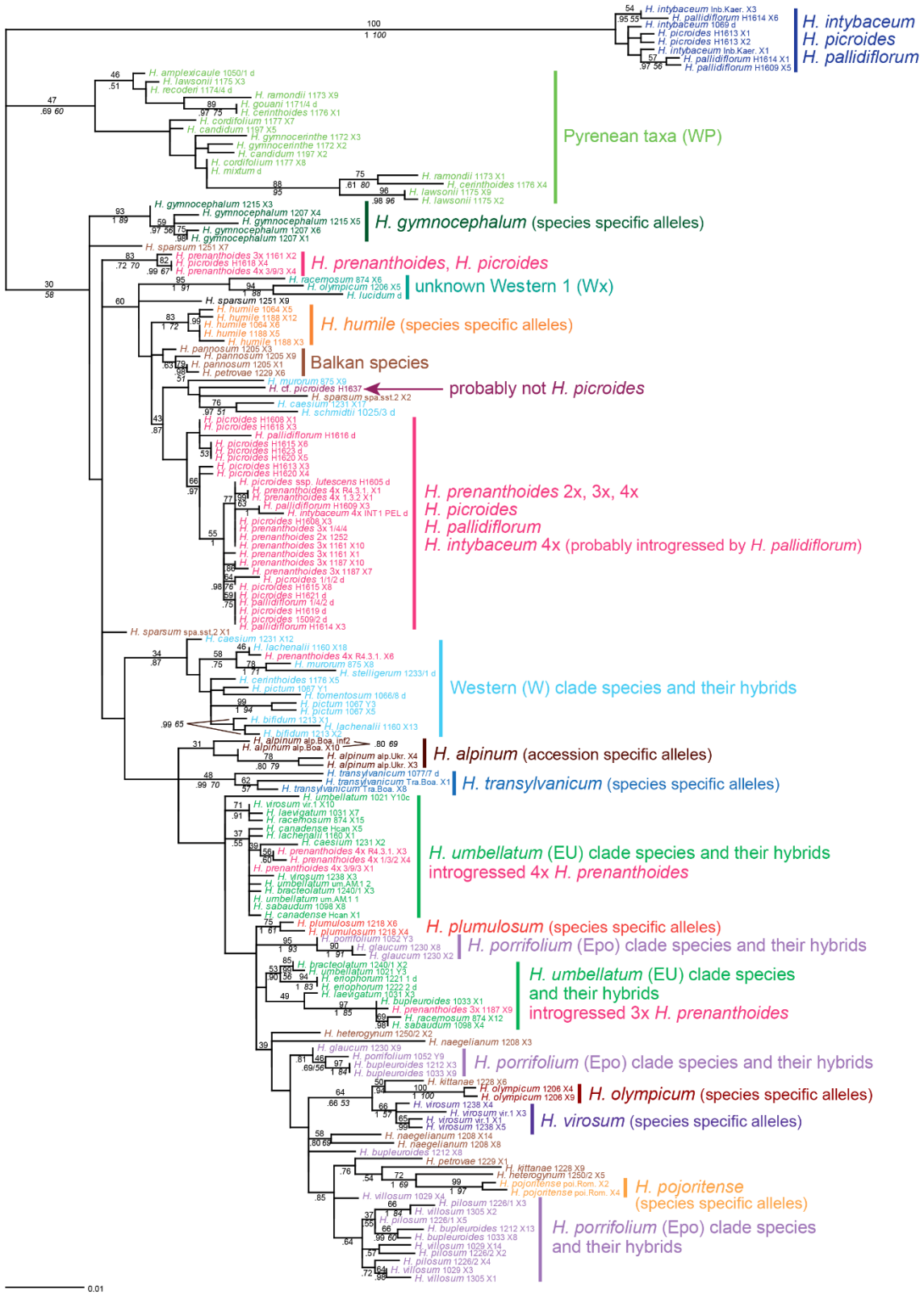


Fig. S3. Detailed *gsh1* tree with species or group specific alleles A ML tree (log likelihood -5197.26) is shown with bootstrap support (BS) larger than 30% above branches. Posterior probabilities from Bayesian analysis and BS of MP analysis (in italics) are given below branches (if available). The backbone of the tree is almost completely unresolved due to strong character conflict in the dataset. Nevertheless, several well supported species specific allele groups are found (e.g. *H. gymnocephalum*, *H. humile*, *H. transylvanicum*, *H. plumulosum*, *H. pojoritense*) as well as species specific alleles of taxa

with two alleles in the 'specific' clade and one in another clade fitting the previously inferred origin of the taxa (*H. olympicum*, *H. virosum*). Most Balkan species are unassigned and interspersed in keeping with their basal position in previous analyses with other markers. Species groups identified with ETS and plastid DNA are also found in the *gsh1* tree albeit with low support or divided into more than one clade (Pyrenean taxa, unknown Western 1, *H. umbellatum* clade, *H. porrifolium* clade, Western clade). A tetraploid accession of *H. prenanthoides* (R4.3.1) has one allele in the Western clade with a sequence almost identical to one allele of *H. lachenalii*. The latter sample has previously been found to have hybrid origin from a Western clade and an *H. umbellatum* clade taxon. Both also have one allele in the largest *H. umbellatum* clade suggesting that *H. lachenalii* may have introgressed this accession of *H. prenanthoides*. For details of sample origin, allelic composition, GenBank accession numbers and a comparison with origins inferred from three other molecular markers, see Appendix S1.

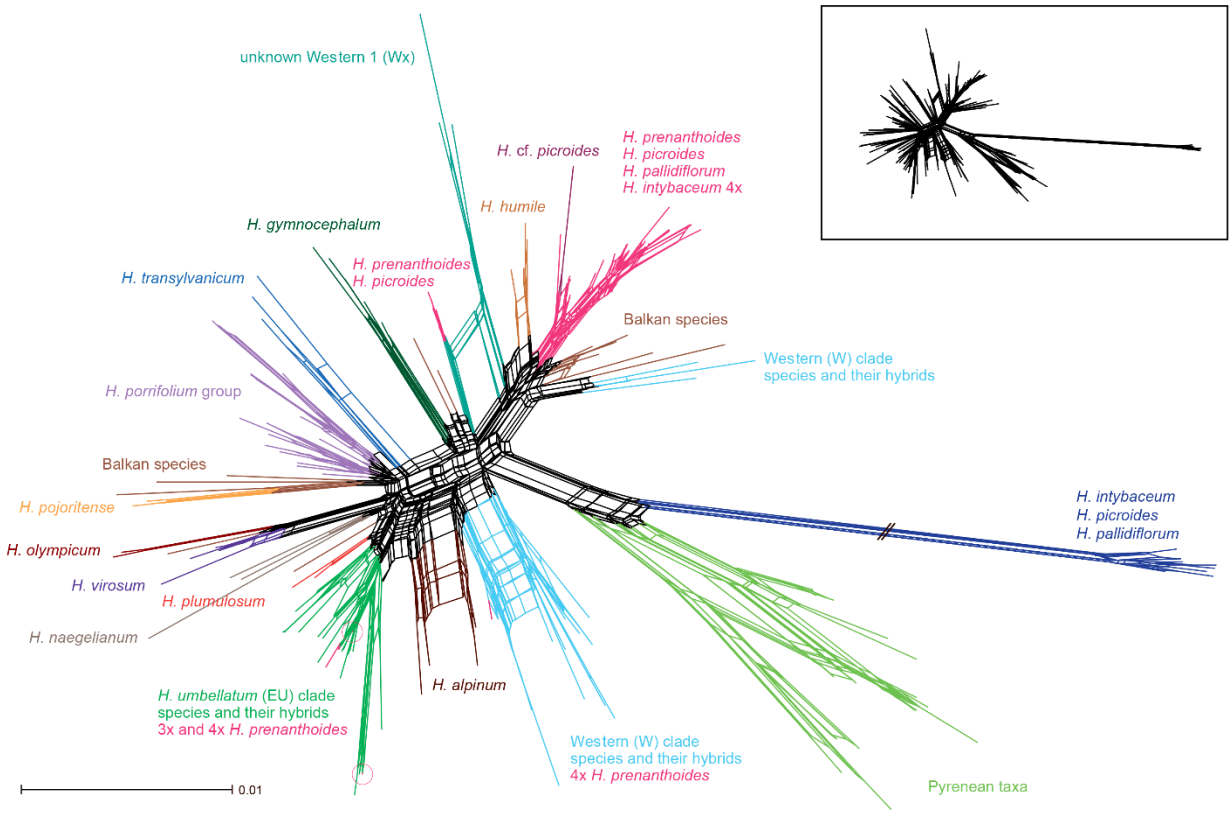


Fig. S4. Neighbor Net of the *gsh1* dataset with species or group specific alleles indicated. The *gsh1* dataset is represented by a network indicating species groups and splits / character incongruence. The centre has a strongly reticulate structure corresponding to the unresolved backbone of the tree. In the figure, the branch leading to *H. intybaceum* is scaled to 50% of its length because of space; the original network is shown in the inset. Balkan species (two times labeled) and individual alleles (additional light brown lines) are interspersed; most other species or species groups are more clearly distinguished than in tree diagrams. Colors correspond to Fig. S3.

Paper II:

Interspecific hybridization and frequency of neopolyploidization in a predominantly polyploid plant genus

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Keywords

apomixis, embryology, flow cytometry, hybridization, mentor effect, polyploidization, unreduced gametes

Abstract

It is generally assumed that interspecific hybridization might stimulate the formation of neopolyploids through the putatively increased production of unreduced gametes. However, experimental studies focused on the frequency of neopolyploidization following interspecific hybridization are scarce. We used control crosses among strictly self-incompatible diploid species of the predominantly polyploid and apomictic genus *Hieracium* s.str. (Compositae) to study the incidence of neopolyploidization.

We performed a series of homoploid crosses and produced F1, F2, BC1 and BC2 plants. Subsequently, we estimated seed set as a proxy of fertility between different types of crosses and determined ploidy levels and origins (autogamously produced versus hybrid) of the progeny. To determine the genomic dosage of parental species in neopolyploid progeny, we applied a cytogenetic approach (GISH and FISH).

In crosses where the maternal plant or both parents were interspecific hybrids, we observed reduced fertility compared to crosses were maternal or both parents were non-hybrids. In certain crosses, we also observed a high frequency of induced autogamy (mentor effect). Overall, we found eight neopolyploids out of 3,739 analysed progeny (0.22%) arisen in six out of 432 crosses (1.4%). Contrary to our expectation, we did not find an increased production of neopolyploids by hybrids (0.11%) when compared to intraspecific and F1 crosses (0.32%). However, the frequency of polyploid progeny formed in crosses where the newly arisen neopolyploids were involved reached 69%.

Our results show that neopolyploidization in *Hieracium* s.str. occurs at a relatively low rate and is independent on the plant origin, i.e. hybrid or non-hybrid. Nevertheless, once formed, neopolyploids can significantly contribute to the production of polyploid progeny and thus stabilize new polyploid lineages.

Introduction

Polyploidization, i.e.,whole-genome multiplication, is a key evolutionary mechanism in vascular plants as it can affect the phenotype and physiology of newly formed polyploids through two, mutually non-exclusive mechanisms – increased cell size and altered gene expression (gene silencing or neofunctionalization) (Otto and Whitton 2000; Comai, 2005). In addition, an increased ploidy level often provides neopolyploids with a strong reproductive barrier compared to the ancestral diploid or lower-ploidy progenitors (Husband et al., 2002; Hörandl and Temsch, 2009; Birchler, 2014). It has been estimated that ca one-third of all vascular plants are evolutionarily young polyploids (Stebbins, 1971; Levin, 2002; Soltis and Burleigh, 2009). Surprisingly, given the ubiquity of polyploidy in angiosperms, we have limited knowledge about the rate of neopolyploid formation and the polyploidization pathways.

It has been suggested that neopolyploids can be formed by direct somatic polyploidization. In that case, somatic polyploidization can occur in the sporophytic meristems resulting in mixoploid chimeras, i.e. tissues with cells composed of two or more ploidy levels (Newton and Pellew, 1929; Skalińska, 1947). Somatic polyploidization can also occur at an early embryonic stage (zygote or several cells-embryo) (Randolph, 1932; Dorsey, 1936). An alternative, supposedly more frequent and thus more important process than somatic doubling involves the production and fusion of unreduced gametes (Ramsey et al., 2003; Ramsey, 2007; Brownfield and Köhler, 2010). These arise due to different mechanisms, which alter meiosis such as anomalous chromosome pairing, exclusion of some phase of meiotic division, synaptic mutation or premature cytokinesis, which causes partially or completely circumvention of meiosis – apomeiosis (Brownfield and Köhler, 2010; Dewitte et al., 2010; Douches and Quiros, 1988; Younis et al., 2014). Observations showed that the frequency of the spontaneous production of unreduced gametes is lineage-specific and can be increased by selection (Parrott and Smith, 1986; Tavoletti et al., 1991; Negri and Lemmi, 1998). The production of unreduced gametes can be spontaneous or enhanced by external factors like chemical treatment (e.g., caffeine, colchicine, nitrous oxide), low or high temperature, causing meiotic spindle abnormalities (Negri and Lemmi, 1998; Lim et al., 2005; Mason et al., 2011; Wongprichachan et al., 2013).

It has been suggested that interspecific hybridization might effectively disrupt normal chromosome pairing and thus alter meiotic division and contribute to the increased frequency of unreduced gamete formation (Genome Collision hypothesis; Carman, 1997). In line with this expectation, many more allopolyploids (which arose from polyploidization involving

hybridization between different species) than autopolyploids (which arose within the same species) have been reported (Soltis et al., 2007). However, the underrepresentation of autopolyploids could be partly influenced by an easier morphological recognition of allopolyploids because of their hybrid phenotype (Soltis et al., 2007). Nevertheless, interspecific hybrids can produce unreduced gametes more frequently than their parental progenitors (Ramsey and Schemske, 1998; Mason et al., 2011; Considine et al., 2012).

In the case of diploid parents, the fusion of one reduced and one unreduced gamete will result in neotriploid progeny while neotetraploid progeny will be formed by syngamy of two unreduced gametes. Because neotriploid plants usually show problems with meiotic pairing of chromosomes due to their uneven chromosome number and unbalanced homology of chromosomes (in allotriploids), they are often considered to represent an intermediate step, a so-called triploid bridge, towards meiotically more stabilized neotetraploids (Husband, 2004; Schinkel et al., 2017). The frequency of unreduced gamete formation has usually been assessed for male gametes (pollen grains) due to their high production and the rather straightforward identification of unreduced pollen using light microscopy (Dweikat and Lyrene, 1988; Ramsey, 2007; Dewitte et al., 2009). In contrast, studies dealing with the frequency of unreduced female gametes are rare as these are produced at considerably lower amounts when compared to pollen (Naumova et al., 1993; Noyes and Givens, 2013) and because the cytoembryological approach assessing unreduced megaspore formation is extremely time-consuming.

Although the rate of unreduced gamete formation is important, because it indicates the potential for neopolyploidization, not every unreduced gamete will participate in this process. Part of the maternal unreduced gametes can simply abort, others can be lost during prepollination transfer as in the case of pollen. Finally, the triploid block, i.e., the abortion of triploid progeny at very early stages due to problems with endosperm development (Yao and Cohen, 1996; Navarro et al., 2003; Köhler et al., 2010), might considerably affect the actual frequency of neopolyploid formation. Therefore, from the evolutionary point of view, the detection of the actual frequency of neopolyploidization is much more important than the assessment of its mere potential by determining unreduced gamete frequency. This goal can be achieved by scoring the number of viable neopolyploid progeny. To the best of our knowledge, there is only one study dealing with the production of neopolyploid progeny by homoploid hybrids or their parental progenitors (Considine et al., 2012). Nevertheless, it has been estimated that the average frequency of unreduced gamete formation is 0.56% in non-hybrid species and 27.52% in hybrids using various unrelated species (Ramsey and Schemske, 1998).

In this study, we report on the frequency and direction of neopolyploidization in the

predominantly polyploid genus *Hieracium* L. s.str. (hawkweeds). This genus (excluding the closely related genus *Pilosella*, which is sometimes treated as a subgenus of *Hieracium*) contains only ca 25 morphologically well-defined, easily recognizable diploid (2n = 2x = 18, based on x = 9) species, but several hundreds to several thousands of polyploid taxa (2n = 3x = 27, and 2n = 4x = 36; Mráz and Szeląg, 2004) depending on the species concept (Majeský et al., 2017). Based on morphology, it has been suggested that most polyploids are of hybridogenous origin as they combine the traits of two or more species (Zahn, 1921–1923). The reticulate pattern of morphological variation has recently been confirmed by molecular approaches, which revealed also that interspecific hybridization occurred repeatedly and frequently during the evolution of the genus (Fehrer et al., 2009; Krak et al., 2013; Chrtek et al., 2020). Interestingly, evidence for recent natural hybridization is rather rare (Mráz et al., 2005; Chrtek et al., 2006; Mráz et al., 2011), mostly because it concerns only diploid, sexually reproducing taxa, which are usually geographically and/or ecologically allopatric (Mráz et al., 2011; Mráz et al., 2020). However, this situation was likely different in the past, when Quaternary climatic oscillations allowed contacts of currently allopatric taxa resulting in frequent interspecific hybridization (Mráz and Zdvořák, 2019; Mráz et al., 2019). Under experimental conditions, diploid taxa hybridize freely with no obvious reproductive barriers, producing frequent interspecific hybrids, which are, however, highly sterile (Mráz and Paule, 2006). In contrast to sexual diploids, polyploids are obligate or almost obligate apomicts (Hand et al., 2005) producing seeds without pollination and fecundation (autonomous apomixis of the *Antennaria* type, Skawińska, 1963). Because of almost strict apomixis, which is frequently coupled with so-called precocious embryony when the parthenogenetically derived embryo starts its development often before flower opening, the role of polyploids as putative maternal plants in interspecific hybridization is rather limited (Koltunow, 1993; Mráz and Zdvořák, 2019). Nevertheless, if polyploids produce functional pollen (polyploid apomicts are frequently pollen sterile; Slade and Rich, 2007; Mráz et al., 2009), in crosses with diploids, they can act as pollen donors (Mráz and Tomčíková, 2004). A high incidence of allopolyploidy, free mating among morphologically different diploid taxa and the preservation of morphological and genetic diversity of interspecific hybrids through apomictic reproduction makes *Hieracium* an excellent model system for testing the role of interspecific hybridization in the formation of neopolyploid plants.

Specifically, we ask the following questions:

i) How frequent is the formation of neopolyploid progeny in interspecific diploid crosses? Given the high frequency of polyploidy in the genus, we suppose a

relatively frequent production of neopolyploid progeny through the frequent production of unreduced gametes in diploid parental taxa.

i) Do F1 diploid hybrids produce neopolyploids more frequently than their parental taxa in intraspecific and F1 crosses? We hypothesize that supposedly meiotic irregularities in diploid F1 hybrids will result in higher production of unreduced gametes, which should lead to a higher frequency of neopolyploid progeny when compared to parental diploid taxa.

ii) If neopolyploids originate from crosses between diploid parental taxa, are they fertile and able to produce a further generation of polyploids? We hypothesize that neopolyploids will suffer from uneven segregation of chromosomes during meiotic division resulting in high sterility and the production of aneuploids.

iii) Which gametes, male or female, contribute more frequently to the formation of neopolyploids? We hypothesize that unreduced female gametes (ovules) contribute more frequently to the production of neopolyploids because of a more suitable embryo:endosperm ploidy ratio for the developing seed.

iv) Do interspecific hybrids have reduced fertility compared to parental species? We suppose that non-homology of chromosomes will cause extensive problems with megasporogenesis and megagametogenesis leading to reduced fertility in hybrids.

To answer these questions, we performed large-scale hybridization experiments involving several diploid taxa of *Hieracium* s.str and their experimental hybrids. Fertility was estimated as a proportion of well-developed seeds per capitulum, i.e. seed set; ploidy of parental plants and their progeny was assessed by flow cytometry; the hybridogenous origin of progeny was determined using morphology. We used also a cytoembryological approach to infer the regularity of megasporogenesis and megagametogenesis.

Materials and methods

Plant material

Eight diploid species of *Hieracium* s.str., namely *H. alpinum*, *H. intybaceum*, *H. pojoritense*, *H. prenanthoides*, *H. stelligerum*, *H. transsilvanicum*, *H. umbellatum*, *H. vranceae* and one natural diploid, presumably F1 hybrid plant of *H. ×krasani* (*H. transsilvanicum* × *H. alpinum*) were used in this study (Table S1). For experimental hybridization, we used cultivated plants grown from seeds or transplanted from the field. Seeds of parental plants and experimental hybrids were stored in paper bags in a fridge at 4°C up to 6 months and then germinated in Petri dishes with soaked filter paper in a growth chamber (PANASONIC MLR-352-PE, Osaka, Japan) with a 12-hour photoperiod and 24/12°C day/night temperature. Seedlings at the cotyledon stage were transferred to seedling trays filled with soil mixture (peat, compost and sand in a ratio of 4:2:1) in the greenhouse. After ca 3 months of cultivation in seedling trays, the ploidy of all seedlings were assessed by flow cytometry (see below). When seedlings reached a size of 5-10 cm in rosette diameter, seedlings were transferred into 0.2 L (*H. alpinum*, *H. ×krasani*, *H. stelligerum* and *H. transsilvanicum)*, or 1 L (*H. intybaceum*, *H. pojoritense*, *H. prenanthoides*, *H. vranceae* and *H. umbellatum)* pots. For further cultivation, we used the same soil mixture as in seedling trays. Plants were fertilized by Kristalon Gold fertilizer (AGRO CS a.s., Czech Republic) in 3-week intervals to stimulate flowering. To prevent fungus gnats, aphids and thrips infestation, experimental plants were treated with biological control agents (nematode *Steinernema feltiae*, mite *Amblyseius cucumeris*, and wasp *Aphidius colemani*) provided by Biocont Laboratory (Modřice, the Czech Republic).

Experimental hybridization

Experimental crosses were performed during four growing seasons from 2014 to 2018 to produce F1, F2 (F1 x F1), F3 (F2 x F2) hybrids as well as BC1 (F1 x P; P x F1) and BC2 (F2 x P, P x F2) introgressants. We performed crosses with those diploid species and plants whose flowering periods were overlapping, but with a higher frequency between one specific pair of diploid taxa, *H. intybaceum* and *H. prenanthoides*, for which we aimed to mimic putative natural hybridization leading to a series of two polyploid taxa (Chrtek et al., 2020). Artificial pollination was performed by rubbing two capitula of two parental individuals together once a day during the whole flowering period (usually 4-6 days). Crossed capitula were isolated in

nylon bags before and during flowering to prevent uncontrolled pollination. Twelve capitula were kept isolated in nylon bags and pollinated artificially between different flowers within a single capitulum using a fine brush to verify the self-incompatibility (SI) system. After artificial pollination, capitula were kept isolated in nylon bags until maturity, then placed in paper bags and stored at room temperature for 3-4 weeks and then in a fridge at 4°C. Seed set was evaluated as the proportion of well-developed seeds from all seeds per capitulum. Well-developed seeds were full (inspected by a dissecting needle) and their seed coat was dark-coloured. In contrast, empty achenes were smaller, squashy and pale. In total, we estimated seed set of 432 successful crosses between nine diploid species and their hybrids. After the flowering season, all well-developed seeds were germinated in Petri dishes, seedlings were cultivated in seedling trays and checked for their ploidy level. Hybrid origin of ca three months old F1 and BC progeny was assessed morphologically in a subset of plants in 2016, as parental diploid species can be easily distinguished from each other. In the case of BC crosses, only progeny from crosses where the diploid species was involved as the maternal plant was evaluated. In summary, the hybrid origin was estimated in 369 progeny of *H. alpinum*, *H. intybaceum*, *H. prenanthoides*, *H. transsilvanicum*, *H. umbellatum* and selected BC hybrids.

Flow cytometry

DNA ploidy level was estimated using flow cytometry. Fresh leaf samples from five individuals were chopped together with internal standard (*Bellis perennis,* 2C = 3.41 pg, Chrtek et al., 2020) using a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0·1 M citric acid, 0·5 % Tween 20; Otto, 1990). The suspension was filtered through a 42- μm nylon mesh and dyed with 1 ml of Otto II buffer (0.4 M Na2HPO4·12H2O), containing DAPI (4′,6-diamidino-2-phenylindole, 4 $\mu g \cdot mL^{-1}$) and β-mercaptoethanol (4 μg·mL-1), and finally left for a few minutes at room temperature. The relative fluorescence intensity of 3000 particles was recorded using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp as UV light excitation source. Histograms were evaluated using the FloMax software, ver. 2.4 (Partec). Usually, five progeny originating from the same cross were analysed as a bulk. In the case of ploidy mixture, each plant was analysed separately again.

Cytoembryological investigation

According to staging series of flower development in the closely related genus *Pilosella*

(Koltunow et al., 1998), capitula of *H. intybaceum*, *H. prenanthoides* and their F1 and BC hybrids were fixed in stages 4 and 6 and stored in FAA fixative solution (ethylalcohol (70%), glacial acetic acid and formaldehyde (38%) in ratios of 7:2:1) and stored at 4°C. Capitula were later treated with hydrochloric acid to remove oxaloacetate crystals according to Koltunow (1998), dehydrated in 100% ethanol and cleared in methylsalicylate according to Tucker et al. (2003). Dehydrated and cleared ovules were dissected and observed by Nomarski differential interference contrast using an Olympus BX51 microscope with an Olympus DP72 camera to determine potential deviation from normal sexual development. The frequency of apomeiosis was scored as the absence of a meiotically derived megaspore tetrad in stage 4, and parthenogenesis was scored as the presence of an embryo in the ovule at stage 6 i.e. just before flower opening (Koltunow et al., 1998; Hand et al., 2015). In both observed stages, also ovule abortion was scored. Aborted ovules were usually smaller, twisted, and all cells of the female gametophyte were missing. In total, 1495 ovules from 69 plants were investigated.

Statistical analysis

We used the Pearson Chi-square test to test for differences in the rate of (i) polyploid formation between diploid × diploid versus neopolyploid × diploid crosses, and (ii) the direction of hybridization between diploids and neopolyploids (whether a neopolyploid was the maternal or paternal plant) in crosses with diploids. Generalized linear models (GLM) with binomial distribution were used to test the effect of parental species and different types of crosses (parental × parental, F1 × F1, F1 x parental, etc.) on seed set. The model testing the effect of the parental species combination on seed set contained the names of parental species in the F1 as a fixed factor and maternal and paternal plant as random factors. Similarly, the model testing the effect of cross type on seed set contains the cross type as a fixed factor and the maternal and paternal plant as random factors. The statistical significance of terms was inferred using a likelihood ratio test (LRT), which compared a fully fitted model to the model from which the tested term was removed. The analyses were performed using the 'glmer' functions in the lme4 package (Bates et al., 2015) within the R environment (R Team, 2013). Tukey multiple comparison posthoc tests were performed to differentiate between different levels of predictors. The non-parametric Kruskal-Wallis test was used to test the effect of hybridization (BC hybrids, F1 hybrids, parental species) on ovule abortion. All analyses and histograms showing relative fluorescence intensities (i.e., ploidy levels) were performed using the R environment (R Team, 2013). All other plots were constructed in Statistica 12 (StatSoft. Inc.).

Results

Frequency of neopolyploidization

We determined ploidy levels in 3,739 progeny originating from 432 experimental crosses. In total, five triploids and three tetraploids representing 0.13% and 0.08% of the progeny resulted from crosses between diploid *Hieracium* taxa. These neopolyploids occurred in six crosses out of 432 with no clear pattern in respect to the type of cross (Figure 1, Table 1 and 2). In detail, parental species produced eight neopolyploids out of 1,899 measured progeny (0.32%) in intraspecific and F1 crosses. Hybrids produced two neopolyploids out of 1,840 measured plants (0.11%; Table 1 and 2). One experimental hybrid from the *H. intybaceum* × *H. alpinum* cross contained diploid and tetraploid tissues in the leaves, which was confirmed by three independent cytometrical analyses (Fig. S2). No aneuploids were detected in experimental crosses between diploids.

Reproduction of neopolyploids

The habitus of neotriploids originating from crosses between diploids was generally less vigorous than in diploid hybrids and most of them did not produce any capitula. Consequently, only two neotriploids were used in three control crosses with an average seedset of 27.7%, from which we obtained 16 offspring. Of these, five were diploid (31%), ten were triploid (63%) and one was hexaploid (6%). Neopolyploids produced polyploid progeny with significantly higher frequency than diploids (χ^2=37.4, P<0.001), and the frequency of polyploid progeny was similar between crosses where neotriploids served as a maternal plant and those where they served as pollen donors (χ^2=1.07, P=0.59, Table 1).

Fertility of hybrids

Although fertility expressed as seed set was fairly similar among different combinations of parental diploid species (χ^2 =6.24, P=0.51, Figure S1, Table S2), we found considerable differences in fertility among different types of crosses (χ^2 =57.9, P<0.001, Figure S3). Specifically, the crosses between hybrid plants (either F1 or BC) and crosses where F1 hybrids were used as maternal plants had significantly reduced seed set when compared to the remaining types of crosses (Figure S3).

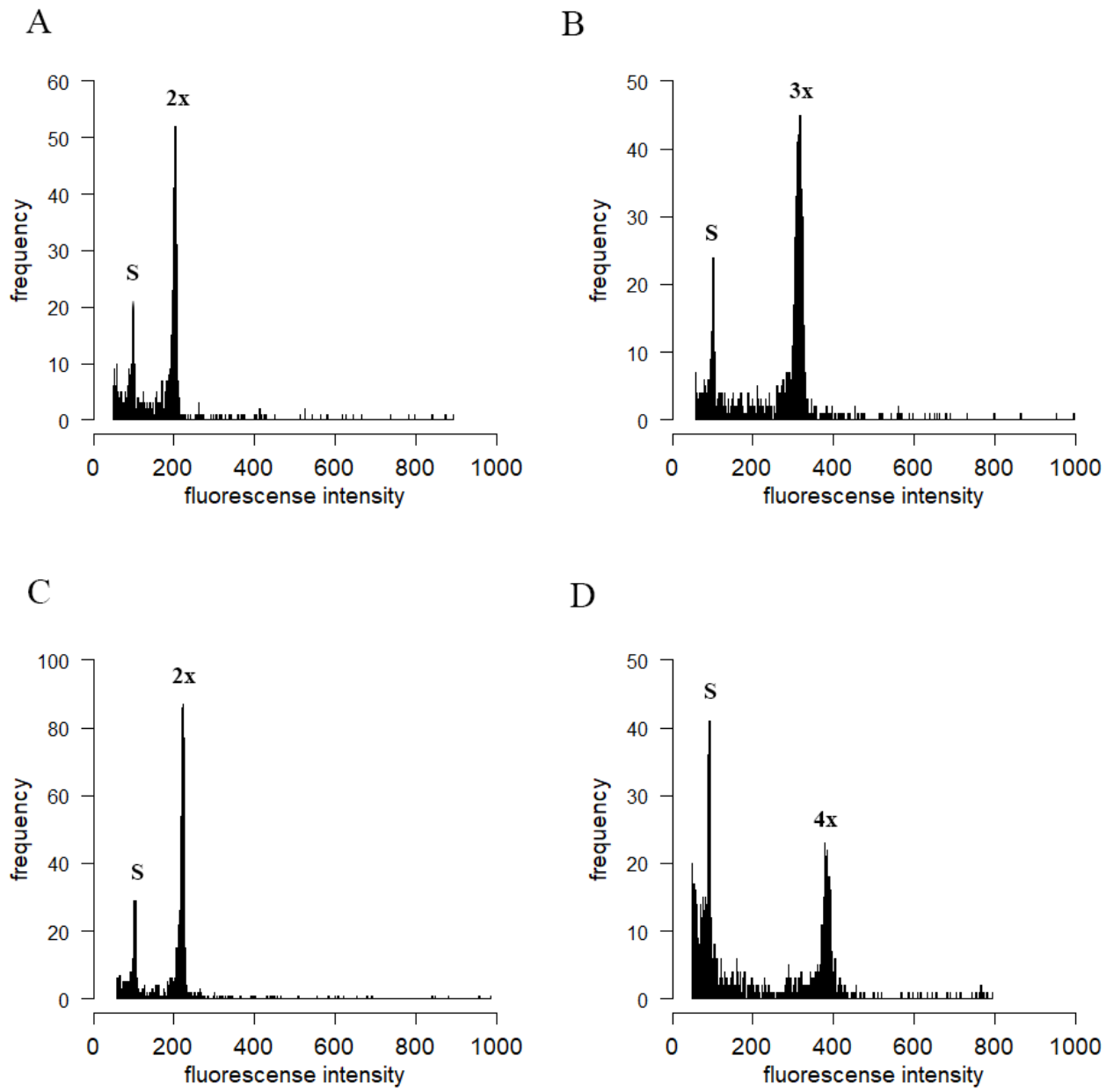

Figure 1. Flow cytometric histograms showing relative fluorescence intensities (i.e. ploidy levels) of nuclei isolated from selected diploid (2x), triploid (3x) and tetraploid (4x) *Hieracium* s.str. hybrid progeny originated from crosses between diploid *Hieracium* taxa. (S) denotes the histograms corresponding to the internal standard (*Bellis perennis*). (A) Diploid progeny (17640_1) originated from the cross between diploid *H. pojoritense* (maternal plant) and diploid F1 hybrid *H. vranceae* × *H. pojoritense* (paternal plant) (cross no. 17640), (B) neotriploid progeny (17640_3) originated from the same cross as in (A), (C) diploid progeny (16401_2) originated from the cross between diploid *H. stelligerum* and diploid *H. intybaceum* (cross no. 16401), (D) neotetraploid progeny (16401_1) originated from the same cross as in (C).

Table 1: Table of the crosses/induced autogamy (†) in which neopolyploidization occurred and crosses with neopolyploids as parental plants (#).

Cross	♀ species / code / ploidy	♂ species / code / ploidy	NO progeny analysed	NO polyploid progeny / ploidy
16293	*H. intybaceum* / 6_14_2 / 2x	*H. alpinum* / 20_out_K_1 / 2x	10	1 / 3x
16159	*H. intybaceum* / 1531_8 / 2x	*H. alpinum* / H63_22_13 / 2x	23	1 / 3x
16401	*H. stelligerum* / 2027 / 2x	*H. intybaceum* / 2039 / 2x	20	1 / 4x
17200	*H. intybaceum* (selfed) / 16135_1 / 2x	*H. (prenanthoides* × *intybaceum)* × *prenanthoides* / 16194_3 / 2x	52	1 / 3x
17640	*H. pojoritense* / 1_7 / 2x	*H. vranceae* × *pojoritense* / 16305_50 / 2x	3	1 / 3x
92_9	*H. prenanthoides* / 1507_2 / 2x	*H. intybaceum* / 1110_2 / 2x	17	1 / 3x
†17010	*H. intybaceum* / 6_14_25 / 2x	-	46	1 / 4x
†17015	*H. intybaceum* / 1531_8 / 2x	-	36	1 / 4x
#16114	*H. prenanthoides* * *intybaceum* / 92_9 / 3x	*H. intybaceum* / 1513_8 / 2x	7	4 /3x
#16125	*H. prenanthoides* / 6_5_2 / 2x	*H. prenanthoides* × *intybaceum* / 92_9 / 3x	4	2 / 3x
#17291	*H. intybaceum***alpinum* / 16293 / 3x	*H. intybaceum* × *pojoritense* / 16139_9 / 2x	5	4+1 / 3x+6x

(♀) maternal plant; (♂) paternal plant i.e. pollen donor; (†) all progeny were produced by induced selfing; (#) crosses with neopolyploids as parental plants; (2x) diploid; (3x) triploid; (4x) tetraploid; (6x) hexaploid.

Table 2: Number and percentage of neopolyploid (triploid and tetraploid) offsprings arisen in several types of controlled crosses between diploid plants of *Hieracium* s.str.

Type of cross	N crossed capitula	N analysed progeny	N neopolyploids (ploidy level)	% neopolyploids
Intraspecific	17+	696	2 (4x)	0.29
Interspecific F1 (P × P)	52	1203	3 (3x) +1(4x)	0.33
Interspecific F2 (F1 × F1)	25	228	0	0
Interspecific BC1 (P × F1)	36	466	1 (3x)	0.21
Interspecific BC1 (F1 × P)	22	293	0	0
Interspecific BC2 (P × BC1)	22	498	0	0
Interspecific BC2 (BC1 × P)	14	190	0	0
Other	19	165	1 (3x)	0.61
Total number	190+	3739	5 (3x) + 3 (4x)	0.22

(+) the exact number of crossed capitula cannot be determined and the listed values are underestimated; (P) parental plants (not hybrids); (3x) triploid progeny; (4x) tetraploid progeny; (Other) combinations of previous types of crosses i.e. F1×BC, F1×F2, F2×F2, F2×P, BC2×P

Embryological observations

All analysed ovules (1495 in total) of *H. intybaceum*, *H. prenanthoides* and their F1 and BC hybrids showed normal sexual development with no irregularities (Figure 2). Apomeiosis, i.e., the formation of an unreduced megaspore, or parthenogenesis, i.e., autonomous embryo and/or endosperm development, was observed in none of the diploid plants. Although the frequency of ovule abortion during the megasporogenetic stage (stage 4) did not differ between diploid parental species and their hybrids ($\chi2=2.25$, $P=0.52$, Table 3), hybrid plants showed a significantly higher rate of ovule abortion during the later stage (stage 6 before flower opening) when compared to the parental taxa ($\chi2=12.48$, $P=0.01$, Table 3). More specifically, diploid F1 hybrids produced significantly more aborted ovules than diploid *H. prenanthoides* (Tukey posthoc test at $P<0.05$). Furthermore, ovule abortion was significantly higher in stage 6 of ovule development compared to the earlier stage 4 ($\chi2=14.07$, $P<0.001$).

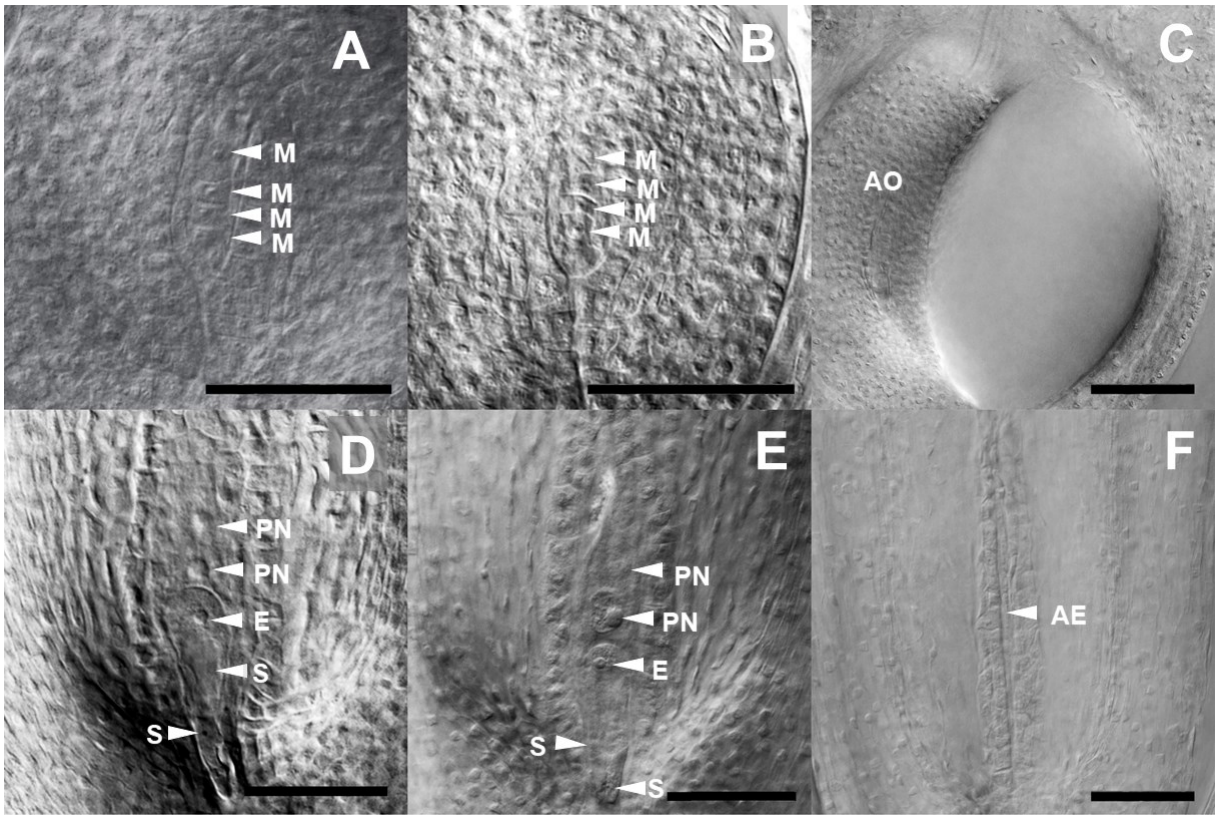

Figure 2. Female gametophyte development in *Hieracium prenanthoides* (A, C, D) and back-crossed hybrid (*H. prenanthoides* × *H. intybaceum*) × *H. prenanthoides* (B, E, F). (A, B) meiotically originated megaspore tetrads at the 4th stage, (D, E) mature embryo sacs at stage 6; (C) aborted ovule in the developmental stage no. 4, (F) aborted embryo sac in the stage no 6, (FM) functional megaspore, (M) megaspore, (PN) polar nucleus, (E) egg cell, (S) synergid, (AO) aborted ovule, (AE) aborted embryo sac. Scale bar = 50 μm.

Table 3: Analysis of embryo sac development in diploid *H. intybaceum*, *H. prenanthoides* and their diploid hybrids.

Taxon	Mode of reproduction	N*	% of ovules undergoing meiosis*	% of aborted ovules*	N**	% of ES containing embryo**	% of aborted ovules**
H. prenanthoides	sexual	40	100.0%	8.8%	547	0.0%	10.3%
H. intybaceum	sexual	100	100.0%	0.0%	168	0.0%	19.2%
F1 hybrids	sexual	5	100.0%	0.0%	20	0.0%	69.7%
BC hybrids	sexual	101	100.0%	0.0%	245	0.0%	24.6%
Kruskal-Wallis test			NT	$\chi2$=2.25, P=0.52		NT	$\chi2$=12.48, P=0.01

(*) Developmental stage 4 (Koltunow et al., 1998), (**) developmental stage 6 (Koltunow et al., 1998)

Frequency of (induced) autogamy

Self-incompatibility was detected in twelve isolated capitula of *H. alpinum*, *H. intybaceum*, *H. umbellatum* and one BC1 hybrid *H. prenanthoides* × (*H. prenanthoides* × *H. intybaceum*). Those plants were seed sterile. However, another two isolated capitula of *H. intybaceum* and one BC1 hybrid (*H. prenanthoides* × *(H. prenanthoides* × *H. intybaceum),* cross 16022) produced a low proportion of well-developed seeds ranging from 7.4 to 16.1%.

Induced autogamy was detected in 48% out of 369 morphologically evaluated offsprings from crosses between parental species and selected backcross hybrids (Table 4). *Hieracium alpinum* and *H. intybaceum* produced a particularly high amount of selfed offsprings when used as maternal parents compared to other species (Table 4). Moreover, *H. prenanthoides* produced 100% selfed offsprings when used as a paternal plant, i.e. pollen donor, but it was used only in crosses with *H. intybaceum*, which already showed a high level of autogamy.

Table 4: Total number of morphologically analysed progeny (N) and percentage of autogamously produced (induced autogamy i.e. mentor effect) progeny from controlled crosses between different diploid *Hieracium* species when used as maternal or paternal plants.

	Maternal plant		Paternal plant	
	N	%	N	%
H. alpinum	67	50.7	25	16
H. intybaceum	175	80.6	67	1.5
*H. prenanthoides**	38	0	80*	100*
H. transsilvanicum	-	-	48	0
H. umbellatum	89	2.2	4	25
hybrids	NA	NA	145	59.3

(NA) not analysed; (*) H. prenanthoides was used only in crosses with H. intybaceum as the pollen donor.

Discussion

In contrary to the Genome Collision hypothesis formulated by Carman (1997), we did not observe increased production of polyploids by interspecific hybrids. Flow cytometry screening showed that hybrids produced 0.11% neopolyploids compared to 0.32% neopolyploids produced by parental species in intraspecific and F1 crosses. Altogether, we found eight neopolyploids out of 3,739 analysed progeny (0.22%) arisen in six out of 432 diploid × diploid crosses (1.4%). Interestingly, we found increased production of polyploids produced by neopolyploids when crossed with diploids. Furthermore, we observed reduced fertility in crosses where the maternal plant or both parents were interspecific hybrids compared to other types of crosses. In certain crosses, we also observed a high frequency of induced autogamy (mentor effect), and in three isolated capitula (*H. intybaceum* and *H. intybaceum* × *alpinum* hybrid), we found selfing without the contribution of foreign pollen.

Frequency of neopolyploidization

Eight neopolyploids were found out of 3,739 progeny. They were produced by hybrids (0.2%) as well as by parental species in intraspecific and F1 crosses (0.29%, Table 2). Aneuploids were not detected. These results are in contrast to the Genome Collisions hypothesis formulated by Carman (1997), suggesting that hybridization might disrupt normal chromosome pairing and alter meiotic division and can, therefore, lead to an increased frequency of unreduced gamete formation and possibly to the production of neopolyploids. Furthermore, Carman (1997) proposed that asynchronous expression of duplicated genes can trigger apomictic reproduction. The formulation of this hypothesis is based on the hybridogenous nature of the majority of polyploids and also apomicts (Beck et al., 2012; Ramsey and Schemske, 1998). Despite these generally accepted assumptions, evidence from studies dealing with homoploid hybridization followed by neopolyploidization and shift to apomictic reproduction was missing.

The role of hybridization in the formation of neotetraploids was tested in a large hybridization experiment performed by Considine et al. (2012) on apples. This experiment provided unclear results, similarly to our data. The authors showed production of triploids and tetraploids at rates of 0.2% and 0.05%, respectively, similarly, like 0.13% of triploids and 0.08% of tetraploids in our experiment (Table 2). Nevertheless, intraspecific crosses of apples produced more euploids than interspecific ones whereas, in *Hieracium*, intraspecific crosses produced a similar number of euploids (0.29%) like interspecific crosses (0.2%, Table 2).

Furthermore, in contrast to our results, Mason et al. (2011) showed that homoploid interspecific hybrids of *Brassica* produce significantly more diploid (1.32%) and abnormal (3.64%) male gametes than their parental species (0.02% and 0.06%). However, frequencies of the formation of unreduced gametes are hardly comparable to the production of neopolyploids in our experiment for several reasons. Firstly, Mason et al. (2011) screened only male gametes, and secondly, not all unreduced gametes give rise to viable neopolyploids.

Considine et al. (2012) also showed that interspecific F1 crosses produced significantly more aneuploids than intraspecific crosses. For that reason, the authors suggested that aneuploidization (0.78% of all progeny) is the leading cause of genome duplication. Moreover, Considine et al. (2012) suggested that aneuploids arose from unreduced pollen only while euploids could be formed by both, male and female gametes. Based on this assumption, we assume that different mechanisms of polyploidization take place in hybridization between diploid species of *Hieracium* s.str. since only euploids (triploids and tetraploids) were detected in our experiment. It seems that unreduced ovules are the main source of polyploid formation in *Hieracium* s.str., similarly like in wild populations of *Achillea borealis* (Ramsey, 2007). On the other hand, we did not directly observe unreduced ovules in our experiment during an embryological screening of 1495 ovules of *H. intybaceum, H. prenanthoides* and their hybrids (see below).

We found also two euploids (tetraploids) in 696 plants of non-hybridogenous diploid species. They originated from *H. intybaceum* by induced selfing, i.e. by a mentor effect. In contrast to this finding, Mráz and Zdvořák (2019) did not find any polyploids among 1,670 seeds of nine diploid non-hybrid species analysed by flow cytometry, including *H. intybaceum* from the same populations as in the present study. Similarly, flow cytometric seed screen of 1,675 seeds produced by diploid *H. alpinum* also did not show any polyploids (Mráz et al., 2019). In conclusion, we can hypothesize that significantly larger sample size is needed to elucidate the cause of neopolyploidization in genus *Hieracium* s.str. Alternatively, cultivation of diploid hawkweeds under experimental conditions may have affected the production of unreduced gametes and be responsible for the different results. Numerous studies have shown that environmental conditions can indeed affect the rate of unreduced gamete production (Yamada et al., 2005; Mason et al., 2011; Zhou et al., 2015; Sora et al., 2016; Wang et al., 2017).

Besides eight neopolyploids, one chimeric plant with two ploidy levels was found among 3,739 progeny (Figure S3). Three independent measurements of different leaves of one *H. intybaceum* × *H. alpinum* F1 hybrid showed a mixture of diploid and tetraploid nuclei, suggesting that somatic duplication can also play a role alongside polyploidization through

unreduced gametes. Nevertheless, this plant did not flower and thus was not able to transfer polyploidy to the next generation. In keeping with this observation, Ramsey and Schemske (1998) suggested that somatic duplication is only a minor mechanism of polyploid formation (Ramsey and Schemske, 1998).

Reproduction of neopolyploids

Our study demonstrated that the neopolyploids arisen from homoploid crosses produces polyploid progeny (when crossed with diploids) in significantly higher frequency than diploid parental species. The majority of the polyploid progeny (one of the parental plants was triploid) were triploids (10), followed by diploids (5) and one hexaploid. We suggest that triploid parent produced reduced haploid, diploid and even aneuploid pollen. Nevertheless, no aneuploid offsprings were observed in our experiment. We propose that diploids derived from triploid parents originated most likely via the fusion of two reduced gametes (n+n). Triploids could originate from either, a fusion of two reduced gametes (2n+n) or by parthenogenetic development of one unreduced gamete (3n), i.e., by apomixis. The hexaploid offspring may have originated from a fusion of two unreduced gametes (3n+3n). However hexaploid and possibly triploid cytotypes could be also explained by double fertilisation of reduced gametes (2n+2n+2n or n+n+n), i.e. by polyspermy (Suárez et al., 1992; Nakel et al., 2017). However, Spielman and Scott (2008) suggested that polyspermy is a rare phenomenon which often leads to the early death of the zygote and thus the variety of mechanisms have evolved to prevent penetration of an egg by more than one sperm cell. Based on this assumption, we conclude that the polyspermic origin of polyploids in our dataset is doubtful (Spielman and Scott, 2008).

In summary, we propose that neopolyploids of *Hieracium* s. str. are reproducing by both, reduced and unreduced gametes. These findings are in contrast to the pattern observed in stabilised polyploids under natural conditions, where residual sexuality, i.e., the production of reduced gametes in polyploids, is extremely rare (Mráz and Zdvořák, 2019; Mráz et al., 2019). Based on the relatively high production of diploids (31%) by neopolyploids, we can hypothesize that the polyploidy does not have to be transferred to the following generations. Alternatively, an unstable polyploid cytotype formed by neopolyploids can be fixed by bypassing meiosis and apomictic reproduction (Carman, 1997). These mechanisms could explain the observed cytotype distribution in the predominantly polyploid genus *Hieracium* s.str (Mráz and Szeląg, 2004; Mráz et al., 2019).

Fertility of hybrids

We observed a reduced seedset in crosses between two hybrids (either F1 or BC) and in crosses where F1 hybrids were used as maternal plants. These results suggest that genome incompatibility between unrelated species can lower seed production in hybrids. In addition, reduced seed production was not observed in crosses when the hybrid plant was the pollen donor. Based on these findings, we can assume that the redundancy of male gametes in comparison to female gametes can compensate for the low viability of hybrid gametes caused by genome incompatibilities. This mechanism where redundancy of pollen increases the fitness of the progeny was already described as pollen competition by numerous authors (Charlesworth, 1988; Klips, 1999; Kalla and Ashman, 2002; Armbruster and Rogers, 2004). Because capitula, which did not produce any viable seeds, were not excluded in our dataset, the seed production of hybrids in our experiment was undoubtedly higher than in a similar experiment performed by Mráz and Paule (2006), but where such capitula were not included in the calculations. A further factor to explain the difference between this and the former study may be that a partially different combination of hybridised species was used.

Embryological observations

We did not observe any deviations from regular sexual development in ovules of diploid *H. intybaceum*, *H. prenanthoides* and their F1 and BC diploid hybrids. These findings are in agreement with those presented by Hand et al. (2015). These authors showed that an absence of meiotic divisions, interpreted as apomeiosis, occurred in 0-7% of ovules depending on the population or species of diploid *Hieracium* s.str., but *H. intybaceum* did not show any apomeiosis, similarly to our observations, and *H. prenanthoides* was not tested. Hand et al. (2015) also did not find any parthenogenetically derived embryos in diploid sexual species. Contrary to our findings, Hojsgaard et al. (2014) demonstrated irregularities in meiosis and megagametogenesis in homoploid *Ranunculus* hybrids. In detail, diploid *Ranunculus* hybrids performed asynchronous meiosis within one flower and the atypical ovule development compared to non-hybrids (Hojsgaard et al., 2014). Those changes in meiosis and megagametogenesis are presumably caused by the alternation of gene expression in hybrids (Carman, 1997; Hojsgaard et al., 2014). However, an arise of functional apomixis, i.e. presence of apomictically derived seeds, was not observed (Hojsgaard et al., 2014). A higher level of ovule abortion observed in *Hieracium* F1 hybrids in our experiment during later development

(fully developed embryo sac in case of non-aborted ovule) also indicate genome incompatibilities in the hybrids. Similar results were observed in hybrids of eggplants (Gowda et al., 1990) or potatoes (Lester and Kang, 1998). This mechanism can explain a lower seed set in particular crosses with hybrids in our experiment.

Selfing

Even though *Hieracium* s.str exhibits sporophytic self-incompatibility (Mráz and Paule, 2006), our results show for the first time that some level of selfing can occur in the genus. We observed selfing in *H. intybaceum* and *H. intybaceum* × *alpinum* hybrids only, suggesting that self-incompatibility is not strict. Fehrer et al. (2007) demonstrated that *H. intybaceum* is, based on the nuclear ribosomal ITS region, genetically most distant to all other species of *Hieracium* s.str. and is sister to all four clades of subtribe Hieraciinae: *Pilosella, Hieracium* s.str., *Chionoracium* and *Andryala*. *Hieracium* s.str. and *Pilosella* were demonstrated to be self-incompatible (Krahulcová et al., 1999; Mráz, 2003), but there are no data about selfing in *Andryala* or *Chionoracium*, which could be theoretically self-compatible (Zahradníček et al., 2018). In conclusion, we suggest that the complicated taxonomical relationship of *H. intybaceum* with self-incompatible *Hieracium* s.str could explain the occurrence of selfing in *H. intybaceum* and *H. intybaceum* × *alpinum* hybrids. Moreover, it has been demonstrated that self-incompatibility can be disrupted by external factors such as high temperature (Hogenboom, 1972; Wilkins and Thorogood, 1992). However, all experimental plants checked for self-incompatibility were cultivated under uniform conditions, therefore the different behaviour may rather be species-specific.

The genus *Hieracium* s.str. is considered to be self-incompatible, but that induced selfing by a mentor effect can occur during hybridization (Mráz and Paule, 2006). In our experiment, we found a noticeably higher amount of selfed progeny in crosses where *H. intybaceum* was used as a maternal plant and in crosses where *H. prenanthoides* was used as the pollen donor. However, *H. prenanthoides* was used as pollen donor only in crosses with *H. intybaceum*. Either *H. prenanthoides* produced non-viable or non-compatible pollen, which subsequently disrupted the self-incompatibility barrier and caused a mentor effect, or *H. intybaceum* as a taxonomically distant species hardly hybridises with *H. prenanthoides* and other species of *Hieracium* s.str.. In addition, selfing (without the presence of foreign pollen, i.e. not by mentor effect) was already detected in isolated capitula of *H. intybaceum* and *H. intybaceum* × *alpinum* hybrids. This can putatively explain the higher amount of selfed progeny in experimental crosses involving this species.

Conclusion

Spontaneous polyploidization occurs in the genus *Hieracium* s.str. in low frequencies, independently of hybrid origin. Moreover, neopolyploids produce polyploid progeny in significantly higher frequencies, and mixed ploidy levels of these progeny suggest a disruption of normal chromosome pairing and meiosis alteration. However, to better understand the role of polyploidization in this genus, the reproduction of neopolyploids should be investigated in more detail for several subsequent generations including experimental crosses, estimation of offspring ploidy and their reproductive mode. Based on the prevalence of polyploids in this genus, we suggest that in this scenario, some of the polyploid cytotypes would be eventually fixed by apomictic reproduction.

Acknowledgments

We thank Susan Johnson from CSIRO, Adelaide for helping to establish the embryological method at the Department of Botany, Charles University, Prague. We also thank Romana Bartošová for help with the experiments. This project was funded by the Czech Science Foundation (GA17-14620S) and the Grant Agency of Charles University (GAUK 907218).

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Supplementary material

Table S1. List of localities of the diploid plants used in experimental hybridization.

Species	Code	Country	Locality	Altitude	Latitude	Longitude	Collector
H. alpinum	H57	Romania	Mt. Pietrosul Broştenilor	702	47.3847	25.5363	PM
H. alpinum	C2	Romania	Mt. Vlădeasa	1828	46.7646	22.7946	PM
H. intybaceum	1110	Italy	Passo dello Stelvio	2472	46.542389	10.422889	JC
H. intybaceum	PM2039	Austria	Arlberg pass	2000	47.1279	10.1953	PM
H. intybaceum	int_2	Switzerlan	Oberalppass	1940	46.6520	8.6790	PM
H. intybaceum	int_6	France	Col du Petit Saint Bernard	2058	45.6580	6.8640	PM
H. intybaceum	int_3	Switzerlan	Furka pass	2118 ± 23	46.5731	8.3931	PM
H. intybaceum	int_4	France	Réfuge des Evettes	2060	44.9420	6.4070	PM
H. pojoritense	PM2012	Romania	Pojorita village	690	47.5340	25.4950	PM
H. prenanthoides	JC_1507	France	Montegenevre	1590,3	44.9235247	6.6962725	JC
H. prenanthoides	JC_1513	France	Modane town	1197	45.2079386	6.7125050	JC
H. prenanthoides	pren_6	Italy	Claviere village	1626 ± 36	44.9464	6.7692	PM
H. prenanthoides	pren_5	France	Ailefroide village	1626 ± 36	44.9050	6.4414	PM
H. prenanthoides	pren_7	France	Fortresse Marie Therese	158	45.2084	6.7356	PM
H. stelligerum	PM2027	France	Jaujac town	415	44.6700	4.2100	PM
H. transsilvanicum	HTRAN_1	Romania	Mt. Pietrosul Bogolin	1460	47.4020	25.5230	PM
H. umbellatum	umb_8	Slovakia	Prakovce village	380	48.8090	20.2100	PM
H. vranceae	PM2011	Romania	Lepşa village	590	45.9430	26.5860	PM
H. ×krasani	HKRA	Romania	Mt. Pietrosul Bogolin	1460	47.4020	25.5230	PM

(PM) Patrik Mráz; (JC) Jindřich Chrtek.

Table S2: Summary of fertility expressed as seed set (percentage of well-developed achenes per capitulum) between different types of diploid crosses in *Hieracium* s.str..

Type of Cross	N	Mean	Minimum	Maximum	Standard deviation
PxP	134	19.02	0.00	92.00	24.66
PxF1	76	23.46	0.00	100.00	22.15
BcxBc	41	6.05	0.00	36.84	8.66
PxBc	27	17.39	0.00	100.00	26.40
BcxP	29	19.79	0.00	78.72	22.71
F1xF1	62	9.67	0.00	69.09	14.53
F1xP	58	9.79	0.00	30.77	9.18

(P) parental species; (F1) F1 hybrid i.e. P × P cross ; (BC) backcross hybrid, i.e. P × F1 or F1 × P cross.

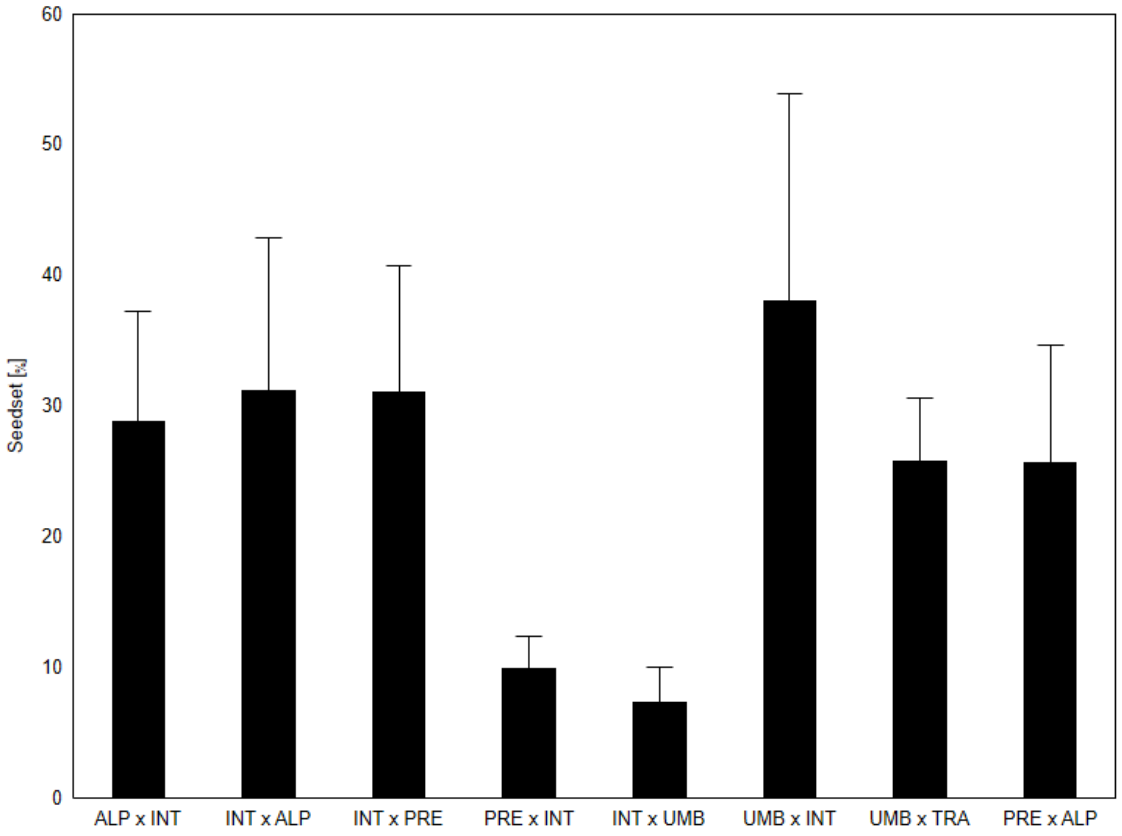

Figure S1. Fertility expressed as seed set [percentage of well-developed achenes per capitulum] between different combinations of diploid species of *Hieracium* s. str. Presented mean and standard errors were computed from raw data. Differences were not significant at P≤0.05. (ALP) *H. alpinum*; (INT) *H. intybaceum*; (PRE) *H. prenanthoides*; (TRA) *H. transsilvanicum*, (UMB) *H. umbellatum*; The order of two abbreviations determines the direction of hybridization, i.e. the species used as the maternal plant is always listed as the first.

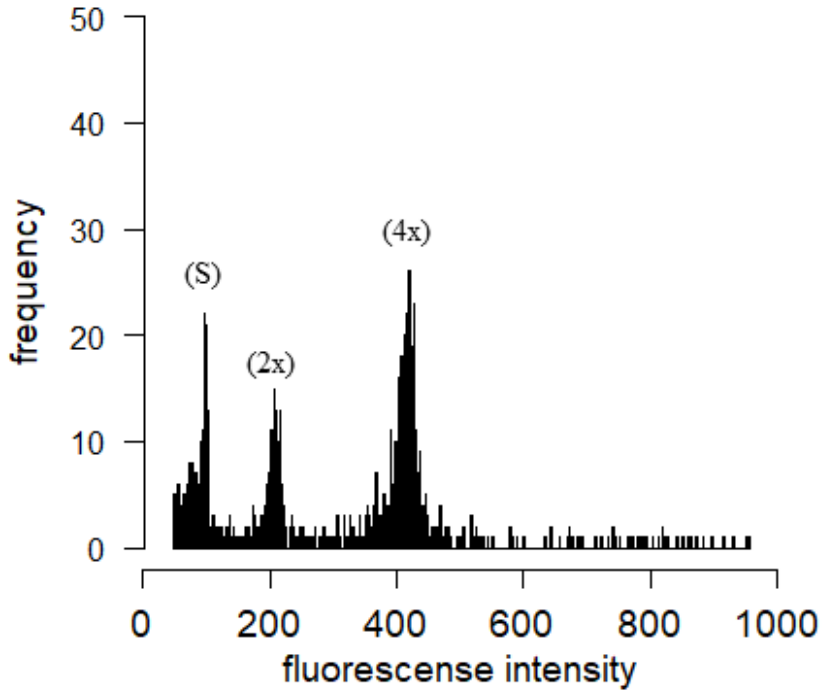

Figure S2: Flow cytometric histograms showing relative fluorescence intensities (i.e. ploidy levels) of nuclei isolated from one F1 hybrid *H. intybaceum* × *alpinum* originated from crosses between diploid plants (cross 16159). The analysis showed peaks corresponding to diploid (2x) and tetraploid nuclei (4x) within a single plant suggesting that this individual hybrid is a chimera containing tissues with two ploidy levels.

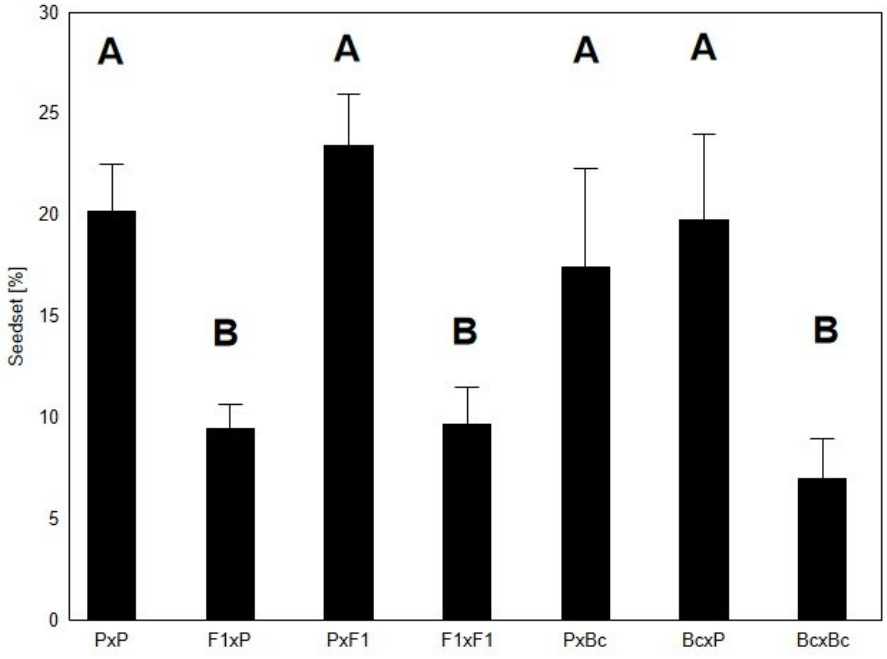

Figure S3: Fertility expressed as seed set (percentage of well-developed achenes per capitulum) between different types of diploid crosses in *Hieracium* s.str. Presented means and standard errors were computed from raw data. Different letters indicate statistically significant differences between categories (at significance level P≤0.05). (P) parental species; (F1) F1 hybrid i.e. P × P cross ; (BC) backcross hybrid i.e. P × F1 cross.

Paper III:

Negative effect of inbreeding on fitness of an arctic-alpine *Hieracium alpinum* (Asteraceae), a species with a geographical parthenogenesis distribution pattern

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Key words: fitness, geographical parthenogenesis, inbreeding, inbreeding depression, interspecific competition, metapopulation hypothesis

Abstract:

Decreased fitness due to inbreeding in small populations can be a considerable disadvantage in sexually reproducing organisms compared to their asexual relatives. Therefore, inbreeding depression of sexuals could contribute to geographical parthenogenesis (GP) distribution patterns, when sexuals have more restricted distribution than their asexual relatives. In this study, we tested whether biparental inbreeding will lead to decreased fitness in sexual diploids of *Hieracium alpinum*, a species showing a GP distribution pattern. We performed three types of crosses (inbred, outbred, interpopulation outbred) and measured eleven fitness traits. A subset of plants was also grown with a natural competitor *Nardus stricta* to increase any negative effects of inbreeding. We found significant effects of inbreeding in two out of eleven measured traits in the F1 generation. Initial biomass was statistically significantly lower by 9.6% in inbred lineages compared to outbred lineages. Similar trends, i.e. lower values in inbred progeny when compared to outbred one, were found also in final biomass and survival rate (values decreased in inbred progeny by 17.5% and 11.5%, respectively), but these differences were statistically non-significant. However, the negative effect of inbreeding on these traits was not increased by the presence of *Nardus stricta* as a competitor. Our results suggest that biparental inbreeding could to some extent explain a lowered colonizing ability of sexual diploid of *H. alpinum*, but the GP pattern observed is also likely to be influenced by other processes and their interactions.

Introduction

Geographical parthenogenesis describes a phenomenon where the geographical ranges of asexually reproducing organisms are wider and often shifted to higher latitudes and altitudes than the ranges of their sexual relatives (Vandel, 1928; Bierzychudek, 1985; Van Dijk, 2003; Hörandl, 2006). Several non-exclusive hypotheses have been proposed to account for geographical parthenogenesis: (i) the advantage of uniparental reproduction because of supposedly superior and more stable reproductive success in apomicts (Baker, 1967; Linder and Barker, 2014); (ii) the advantage of increased genome dosage / allelic richness per locus since almost all apomicts are polyploid (Bierzychudek, 1985); (iii) better exploitation of the niche by asexuals because of the existence of either a general-purpose genotype (Lynch, 1984) or multiple genotypes occupying correspondingly narrow niches (frozen niche variation model, Vrijenhoek 1984); (iv) context-dependent advantage under the differential level of biotic interactions (the Red Queen hypothesis, van Valen 1973); (v) colonization advantage of asexuals through avoidance of genetic load and inbreeding in small founder populations (the metapopulation hypothesis, Haag and Ebert 2004).

The metapopulation hypothesis proposes that in small, subdivided populations, which typically occur in marginal habitats or range margins and undergo frequent extinctions and recolonizations, sexuals will frequently be exposed to biparental inbreeding (Haag and Ebert, 2004). Consequently, this might have a negative effect on the fitness of inbred progeny, a pattern referred to inbreeding depression, what might ultimately lead to the lowered colonization / establishment potential in sexuals compared to asexuals.

Inbreeding depression is presumably linked to the expression of recessive deleterious alleles (Charlesworth and Willis, 2009). These alleles, which are usually masked by dominant alleles in heterozygous state, could be expressed under homozygous constitutions which frequencies will dramatically increase as a consequence of biparental inbreeding (Wright, 1984). Inbreeding depression can be manifested in various traits, including those which are essential for population establishment and survival (e.g. biomass production, reproductive output, survival rate) and has been confirmed in many plant species (Molina-Freaner and Jain, 1993; Cheptou *et al.*, 2000; Richards, 2000; Nielsen *et al.*, 2007). The level of inbreeding depression also depends on various intrinsic or extrinsic factors. For example, little or no negative effects of inbreeding have been recorded in predominantly selfing plants (typically annual weeds) which are able to effectively purge deleterious alleles (Dole and Ritland, 1993). Reduced inbreeding depression has been recorded also in polyploids when compared to closely related diploids

presumably because of higher level of heterozygosity in the latter (Rosche *et al.*, 2017). The effects of inbreeding can significantly increase in subsequent generations of inbred crosses (Dudash *et al.*, 1997). Furthermore, some studies reported that inbreeding depression might be enhanced under the presence of environmental stress or competition (Antonovics, 1968), whilst others suggested that inbreeding depression might not be detectable under favorable experimental conditions (Schmitt and Ehrhardt, 1990; Cheptou *et al.*, 2000). In contrast to sexuals especially those with strict self-incompatibility system, their apomictic congeners do not suffer from biparental inbreeding as the embryo is formed fully autonomously, i.e. without syngamy. Because of this, and the fact that apomictic plants are almost exclusively polyploid (Gustaffsson 1935; Asker and Jerling 1992) and often of hybridogeneous origin (Hörandl 2004; Fehrer et al., 2009; Sochor et al. 2015; Chrtek et al. 2020), apomictic progeny not only fixes maternal level of heterozygosity (Gornall 1999) but this level is also usually higher when compared to diploid sexuals (Hörandl et al. 2000; Hörandl and Greilhuber 2002; Šingliarová et al. 2011). Consequently, the fitness of apomicts is under stable conditions maintained over the generations and cannot be lowered by genetic processes like biparental inbreeding especially in small and isolated populations. Indeed, decreased observed heterozygosity has been observed in small / isolated sexual populations of various apomictic complexes (Šingliarová et al. 2008; Šingliarová et al. 2011; Šarhanová et al. 2017).

In addition to the negative effect of biparental mating on overall fitness, in strictly self-incompatible plants, inbreeding might decrease the level of cross-compatibility in mating partners. The self-incompatibility (SI) is controlled by S locus expressed in forms of S alleles in both pistils and pollen (Brennan *et al.*, 2013). This mechanism hampers not only self-fertilization but also fertilization between individuals sharing the same S-alleles (Wagenius *et al.*, 2007). Therefore, both, biparental inbreeding and limited number of S-alleles in a small founder population might increase the risk of non-compatible crosses which will ultimately reduce the reproductive output (i.e. fertile seeds). Importantly, it is not easy to distinguish which of the two processes, i.e. inbreeding depression or incompatible mating, are involved in seed abortion (Brennan *et al.*, 2006; Wagenius *et al.*, 2007).

Although the impact of inbreeding on fitness has been studied many times in various angiosperms (e.g. Cheptou *et al.*, 2000; Picó and Koubek, 2003; Brennan *et al.*, 2005; Rosche *et al.*, 2017), we are not aware of any experimental study focused on the effect of biparental mating on fitness of sexual relatives in plant apomictic complexes with GP patterns. To fill this gap, we compared various fitness traits between inbred and outbred lineages of

Hieracium alpinum L. (Asteraceae). This species encompasses two cytotypes which exhibit two different reproductive strategies; diploids are strictly sexual and triploids are strictly apomictic (Skawińska, 1963; Chrtek, 1997; Mráz *et al.*, 2019). Importantly, asexual triploids have considerably larger distributional range than sexual diploids (Mráz *et al.*, 2019). Therefore, we suppose that the negative effect of inbreeding in metapopulation like structure during glacial and postglacial colonization could contribute to the observed geographical patterns of sexuals and asexuals. Specifically, we ask the following questions: (i) Is seed-set and germination rate reduced in inbred crosses when compared to outbred crosses? (ii) Have inbred plants lower fitness than outbred plants and is the negative effect increased by the presence of natural competitor?

To answer these questions we performed experimental crosses with the diploid cytotype to produce plants with different levels of inbreeding. Subsets of plants were exposed to environmental stress provided by their natural competitor *Nardus stricta*. Plants were subsequently assessed for 11 fitness and performance traits in early ontogenetic stages (e.g. seed-set, seed weight, seed germination) and in later ontogenetic stages (e.g. biomass, time to first inflorescence, capitulum size, flowering probability and survival rate).

Materials and Methods

Study species

Hieracium alpinum (Asteraceae) is a perennial herb with an arctic-alpine distribution through Europe. Within the species, two cytotypes with allopatric distribution have been confirmed. Strictly sexual diploids (2n = 2x = 18) occur in the Eastern and Southern Carpathians while asexually reproducing triploids (2n = 3x = 27) occupy the remaining part of the distributional range including Scandinavia, Scotland, Iceland, Greenland, northwest Siberia, northern Ural and high mountain ranges in central Europe (Skawińska 1963, Mráz *et al.*, 2009, Mráz *et al.*, 2019). This geographical distributional is an excellent example of geographical parthenogenesis distributional pattern. *Hieracium alpinum* is strictly self-incompatible, insect-pollinated and produces seeds adapted to long-distance dispersal (Mráz *et al.*, 2009; Chrtek *et al.*, 2018).

Plant material

Mature seeds of *H. alpinum* were collected from several mother plants (= seed families) in four sexual diploid populations from the Eastern Carpathians in 2014 and 2015. Seeds within one seed family can represent either full siblings having only one pollen donor or half-siblings assuming contribution of different pollen donors. Seeds were left for c. one month at room temperature and then stored in the fridge at 4°C to imitate natural conditions. Seeds from 5 seed families of each population were sown in the seedling trays filled with soil mixture (4 parts peat, 2 parts compost and 1 part sand) at the beginning of March 2015. At the end of April 2015, 10 plants per seed family were transferred into c. 0.2 L pots and grown in the experimental field of the Institute of Botany in Průhonice, Czech Republic.

Our original experimental design included two large and two small isolated populations of *H. alpinum;* i) H57 (Romania, Mt. Pietrosul Broştenilor, 47.3847 N, 25.5363 E) – relatively small and isolated population within one very short mountain range composed of several hundreds of individuals; ii) H67 (Ukraine, Mt. Pikuï, 48.8253 N, 22.9908 E) is an extremely small population (up to 100 individuals in total, but regularly flowering c. 30-40 individuals only) and the isolated population at the very diploid cytotype‘s range margin; iii) C1 (Romania, Mt. Vărful Musceta, 47.5806 N, 24.8019 E) and iv) H63 (Ukraine, Mt. Blyznytsya, 48.2217 N, 24.2333 E), both forming large continuous metapopulations spreading throughout several tens of kilometres consisted of many thousands / millions of individuals) to assess putative differences in inbreeding depression between small and large populations. However, due to extremely low germination rate resulting in the insufficient number of parental plants, the population H67 had to be completely excluded from the experiment, while the population H57 was used in interpopulation outbred crosses only. We can hypothesize that low germination of seeds originating from these two small populations could be caused by strong inbreeding depression, especially in H67. Indeed, extremely low intrapopulation diversity assessed with AFLPs markers was found in H67 (Hartmann et al., 2017, Supplementary data: Table S2) but not in H57. In total, we cultivated 200 plants which were dunged 3 times at 3-week intervals using Kristalon Gold fertilizer (AGRO CS a.s., Czech Republic).

Experimental crosses

Experimental crosses were performed in July and August 2015 in the experimental field of the Institute of Botany in Průhonice, Czech Republic. Capitula were isolated by nylon bags before and during flowering to prevent uncontrolled cross-pollination. Artificial pollination was performed by rubbing two capitula of two parental individuals together twice a day during the flowering period (usually 4-6 days). Isolated capitula of 10 plants were left as a control to verify strict self-incompatibility in this species. We performed three types of crosses: (i) between the progeny of the same seed family (inbred cross); (ii) between the progeny of different seed families originated within the same population (outbred cross); and finally, (iii)

between the progeny from seed families originated from two different populations (interpopulation outbred cross). From 200 cultivated plants, 154 plants were used in experimental crosses. After experimental hybridization, capitula were kept in the nylon bags until harvesting of mature capitula into paper bags.

Cross-compatibility and seed germination

We estimated seed-set as proportion of well-developed seeds from all seeds per capitulum. Well-developed seeds were full (inspected by dissecting needle) and their seed coat was dark-coloured. Subsequently, we weighed 10 randomly chosen well-developed seeds per capitulum using Sartorius analytical balance (EP 2255m-DR) with a precision of 0.1 mg. Seed samples were then stored in the fridge (4°C) until early spring 2016. In February 2016, 20 to 50 well-developed seeds per capitulum were sown in Petri dishes with soaked filter paper. Seeds were germinated in the growth chamber (PANASONIC MLR-352-PE, Osaka, Japan) with 12-hour photoperiod and 24/12°C day/night temperature. The number of germinated seeds was counted every second day and seedlings were regularly transferred to seedling trays in the greenhouse. The germination experiment finished after 2 months when germination curve achieved a plateau phase. Subsequently, we estimated three parameters related to germination. (i) Germination rate was calculated as a ratio of germinated seeds to the total number of sown seeds. (ii) Germination rate index (Al-Mudaris, 1998), which reflects both the germination speed and the germination rate, calculated using following formula GRI = G1/1 + G2/2 + Gx/x where G1 stands for germination rate in the first day x100, G2 stands for germination rate in the second day x100 etc. (iii) Seed dormancy was expressed as proportion of dormant seeds (viable but not germinated) at the end of the germination experiment.

Cultivation experiment

After 10 weeks seedlings of c. 5cm height was transferred from seedling trays to 0.2 L pots filled with the same soil substrate. A subset of the plants from those crosses with sufficient progeny were cultivated together with seedlings of *Nardus stricta* (Planta naturalis, www.plantanaturalis.com, Czech Republic) under the same conditions as control plants. Appropriate light conditions were provided by adding artificial lighting using LumiGrow Pro 325 (Lumigrow, Emeryville, California, USA) for 12 hours. All plants were dunged at two-week intervals using Kristalon Gold fertilizer to enhance flowering**.** Because of fungus gnats, aphids and thrips infestation, all experimental plants were treated with biological control agents (nematode *Steinernema feltiae*, mite *Amblyseius cucumeris*, and wasp *Aphidius colemani*) provided by Biocont Laboratory (Modřice, Czech Republic).

In total, we used 366 plants in the experiment. Of these, 184 were assessed in the inbreeding treatment (72 outbred, 68 inbred and 44 population outbred plants) and 182 were involved in inbreeding x competition treatment (71 outbred, 63 inbred and 48 population outbred plants). All experimental plants were randomized every two weeks to provide them with the same cultivation condition. Initial biomass of freshly potted seedlings was expressed as the product of number of rosette leaves and measured length and width of the longest leaf of each plant [number of rosette leaves × π × (length of the longest/2) × (width of the longest/2)]. The product of these traits was a reliable proxy for biomass (Pearson correlation test: N=20, R^2=0.795, P<0.001). After five months from the beginning of the experiment, final biomass was measured in the same way as initial biomass. From initial and final biomass, relative growth rate (RGR) was calculated [RGR=log(final biomass)-log(initial biomass)/t, where t is time in days between two measurements]. We measured also two generative traits, the time to the first inflorescence expressed as the number of days from sowing, and capitulum size expressed as the total number of florets per capitulum. Survival rate was measured at the end of March 2017 when the experiment finished.

Statistical analyses

Statistical analyses were performed using linear mixed-effect models (LMM) with the following response variables: seed weight, germination rate index, initial biomass, final biomass and relative growth rate. Generalized mixed-effect models (GLM) with binomial distribution were used for seed-set, germination rate, flowering probability and survival rate. A zero-inflated linear mixed effect model with Poisson distribution was used to analyze seed dormancy. The models testing the differences in the seed-set and seed weight among the different types of cross (i.e. inbred, outbred, interpopulation outbred) were fixed factors, while maternal seed family and paternal pollen family, both nested in particular cross pair, were used as random factors. The same random structure was applied in the models testing the effect of type of cross (fixed effect) on germination rate, germination rate index and seed dormancy (response variables). In the models testing the effect of competition and type of cross (fixed factors) and their interactions on initial biomass, final biomass, relative growth rate, flowering probability and survival (response variables), particular cross pair was used as a random factor. The analyses were performed using the 'lmer' and 'glmer' functions in the lme4 package (Bates et al., 2015) and 'zeroinfl' function in the pscl package (Jackman, 2017) within the R environment (R Team, 2018). The statistical significance of terms was inferred using a likelihood ratio test (LRT) which compared a fully fitted model to the model from which the tested term was removed. To test the possible influence of mother plant's fitness on resulted progeny (Wolfe, 1993; Picó et al., 2003), we performed Pearson correlation tests between seed weight and initial and final biomass. Finally, we used ANOVA, function 'aov' in R, to test the differences among treatments

in those traits which had limited number of observations, i.e. the number of days to first inflorescence and capitulum size, since the random structure could not be applied. Tukey multiple comparison post-hoc tests were performed to differentiate between different levels of predictors. All plots were constructed in Statistica 12 (StatSoft. Inc.).

Results

Effect of biparental inbreeding on cross-compatibility and fitness in early ontogenetic stages

Ten isolated capitula, which were isolated by nylon mesh and served as a control, did not set any viable seeds. The type of cross had no statistically significant effect on seed-set, seed weight, germination rate, germination rate index and seed dormancy (Table 1, Figure S1).

Effect of biparental inbreeding on fitness traits

From seven measured fitness traits, we found statistically significant effects of the type of cross only on initial biomass and capitulum size (Table 2, Figs. 1 and,S2). Inbred plants had significantly lower (9.6%) initial biomass than outbred plants. Outbred plants had significantly larger capitula than interpopulation outbred ones, but inbred plants did not differ from either. Final biomass was reduced by 17% and survival rate by 11.5% in inbred progeny compared to both types of outbred progeny (Table 2, Figs. 2, 3). There was no correlation of seed weight with initial biomass ($R_=$-0.01, P=0.9) or final biomass (R=0.05, P=0.64).

Effect of interspecific competition on the extent of inbreeding depression in *Hieracium alpinum*

Interspecific competition with *Nardus stricta* strongly reduced relative growth rate, final biomass, flowering probability and survival rate equally in all three types of progeny (Table 2, Figs. 2, 3, S3 and S4).

Effect of interspecific competition on the extent of inbreeding depression in *Hieracium alpinum*

Interspecific competition with *Nardus stricta* strongly reduced relative growth rate, final biomass, flowering probability and survival rate equally in all three types of progeny (Table 2, Figs. 2, 3, S3 and S4).

Table 1 Results of LMM, GLM and ZEROINFL models testing the effect of type of cross (inbred, outbred, interpopulation outbred) on reproductive output and four seed traits in diploid cytotype of *Hieracium alpinum*. Maternal seed family, paternal pollen family, both nested in particular cross pair were used as random factors. Numbers are χ^2 values obtained from LRT tests. Type of cross had no statistically significant effect on any of the measured variable.

	Seedset	Seed weight	Germination rate	Germination rate index	Seed dormancy
Model used	GLM	LMM	GLM	LMM	ZEROINFL
Type of cross	2.8	4.06	0.96	4.17	3.19
Residual degrees of freedom	149	41	139	144	136

Table 2 Results of one way ANOVA, LMM and GLM models with logit link function testing the effect of type of cross (inbred, outbred, interpopulation outbred), competition treatment and their interaction on several fitness parameters in diploid cytotype of *Hieracium alpinum*. The parental cross was treated as a random factor in LMM and GLM models. Numbers are either χ^2 values obtained from LRT tests (LMM and GLM) or F values (ANOVA).

	Initial biomass	Final biomass	Relative growth rate	Time to first inflorescence	Capitulum size	Flowering probability	Survival rate
Model used	LMM	LMM	LMM	ANOVA	ANOVA	GLM	GLM
Type of cross	3.67*	8.4+	7.48	1.51	3.41*	7	8.08+
Competition treatment	NA	52.96***	79.98***	NT	NT	7.86*	28.36***
Type of cross : Competition treatment	NT	2.25	0.84	NT	NT	<0.01	0.99
Residual degrees of freedom	366	362	362	28	30	245	366

$^{+}$P<0.1; *P < 0.05; **P < 0.01; ***P < 0.001; NA – not applied, NT – not tested

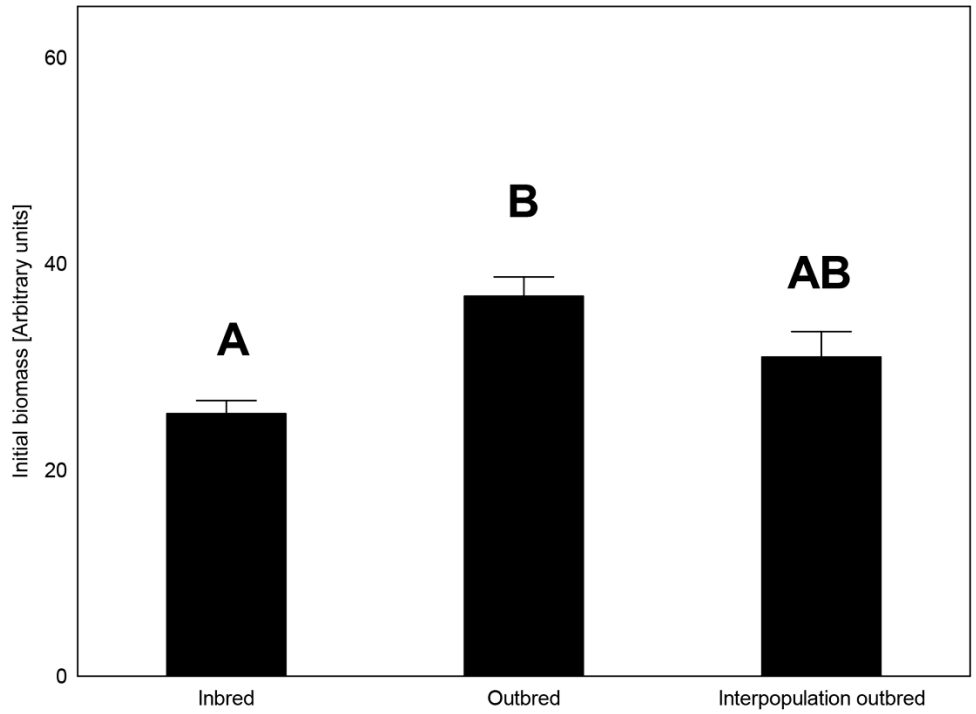

Fig. 1 Effect of type of cross (inbred, outbred, interpopulation outbred) on initial biomass (mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories based on Tukey's post hoc tests (at significance level P=0.05).

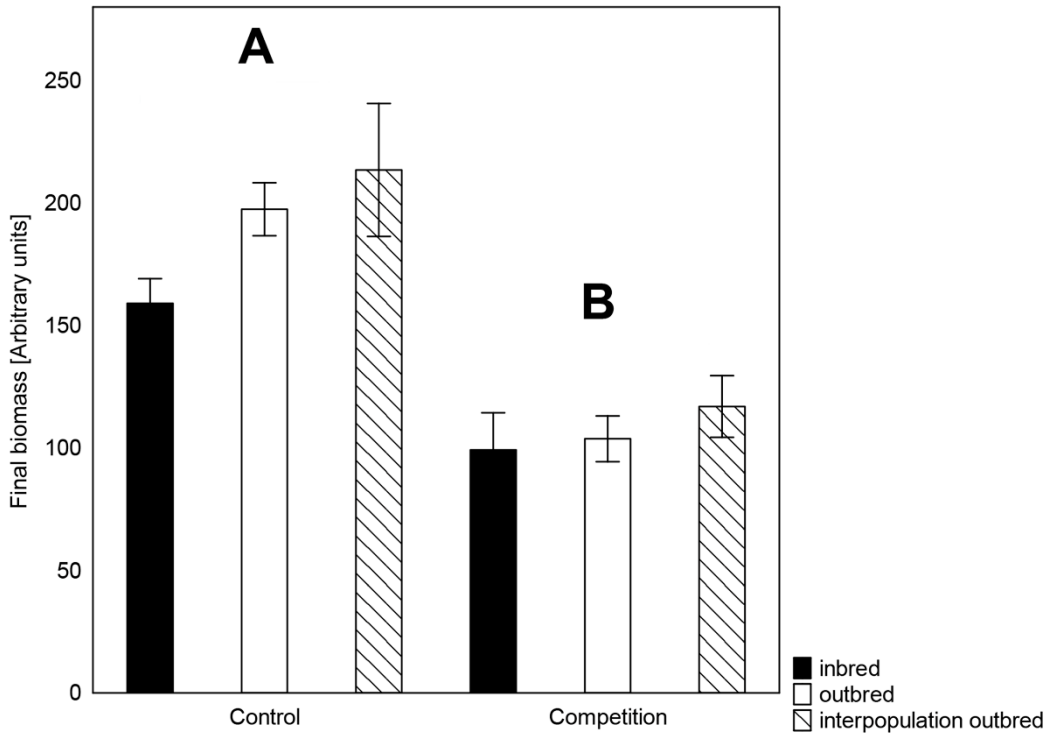

Fig. 2: Effect of interspecific competition and type of cross (inbred, outbred, interpopulation outbred) on final biomass (mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories (at significance level P=0.05).

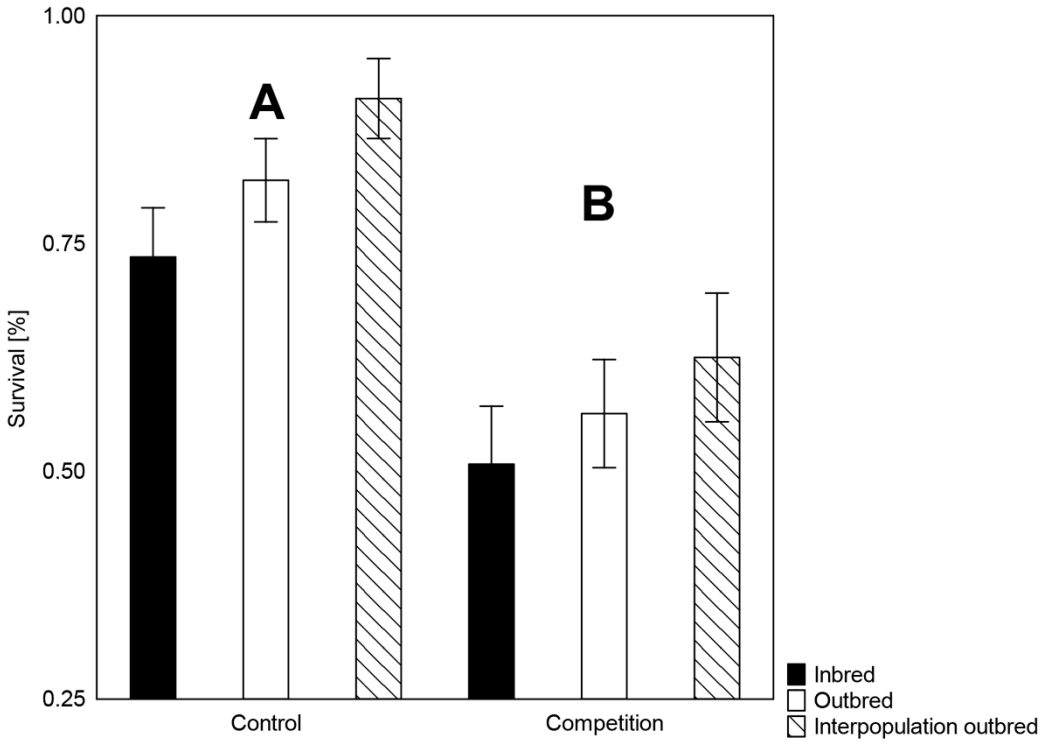

Fig. 3: Effect of interspecific competition and type of cross (inbred, outbred, interpopulation outbred) on survival (mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories (at significance level P=0.05).

Discussion

Inbreeding in strict outbreeding species can negatively affect fitness in different stages of a plant's lifecycle (Dudash et al., 1997; Cheptou et al., 2000; Picó and Koubek, 2003; Rosche et al., 2017). Firstly, seed production can be substantially lowered or completely suppressed by sharing the same (i.e. incompatible) S-alleles hampering successful fertilization (Bartlewicz et al., 2015; Rosche et al., 2017). Secondly, the performance of inbred lineages can be reduced due to the expression of recessive alleles (inbreeding depression) (Charlesworth and Willis, 2009).

Effect of biparental inbreeding on cross-compatibility

Since *Hieracium alpinum* exhibits strict sporophytic self-incompatibility (Chrtek 1997; Mráz 2003) which is the predominant system in Asteraceae (de Nettancourt 2001), we hypothesized that seed-set (i.e. the percentage of well-developed seeds in capitulum) in diploid *H. alpinum*, would be strongly reduced in inbred crosses compared to outbred ones because of

substantially higher incidence of sharing the same S-alleles originated from closely related parents, as demonstrated for example in *Centaurea stoebe* (Rosche *et al.*, 2017) or *Rutidosis leptorrhynchoides* (Young and Pickup, 2010).

Despite our expectations, the seed-set did not differ between outbred and inbred crosses in *H. alpinum*. Our results might, therefore, suggest that the relationship between S-alleles in this species might not be strictly co-dominant since in that case the cross-fertilization between full or half-siblings will be completely hampered because of sharing of at least one of the two S-alleles present in both parental individuals (De Nettancourt, 2001). In contrast, in the case of the dominant or partially dominant relationship between S-alleles, the dominant S-allele will suppress the expression of the recessive one, which in turn will lead to successful fertilization (Thompson and Taylor, 1966; Sampson, 1967). Such dominant or partially dominant mechanism has been found in plants with sporophytic SI systems such as *Arabidopsis lyrata* (Mable *et al.*, 2003), *Brassica campestris* (Hatakeyama *et al.*, 1998), *Brassica oleracea* (Ockendon, 1975) and *Senecio squalidus* (Brennan *et al.*, 2006). Nason (1995) furthermore showed that seed-set was not correlated with the level of inbreeding in self-incompatible *Raphanus sativus*.

Effect of biparental inbreeding on fitness traits

We found a negative effect of inbreeding in three (initial biomass, final biomass and survival rate) out of ten measured fitness traits in later ontogenetic stages but not in early ontogenetic stages. Similarly, other studies in Asteraceae have shown delayed inbreeding depression effects. Picó and Koubek (2003) showed that inbreeding in the perennial herb *Leontodon autumnalis* negatively affected primarily traits in later ontogenetic stages such as growth rate and biomass at flowering. Cheptou et al., (2000) also found in perennial *Crepis sancta* that inbreeding negatively influenced the traits of later ontogenetic stages (such as survival or number of capitula) to a considerably larger extent than traits of earlier ontogenetic stages (such as seed production and germination).

Husband and Schemske (1996) showed a bimodality of negative effects of biparental inbreeding in angiosperms during their life cycles, in some species inbreeding depression was expressed mostly at early ontogenetic stages, while in other species it was delayed. *Hieracium alpinum* belongs to the second group of plants as we did not find a significantly negative effect of inbreeding on traits of early ontogenetic stages but only later ontogenetic stages. Therefore,

our data might suggest that negative effects of inbreeding are likely caused by weakly deleterious mutations, which can be difficult to purge, in contrast to strong deleterious mutations which are supposedly purged in earlier ontogenetic stages (cf. Husband and Schemske, 1996). Consequently, this means that rather weak or moderate negative effects of inbreeding recorded in the first generation of inbred progeny of *H. alpinum* could accumulate and increase over subsequent generations.

The values of traits related to early ontogenetic stages, such as seed weight and seed germination were not reduced in inbred lineages when compared to the outbred lineages, similarly in several fitness traits related to later ontogenetic stages like relative growth rate, time to first inflorescence and flowering probability. The lack of effect of inbreeding on these traits could be explained by several mutually non-exclusive phenomena. Firstly, we can hypothesize that inbreeding depression was marginal and recordable only in some traits because we use only one generation of inbred crosses; it is well known that inbreeding depression is increasing with subsequent generations of inbreeding due to increasing level of homozygosity (Dudash et al., 1997). Secondly, early ontogenetic stages could be affected by the maternal effect, as demonstrated by Wolfe (1993) who showed that variable seed sizes influenced seedlings growth in *Hydrophyllum appendiculatum* and thus may postpone inbreeding depression. In our case, however, the maternal plants were cultivated under the same conditions and therefore this effect, if present from previous generation(s), should be small in agreement with a very weak correlation between seed weight and biomass parameters. Lastly, the weak effect of inbreeding could be eventually caused by using already inbred populations with purged deleterious alleles, as demonstrated for example in perennials such as *Brachypodium sylvaticum* (Marchini et al., 2015),*Senecio integrifolius* (Widén, 1993) or *Turnera ulmifolia* (Belaoussoff and Shore 1994). However, populations used in our study were genetically highly variable, as tested using amplified fragment length of polymorphism and three plastid loci (Mráz et al., unpubl. results).

Effect of interspecific competition on the extent of inbreeding depression in *Hieracium alpinum*

Originally, we hypothesized that the negative effects of inbreeding would be greater in the presence of a natural plant competitor than in ideal cultivation conditions. Competitors create more stressful environments by reduced availability of resources such as light or nutrients, and inbreed progeny with presumably lower fitness should react more negatively to that stress than

outbreed progeny (Antonovics, 1968). Several studies have confirmed such pattern, for example, Schmitt and Ehrhardt (1990) demonstrated that negative effects of inbreeding in annual *Impatiens capensis* (Balsaminaceae) were enhanced under interspecific competition, and similarly, Cheptou et al., (2000) found the more pronounced dro in fitness in inbred lines of *Crepis sancta* under natural conditions, i.e. in the presence of interspecific competition, than in inbred lines cultivated in natural conditions alone. However, our results did not confirm these findings, as the competition with *Nardus stricta* significantly similarly reduced fitness traits in all types of progeny. It is possible that the effect of the competitor *N. stricta* was probably too strong and thus could hide subtle differences in fitness traits among the three types of progeny (Table 2). The pronounced effect of inbreeding depression in the presence of competitor as a proxy for a more stressful environment can be population (and/or species?) specific, as demonstrated by Cheptou et al., (2000).

Geographical parthenogenesis in *Hieracium alpinum*

Our results showed that inbreeding depression can occur in *H. alpinum* already in the first generation of inbred crosses. Consequently, according to the metapopulation hypothesis formulated by Haag and Ebert (2004), reduced fitness and loss of heterozygosity caused by inbreeding could disadvantage sexuals against asexuals in metapopulation-like structures and explain GP distribution pattern in *H. alpinum* (Hörandl 2010). In addition to metapopulation hypothesis, other alternative mechanisms underlying GP patterns, like the advantage of uniparental reproduction (Baker 1967; Linder and Barker 2014), the advantage of increased genome dosage (Bierzychudek 1985) and/or heterozygosity due to an (allo-)polyploid state potentially leading to niche shifts (Mau et al., 2015; Kirchheimer et al., 2016; Karunarathne et al., 2018; Paule et al., 2018), general-purpose genotype hypothesis (Lynch 1984), frozen niche variation model (Vrijenhoek 1984), avoidance of minority cytotype disadvantage (Levin 1975; Mártonfiová 2015) or Red Queen hypothesis (van Valen 1973) have been proposed. If we consider that all these processes might influence the distributional patterns in various extent and might interact with each other and the environment, the explanation of GP might be very complex (Hörandl 2006). For example, Mráz et al., (2019) discussed whether triploid apomictic populations of *H. alpinum* has increased and had more stable reproductive success when compared to strictly sexual diploid populations. While reproductive assurance was consistently more stable in apomicts under both natural and pollinator-restricted environment, the level of

seed-set was fairly similar under natural conditions, suggesting strong context-dependency of this trait. However, in the common garden experiment, when there was a low density of compatible sexual plants, like in small founder populations, *Hieracium alpinum* apomicts had considerably higher seed sets than co-occurring sexual diploids (Mráz and Mrázová, unpubl.).

Conclusions

Overall, our results show that diploid, sexually reproducing populations of *Hieracium alpinum* are susceptible to biparental inbreeding. Decreased fitness could reduce the chance of small founder populations to successfully establish and spread further. This decreased fitness could be important especially when compared to asexual triploid populations which, by their nature, are not negatively affected by genetic processes acting in small or isolated populations. The question remains, however, whether this process could contribute, and if so to which extent, to the geographical parthenogenesis observed in this species (Mráz et al., 2009). A reduced colonizing ability due to biparental inbreeding is not the only factor which could underlie the GP distributional patterns in *H. alpinum* (see Introduction). Ideally, the fitness and fate of sexual and asexual populations should be tested under natural conditions in sympatry and over several generations, but this approach is not possible due to an ethical issue as the cytotypes currently grow in strict allopatry. Alternatively, the establishment of artificial populations of contrasting size (e.g. small vs medium) composed of both sexuals and asexuals in the experimental field created outside of the species range but in similar climatic conditions could provide answers related to fitness and demographic trends in both reproductive modes, and importance of underlying mechanisms of GP, including biparental inbreeding.

Acknowledgements

We thank M. Hartmann, V. Mrázová and M. Štefánek for their help with the greenhouse experiment and Tim Rich for language revision. The work was financially supported by the Czech Science Foundation (GAČR grant no. GAČR 14-02858S to PM and 17-11281S to VL) and Grant Agency of Charles University (GAUK 907218). The authors declare no conflicts of interests. Datasets generated and analyzed during the current study are available from the corresponding author on request.

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Supplementary material

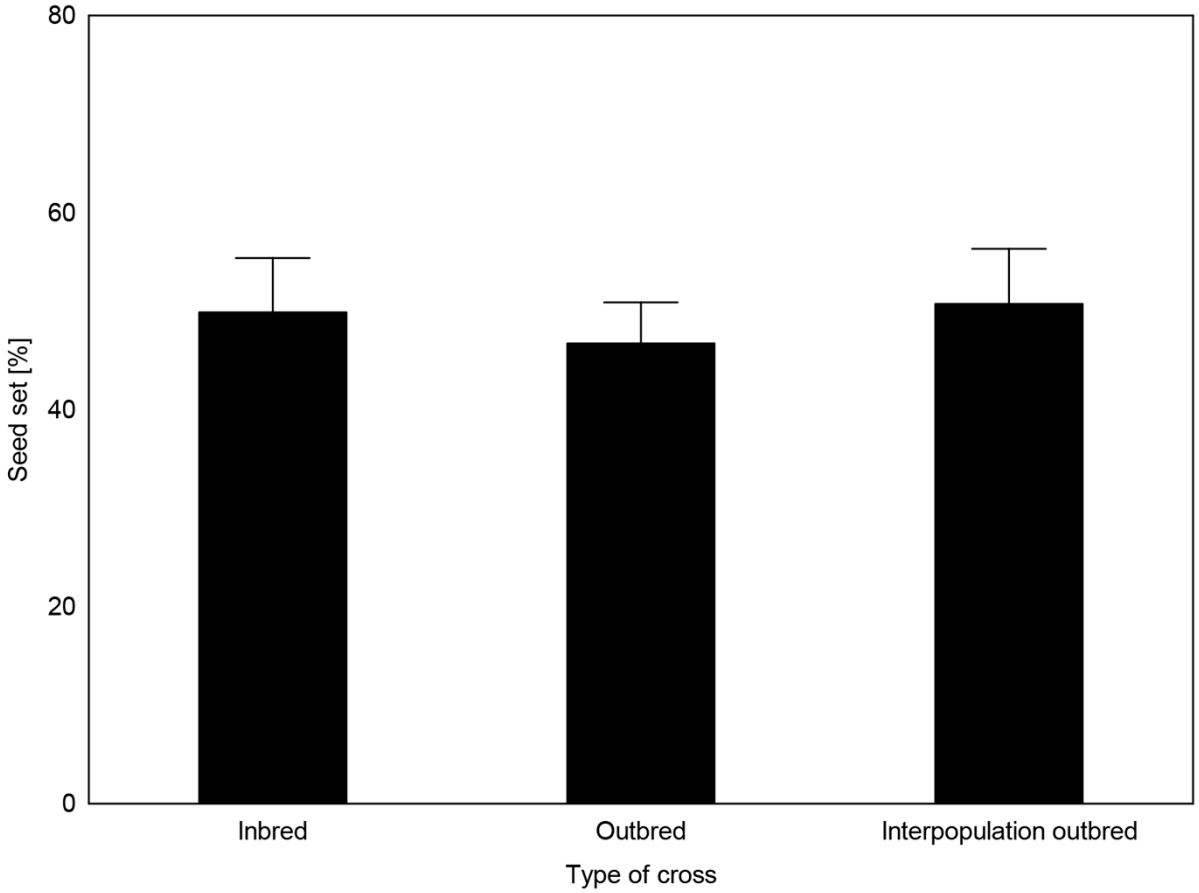

Figure S1: Effect of type of cross (Inbred, Outbred, Interpopulation outbred) on seed-set (mean and standard errors) in diploid cytotype of *Hieracium alpinum*.

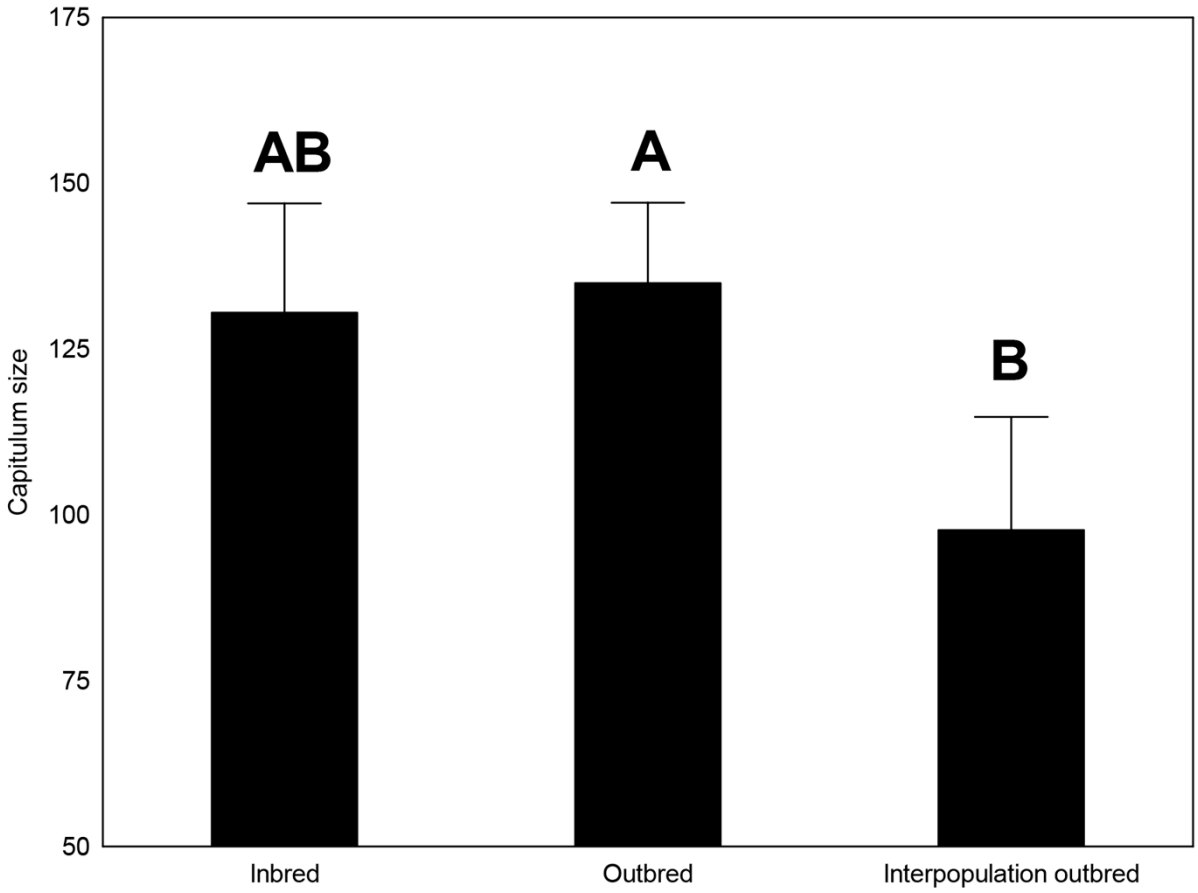

Figure S2: Effect of type of cross on capitulum size (number of flowers per capitulum; mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories (at significance level P=0.05).

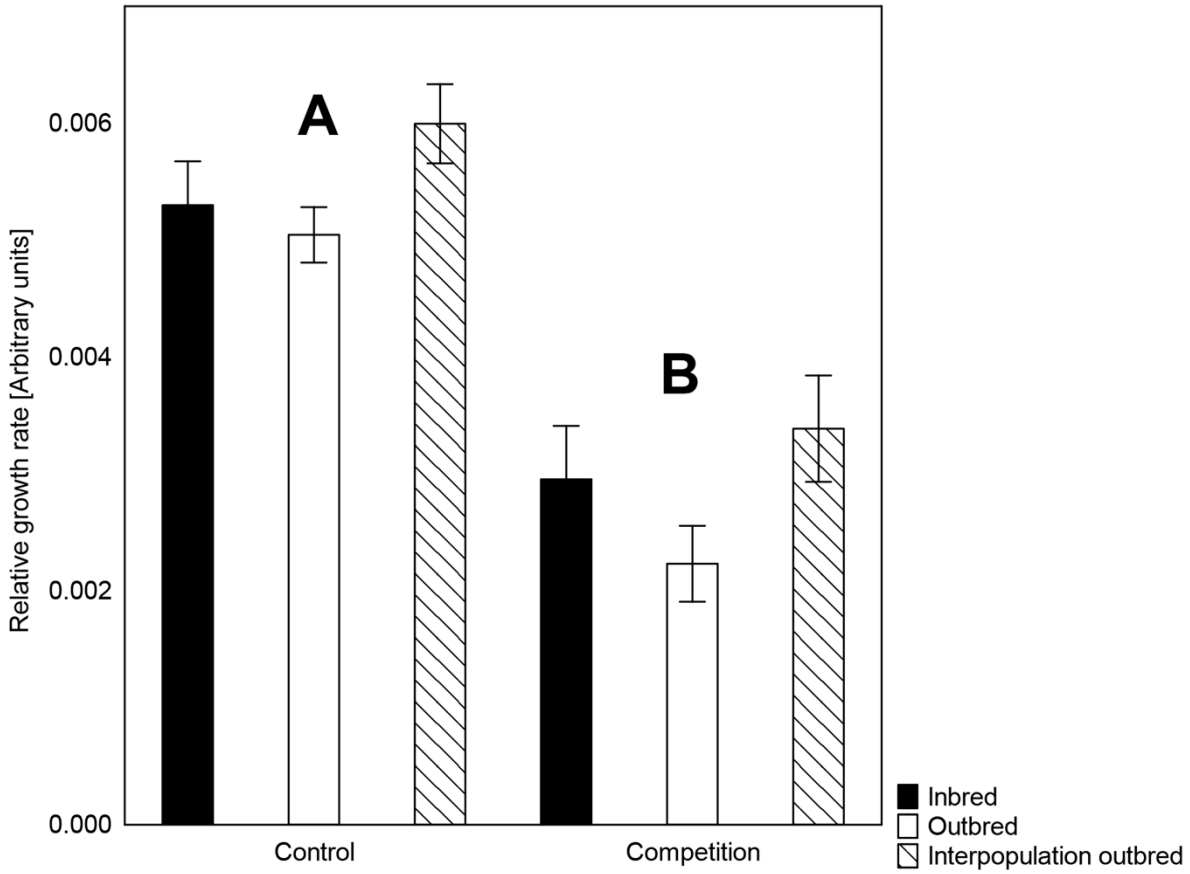

Figure S3: Effect of type of cross and competition treatment on relative growth rate (mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories (at significance level P=0.05).

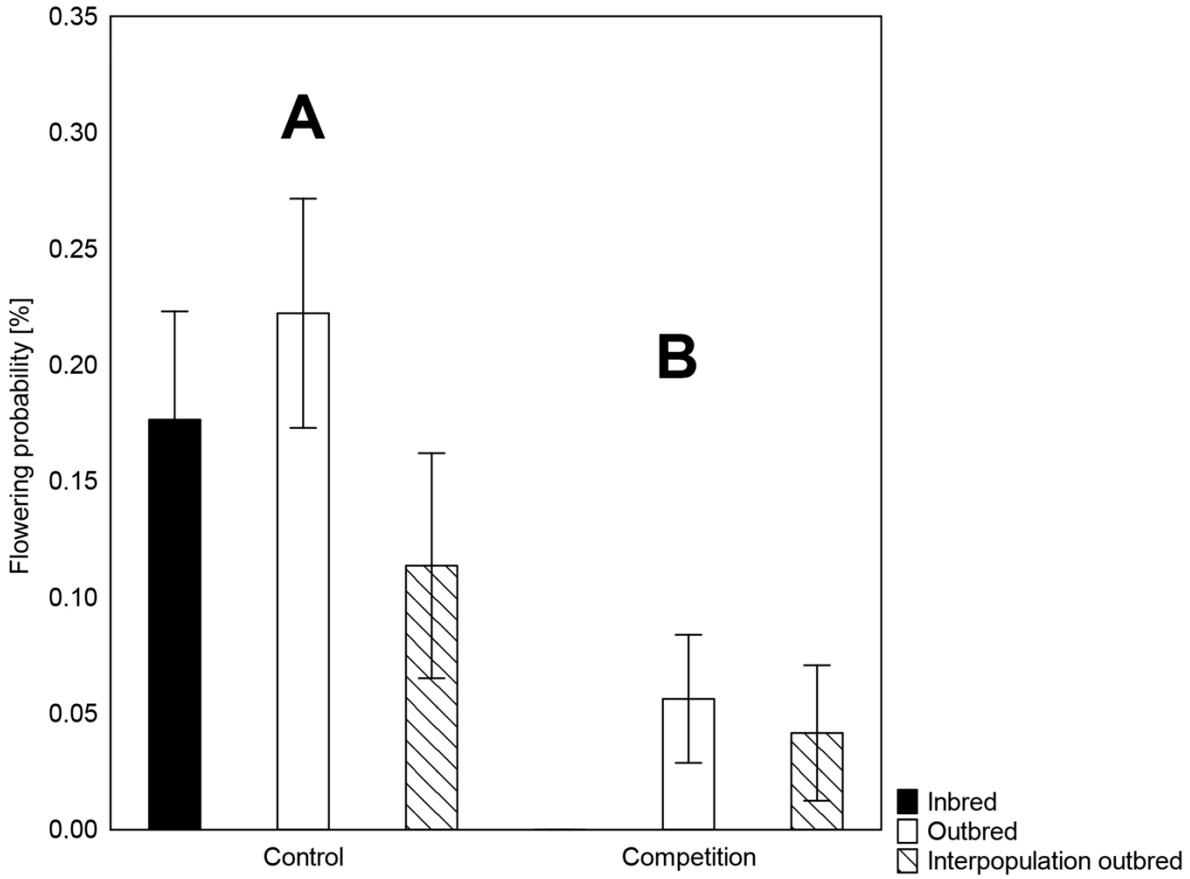

Figure S4: Effect of competition and type of cross on flowering probability (mean and standard error) of diploid cytotype of *Hieracium alpinum*. Different letters indicate statistically significant differences between categories (at significance level P=0.05).

Paper IV:

Testing experimental demethylation in plants: Effect, efficiency and temporal stability

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Keywords: 5-azacytidine, biomass, experimental demethylation, HPLC, leaf carbon content, leaf nitrogen content, methylation, seed dormancy, zebularine

Abstract

Experimental demethylation is an easy, cheap and straightforward method to test the importance of epigenetic variation in evolutionary and ecological processes in plants. Experimental demethylation is achieved by the application of demethylation agent which is indiscriminately incorporated into DNA and causing the lowering of the global level of DNA methylation (%mC). However, little is known about the effect of different application methods, the effect of the total amount of %mC in untreated plants on the efficiency of experimental demethylation and temporal stability of induced changes in %mC.

We compared different application approaches (germinating and spraying leaves with different frequencies and concentrations) and different demethylation agents (5-azacytidine and zebularine). Moreover, we tested the effects of experimental demethylation on seed germination, plant survival, amount of biomass, leaf nitrogen and carbon contents and temporal stability of %mC. Furthermore, we tested if genome size and %mC of untreated plants can affect induced decrease of %mC. Finally, we tested the accuracy of %mC estimation by HPLC.

We found that 50μM solution of 5-azacytidine applied on seeds followed by spraying caused the largest drop of %mC (by 9.9%). In contrast, 50μM zebularine solution applied to germinating seeds caused high mortality of seedlings. Application of demethylation agent on germinating seeds followed by spraying lowered %mC significantly more than only spraying. Furthermore, we found that %mC in demethylated plants increased into the insignificant level without a continuous supply of demethylation agent. The total amount of %mC affects the efficiency of experimental demethylation and averaged accuracy of %mC measurements assessed by HPLC was found to be around 1.03%.

We conclude that different application of demethylation agents causes different outcomes. The highest concentration of 5-azacytidine caused the largest drop of %mC while the same concentration of zebularine was highly toxic to seedlings. The most efficient was an application of demethylation agent on germinating seeds followed by spraying leaves. We showed also that lowering of %mC was not stable in time and DNA without the further supply of demethylation agent remethylated to its original level. HPLC seems to be a relatively accurate and cheap method for %mC estimation in demethylation experiments.

Introduction

A growing body of evidence suggests great importance of epigenetic mechanisms in the adaptability of plants (Verhoeven and Preite 2014). Epigenetic mechanisms consist of several DNA modifications from which, cytidine methylation is one of the most important in plants' environmental responses (González et al*.,* 2016; Herman and Sultan 2016). One of the methods to test the importance of DNA methylation on plants performance is the alternation of DNA methylation using demethylation agents. Experimental demethylation is a simple, cheap and straightforward method and therefore is widely used in ecological and evolutionary studies (Vergeer and Ouborg 2012; González et al., 2016; Latzel et al., 2016; González et al., 2017; Münzbergová et al., 2019). However, different authors dealing with experimental demethylation are using different application methods and different species, which can presumably result in highly variable outcomes (Vergeer and Ouborg 2012; González et al., 2016; Puy et al., 2018; Münzbergová et al., 2019). In the present study, we aim to identify the most efficient method of experimental demethylation, i.e. the method causing the largest decrease in global cytidine methylation (%mC hereafter) in our study system – one clone of alpine hawkweed, *Hieracium alpinum,* and test the stability of induced changes in DNA methylation. Furthermore, we aim to elucidate possible sources of variation in the global level of DNA methylation reported in demethylation studies. We specifically test whether the absolute genome size of treated species and global level of DNA methylation in untreated plants affects the amount of %mC in demethylated plants.

Demethylation agents are small biomolecules, analogues of nucleoside cytidine, which are indiscriminately incorporated into DNA during replication (Pecinka and Liu 2014). The presence of cytidine analogues in DNA strand causes degradation of DNA methyltransferases, enzymes responsible for incorporating methyl groups into DNA (Jones and Taylor 1980; Creusot et al., 1982; Finnegan et al., 1996). As a result, demethylation agents lower the level of methylation of the whole genome i.e. methylome (Griffin et al., 2016). Moreover, if the methylation mark is once erased from DNA, it disables methylation transfer on the newly synthesized DNA strand during DNA replication. Subsequently, cytosine is demethylated in all-new DNA strands originated from the one which was once demethylated (Stresemann and Lyko 2008). However, demethylated DNA can be to some extent methylated back i.e. remethylated, even without the previous epigenetic mark. Yet, studies dealing with remethylation of demethylated DNA in plants are scare and limited only on tissue cultures

(Kumpatla and Hall 1998) except one performed on in vitro cultivated seedlings of *Arabidopsis thaliana* (Baubec et al., 2009).

Among several demethylation agents, 5-azacytidine and zebularine are the most frequently used in experimental studies (Baubec et al., 2009; Griffin et al., 2016). Both agents have the same mechanism of action leading to similar epigenetic changes (Stresemann and Lyko 2008; Champion et al*.,* 2010; Griffin et al., 2016). However, these agents differ in their stability in water solutions. Based on their half-life in standardised conditions (37°C, pH 7), zebularine is almost 73 times more stable than its analogue 5-azacytidine which half-life is only seven hours (Yoo et al., 2004; Stresemann and Lyko 2008). Therefore, zebularine should be more efficient demethylation agent i.e. causing a larger decrease in %mC than its less stable analogue 5-azacytidine. However, comparison studies of 5-azacytidine and zebularine brought inconclusive results (Baubec et al., 2009; Griffin et al., 2016).

Demethylation agents are usually applied directly on germinating seeds when water in Petri dishes is replaced by a solution of particular demethylation agent (Fieldes and Amyot 1999; Vergeer and Ouborg 2012; Puy et al*.,* 2018). However, it is well known that both, 5-azacytidine and zebularine can induce serious problems with root system development leading to dwarfism, reduced biomass and reduced viability of plants in early ontogenetic stages, i.e. germinating seeds and young seedlings (Baubec et al., 2009; Puy et al., 2018). For that reason, it has been suggested an alternative application of demethylation agents by spraying of experimental plants, which can diminish the problems with root system development (González et al., 2016; Puy et al., 2018). Spraying of demethylation agent can be applied also on clonal plants which are not easily reproducing by seeds (Baubec et al., 2009; Puy et al., 2018). Besides the negative impact on seedlings' development, experimental demethylation is known to have an impact on the plant's adaptability suggesting the importance of DNA methylation in plants' environmental response. In detail, experimental demethylation can affect plant performance by reducing stress responses (Verhoeven and van Gurp 2012), transgenerational adaptability (Boyko et al*.,* 2010), phenotypic plasticity (Bossdorf et al., 2010; Gao et al., 2010; Zhang et al., 2013), the manifestation of inbreeding depression (Vergeer and Ouborg 2012), affecting plant phenology and seed or bud dormancy (Ríos et al., 2014).

Application of demethylation agent might lead to the decrease of %mC from 4% to 35 % depending on application method, demethylation agent, exposure time and target species (Table 2). It can be assumed that response to demethylation agent (demethylation efficiency) can differ between species having a different level of %mC in untreated plants or/and different genome size due to simple requirement of different amount of substance (demethylation agent). Indeed,

it has been reported that the genome size and %mC in untreated plants are strongly positively correlated (Alonso et al., 2015). However, the correlation between the efficiency of experimental demethylation and %mC in untreated plants or genome size has never been tested before.

Although experimental demethylation is a commonly used method to study the impact of epigenetic variation on plants performance, studies using demethylation agents often brings highly variable results (Table 2). Moreover, to the best of our knowledge, temporal stability of experimental demethylation was studied only once in a model species *Arabidopsis thaliana* in vitro (Baubec et al., 2009), providing evidence that demethylated DNA can be methylated back i.e. remethylated. The main aim of this study is to determine the best method of experimental demethylation for the alpine hawkweed, *Hieracium alpinum*, and to better understand the sources of variation reported in experimental studies dealing with demethylation approach. *Hieracium alpinum* is suitable species to test these aims, because besides sexually reproducing diploid cytotype (2n = 2x = 18), it contains also triploid cytotype (2n = 3x = 27), which reproduces by obligate apomixis (Mráz et al., 2019). This reproductive pathway, which includes complete circumscription of meiosis and double fecundation, results in the production of genetically identical seed progeny thus providing the material with the same genetic background.

More specifically, we ask following questions: i) Which application method of which demethylation agent reduces global cytidine methylation of clonally reproducing alpine hawkweed, *Hieracium alpinum*, the most, while it has the smallest negative impact on plant viability? ii) Is the induced decrease of the global amount of DNA methylation stable in time or DNA will be remethylated after cessation of application of demethylation agent? iii) Is the efficiency of experimental demethylation, i.e. decrease of the global amount of DNA methylation, species-specific, and if so, is there any association between efficiency of experimental demethylation, genome size and %mC of untreated plants?

To answer these questions we performed germination and greenhouse experiments where seeds and young seedlings of one triploid clone of *Hieracium alpinum* were exposed to two different demethylation agents, i.e., 5-azacytidine and zebularine, which were applied in form of aqueous solutions with different concentrations and frequencies on seeds during their germination stage and seedlings / young plants. In the latter case, we used a spraying approach. The effect of demethylation treatments was measured on several physiological and performance traits, namely germination rate, seedling survival rate, amount of biomass, leaf carbon and

nitrogen contents. The level of global cytidine was measured using high-performance liquid chromatography (HPLC; Rozhon et al., 2008).

Materials and methods:

Origin of plant material

We used apomictically (parthenogenetically) derived seeds of one triploid plant (H82-102) of *Hieracium alpinum* collected by J. Chrtek on Mt. Studničná hora, Krkonoše Mts., Czechia (50.7233ºN, 15.7040º E) in 2014. Triploid level and strict apomixis in a subset of seeds originated from this plant have been confirmed by flow cytometric seed screen (Mráz et al., 2019). The seeds from H82-102 plant were sown in winter 2015 directly into the pot filled up with soil mixture (peat, compost and sand with ratio 4:2:1). Young seedlings were subsequently transferred into in 0.2 L pots filled up with the same soil mixture and cultivated under the same conditions in the experimental greenhouse until autumn 2015 when they were transferred into the experimental garden at the Institute of Botany in Průhonice, The Czech Academy of Sciences. In the next year, we collected the seeds from the cultivated plants. The seeds were subsequently stored for ca 1 month at room temperature and then in the fridge at 4°C until germination experiment (Table 1).

Germination experiment and demethylation treatments

In April 2018 the seeds were sown on Petri dishes of 9 cm in diameter and exposed to different treatments and germinated in a growth chamber (PANASONIC MLR-352-PE, Osaka, Japan) with 12-hour photoperiod and 24/12°C day/night temperature. In summary, 35 seeds were sown per single Petri dish, three Petri dishes were used per one treatment and six different treatments were used during germination experiment. Filter papers on Petri dishes were saturated either by distilled water in the control treatment, or by 5μM, 20 μM, 50 μM, daily refreshed 50 μM solution of zebularine (Sigma-Aldrich, Saint Louis, Missouri, USA), or daily refreshed 50 μM solution of 5-azacytidine (Sigma-Aldrich, Saint Louis, Missouri, USA). Only one concentration

Table 1: Schedule of the germination and cultivation experiments which started on the 26.4 2018 and terminated after 35 weeks on the 2.11 2018

	Week of the experiment																																	
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.	31.	32.	33.	34.	35.
Germination experiment	x	x	x	x																															
Cultivation experiment in seedling trays					x	x	x	x																											
Cultivation experiment in pots									x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Application of demethylation agents on germinating seeds	x	x	x	x																															
Application of demethylation agents on plants by spraying					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x										
Estimation of initial biomass								x																											
Estimation of final biomass																									x										
Collection of leaf material (for DNA extraction)																									x										x

Table 2: Summary of different studies dealing with experimental demethylation using two of the most popular demethylation agents, I .e.5 azacytidine and zebularine, different application methods and different estimation of %mC. G, germination in water solution of demethylation agent; S, spraying by water solution of demethylation agent; H, hydroponic cultivation using water solution of demethylation agent; HPLC, high-performance liquid chromatography; TLC, two-dimensional thin-layer chromatography; MSAP, methyl-sensitive amplification polymorphism; MDQK, colorimetric methylated DNA quantification kit. (NE) not estimated; (a) Schmuths et al., 2004; (b) Chrtek et al., 2009; (c) Pustahija et al., 2013; (d) Bennett and Smith 1976; (e) Temsch and Greilhubert 2010; (f) Kirschner et al., 2013; (g) Baubec et al., 2009; (h) Sano et al., 1990; (i) Vergeer and Ouborg 2012; (j) Puy et al., 2017; (k) González et al., 2016; (l) Horváth et al.,. 2003; (m) Sano et al., 1989.

species	genome size in pg (reference)	demethylation agent	application method	%mC estimation method	averaged %mC in untreated plants	decrease of %mC (relative value)
Arabidopsis thaliana	2C = 0.89[a]	Zebularine	G	HPLC	6.2[g]	35.5[g]
Arabidopsis thaliana	2C = 0.89[a]	5-azacytidine	G	HPLC	6.2[g]	22.6[g]
Hieracium alpinum	2C= 11.87[b]	5-azacytidine	G+S	HPLC	32.5	9.9
Hieracium alpinum	2C= 11.87[b]	Zebularine	G+S	HPLC	32.5	3.8
Medicago sativa	2C = 3.44[c]	Zebularine	G	HPLC	20.6[g]	14.6[g]
Oryza sativa	2C= 1[d]	5-azacytidine	G	TLC	14[h]	16[h]
Scabiosa ochroleuca	2C= 2.1[e]	5-azacytidine	G	MSAP	42[i]	11[i]
Taraxacum brevicorniculatum	2C = 3.63[f]	5-azacytidine	G	MDQK	4.7[j]	34[j]
Taraxacum brevicorniculatum	2C = 3.63[f]	5-azacytidine	S	MDQK	4.7[j]	21[j]
Trifolium repens	2C = 2.12[c]	5-azacytidine	S	HPLC	31.1[k]	4.5[k]
Triticum aestivum	2C= 34.6[d]	5-azacytidine	G+H	HPLC	30.7[l]	23.3[l]
Zea mays	2C= 5.5[d]	5-azacytidine	G	TLC	NE	8[m]

of 5-azacytidine was used because of its supposedly low stability in water solution (Stresemann and Lyko 2008; Baubec et al., 2009). After four weeks (Table 1), the germination rate (percentage of germinated seeds of all sown seeds) was assessed. Subsequently, germinated seedlings were transferred into seedling trays with 2×2 cm large cells filled with soil mixture (peat, compost and sand in ratio 4:2:1). Stability of zebularine in 50 μM water solution was verified using high-performance liquid chromatography (HPLC). Zebularine solution in Petri dish was placed in the cultivation chamber with the same settings as for the germinating seeds (see below). 1 ml of the zebularine solution was collected after 12, 24, 48, 72 and 96 hours in the Eppendorf tube and stored in -20°C until measuring by HPLC (Table S1).

Cultivation experiment and demethylation treatments

Seedlings in seedling trays were sprayed by water, zebularine or 5-azacytidine solutions according to a particular treatment in three different time intervals: once in two weeks (f1), once a week (f2) or twice a week (Monday and Thursday, f3). Control plants were sprayed twice a week by water instead of water solution of demethylation agent. After four weeks of treatment, the survival rate was assessed and 15 randomly chosen seedlings from each treatment were transferred from seedling trays into the 0.2 l pots filled with the same soil mixture as given above (Table 1). Because of high mortality of seedlings in seedling trays (79%) treated with 50 μM zebularine, they were replaced by back up plants from the control treatment (previously spraying with water), and were subsequently sprayed according to the particular treatment (50 μM zebularine), similarly to the remaining plants from other treatments (Table 1). At this step of the experiment, initial biomass of all transplanted seedlings was determined as a product of following traits: the number of rosette leaves (N), length (L) and width (W) of the longest leaf using formula $N \times \pi \times (L/2) \times (W/2)$. This parameter is strongly correlated with real dry biomass (Pearson correlation test: N=20, R^2=0.795, P<0.001, Pinc unpubl.). Position of plants in the experimental greenhouse was randomized in two weeks intervals during the experiment. Two biocontrol agents, i.e. a mite *Amblyseius cucumeris* and a wasp *Aphidius colemani* (Biocont Laboratory, Modřice, Czechia), were applied to eliminate spider mites, thrips and aphids and all cultivated plants were fertilised in 2 weeks interval by commercial fertiliser (Kristalon Gold fertilizer, AGRO CS a.s., Czech Republic). After 17 weeks of greenhouse cultivation, application of demethylation agents was terminated, final biomass was estimated in the same way as initial biomass and plants were further cultivated to estimate temporal stability of experimental demethylation (Table 1).

Estimation of demethylation efficiency

To estimate demethylation efficiency after 17 weeks of greenhouse cultivation, we sampled two to three youngest fully developed leaves from all cultivated plants into silicagel (Table 1). After further ten weeks without application of demethylation agent, another two to three youngest fully developed leaves from all plants were sampled into silicagel to assess %mC (Table 1). Off these samples, we randomly selected five samples from each treatment collected at the cessation of demethylation treatment to measure %mC. To test temporal stability of experimental demethylation, %mC was measured in the same plants twice, for the first time at the cessation of demethylation treatment and for the second time after further 10 weeks without any treatment. This procedure was done in the plants from 50 µM 5-azacytidine f3 (sprayed twice a week) treatment, in which the most pronounced decrease of %mC was recorded at the cessation of the application of demethylation agents (Table 1). The same has been done with five randomly selected plants from the 5 µM zebularine treatment which were used as a control. Originally, we planned to use true control plants, i.e. those treated by water only, but we were not able to collect sufficient amount of leaf material for DNA extraction from them at the end of the experiment, i.e. 35 weeks after sowing. Importantly, %mC level of plants in the 5 µM zebularine treatment did not significantly differ from the control treatment (Fig. 1). DNA from silicagel dried leaf samples was extracted by sorbitol extraction with minor modifications (Štorchová et al., 2000). In contrast to the original protocol, we used 8 µl of RNase A (10 $mg.mL^{-1}$, Biogen, Cambridge, USA) and we repeated chloroform-isopropylalkohol extraction twice to ensure higher purity of isolated DNA. Extracted DNA was diluted in water and its concentration was measured by Qubit dsDNA high-sensitivity assays (Thermo Fisher Scientific, Massachusetts, USA). DNA was subsequently digested according to Rozhon et al. (2008); 1 µg of DNA diluted in 44 µl of water was mixed with 5 µl of 10× digestion buffer (200 mM acetic acid, 200 mM glycine, 50 mM $MgCl_2$,5 mM ZnCl2, and 2 mM $CaCl_2$ adjusted with NaOH to pH 5.3) and 1µl of nuclease mix (2.5 U/ml nuclease P1 (Sigma, St. Louis, MO, USA) and 500 U/ml DNase I in 50% glycerol) and incubated at 37°C overnight. Then, 5 µl of 100 mM NaOH and 1µl of calf intestine alkaline phosphatase (1 U/µl) was added and the mixture was incubated at 37°C for further 12-24 hours. Digested samples were diluted with 44 µl of 12 mM HCl and analysed using HPLC system equipped with a 125 · 4 mm Nucleosil 100-10 SA column (Macherey-Nagel, http://www.macherey-nagel.com/)

Reproducibility of HPLC assessment of %mC was verified in two ways; (i) the products of hydrolysis of 17 samples were analysed chromatographically two times to test the accuracy of HPLC measurement, and (ii) DNA extraction and hydrolysis were done two times independently in five samples to test for accuracy of both, sample preparation and HPLC measurement. In each pair of measurements, we determine absolute deviation and coefficient of variation, i.e. relative standard deviation calculated, as SD/mean × 100 according to Rozhon et al. (2008).

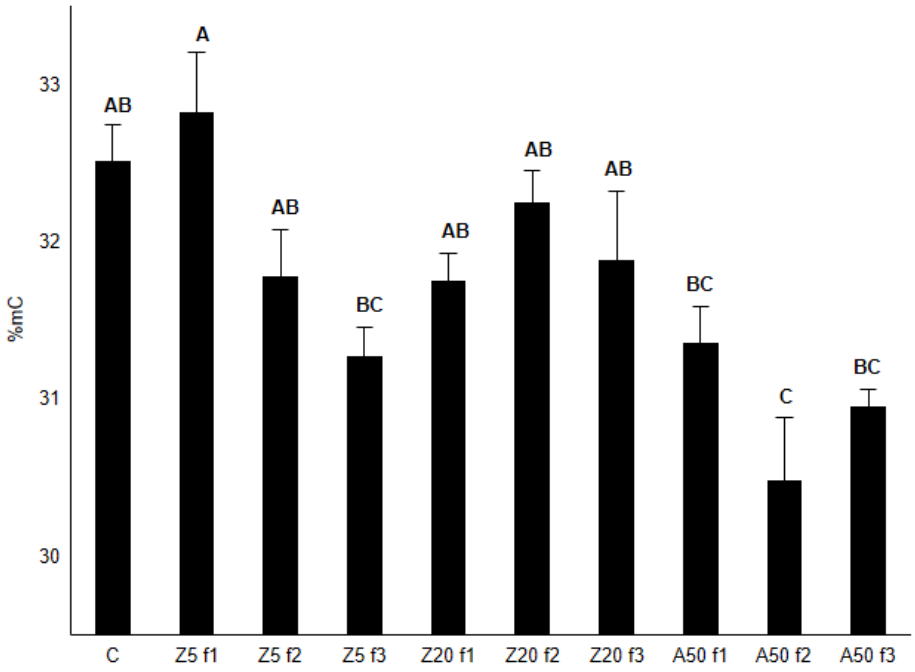

Fig. 1: The effect of different demethylation treatments on a global amount of methylated cytidine (%mC) in leaves of *Hieracium alpinum* collected at the cessation of demethylation treatment, after 25 weeks from sowing. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling and young plants for subsequent 21 weeks was applied. Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level $P \leq 0.05$). (C) control plants; (Z5) 5 µM zebularine solution; (Z20) 20 µM zebularine solution; (A50) 50 µM 5-azacytidine solution; (f1) spraying once in two weeks; (f2) spraying once a week; (f3) spraying twice a week.

Foliar nitrogen and foliar carbon assessment

Because we found a larger amount of biomass in plants treated with higher concentrations of zebularine and 5-azacytidine (Fig. 2) when compared to lower concentrations of zebularine and control plants, we tested whether demethylation agents could serve as additional nitrogen source of plants. Alternatively, no positive association might suggest the direct effect of demethylation agents through the alternation of the expression of genes involved in plant growth. To assess foliar nitrogen and carbon contents, we used silicagel dried leaf samples as for DNA methylation study, but only for those plants for which we had sufficient amount of silicagel-dried material, i.e. at least 3 mg of dried mass. These samples were homogenised using a mixer mill (MM 400, Retsch, Germany) in 2 ml Eppendorf tubes and ca 2 mg of material per sample were weighed into tin capsules using Metler Toledo MX5 scales. The samples were analysed by mass spectrophotometer (FlashSmart Elementar Analyser, Thermo Scientific, Bremen, Germany) in the Center for Stable Isotope Research of the Charles University (Prague, Czechia). The analytical precision was ± 0.01%.

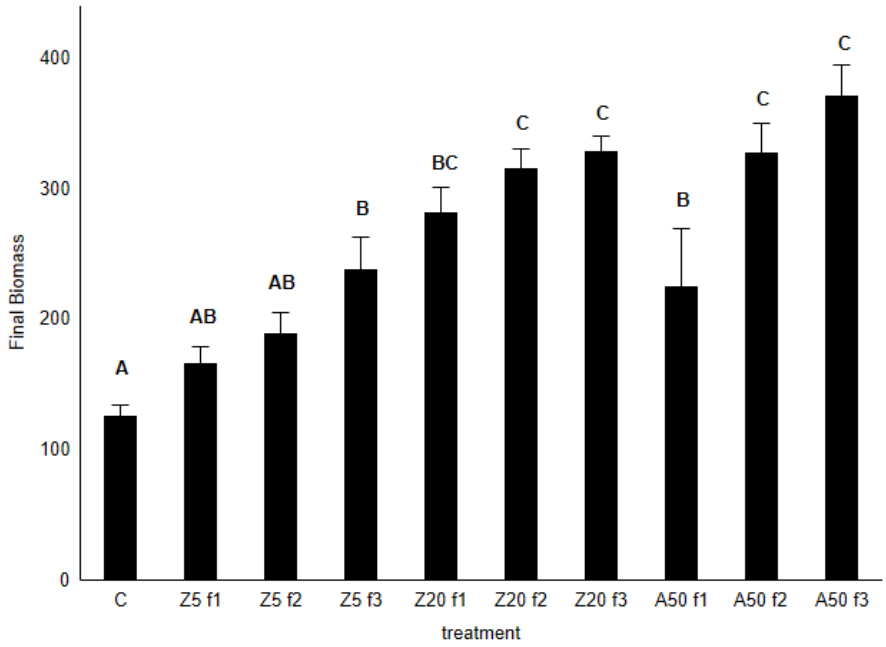

Fig. 2: The effect of different demethylation treatments on the final biomass of *Hieracium alpinum* at the cessation of demethylation treatment, after 25 weeks from sowing. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling and young plants for subsequent 21 weeks was applied. Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level $P \leq 0.05$). (C) control plants; (Z5) 5 µM zebularine solution; (Z20) 20 µM zebularine solution; (A50) 50 µM 5-azacytidine solution; (f1) spraying once in 2 weeks; (f2) spraying once a week; (f3) spraying twice a week.

Statistical analysis

To test the effect of a particular treatment (defined as a combination of demethylation agent, concentration and spraying frequency; in total 12 different demethylation treatments plus control treatment) on germination rate, the survival rate of seedlings in seedling trays, amount of initial biomass, amount of final biomass, %mC (at the cessation of demethylation agent application), leaf nitrogen and carbon contents (all response variables), we used one-way ANOVA. The temporal stability of induced changes in %mC was also tested by ANOVA using treatment (demethylation versus control) and sampling time (at cessation of demethylation agent application versus after 10 weeks after this cessation) as a fixed factor and %mC as the response variable. If the effect was significant, we performed a posthoc Tukey test to identify which levels of predictors differ significantly at $P \leq 0.05$. Multiple regression was performed to test the effect of genome size and %mC level of untreated plants on demethylation efficiency (expressed as a decrease of %mC). Statistical analyses and plots were performed using Statistica 12 (Statsoft. Inc., Tulsa, Oklahoma, USA).

Results

Accuracy of %mC estimation by HPLC

Accuracy of HPLC estimation of %mC ranged from 0.03 % / 0.07% (the first value is absolute deviation and the second one coefficient of variation (relative standard deviation, Table S2) to 1.33 % / 3.03% with mean values 0.4 / 0.78% for two measurements of the same hydrolysis product; and from 0.31 % / 0.67% to 0.62 / 1.4% with mean values 0.48 / 1.03% (Table S2) for two measurements of the same sample prepared independently two times (independent DNA extraction and hydrolysis).

Efficiency and temporal stability of experimental demethylation

The type of demethylation agent and its concentration had a significant effect on %mC in leaves measured at the cessation of demethylation treatment ($F_{12,52}=5.2$, $P<0.001$, Figs. 1 and S1). We found the highest decrease of %mC (9.9%) in leaves of plants treated with 50 μM solution of 5-azacytidine applied once a week (30.5 % of %mC on average) compared to control plants (32.5% of %mC on average). In general, %mC decreased with increasing concentration of demethylation agent and spraying frequency but the application of 50μM zebularine solution

did not differ from the control treatment (Figs. 1 and S1). However, the treatment using 50µM zebularine differ from the other treatments because of replacement of treated seedlings by back-up plants in the initial phase of the spraying experiment due to their high mortality in the early ontogenetic stages (see Materials and Methods).

In respect of stability of demethylation pattern over time, the plants treated with the largest decrease of %mC at the cessation of demethylation treatment showed a significantly higher level of %mC after 10 weeks without further demethylation agent application suggesting remethylation of their genomes ($F_{3,16}$=6.36, P=0.005, Fig. 3, Table S3). Amount of %mC of treated plants did not differ from 5 µM zebularine treatment, which was used instead of controls (see Materials and methods). 10 weeks after cessation of demethylation treatment (Fig. 3, Table S3).

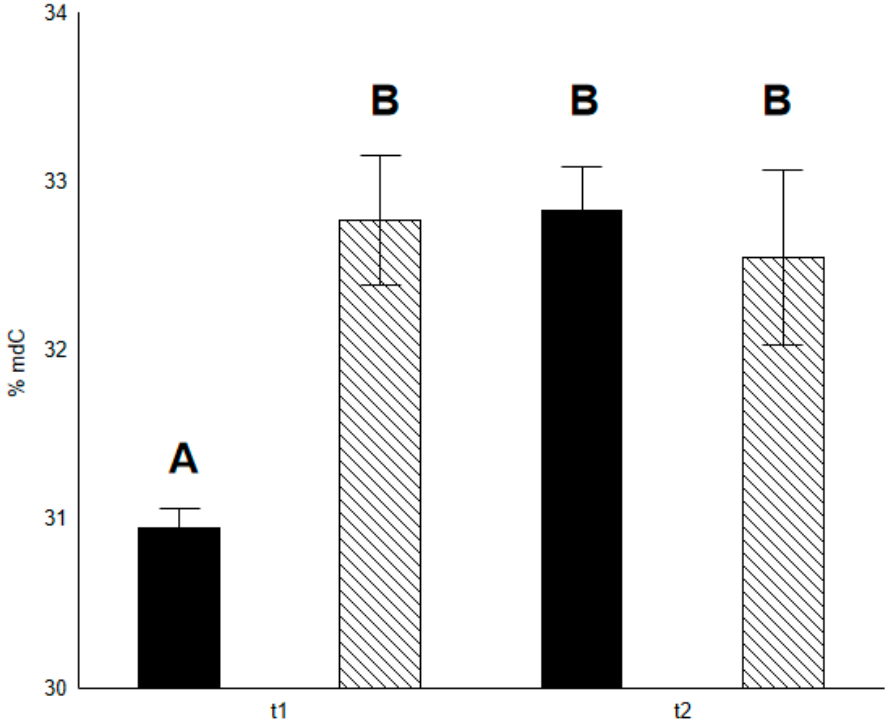

Fig. 3. The effect of different treatments and sampling time on the amount of methylated cytidine (%mC) in leaves of *Hieracium alpinum*. Presented are means and standard errors, five plants for each treatment were measured. Different letters indicate significant differences between treatments (at significance level P≤0.05). (t1) DNA material (leaves) sampled immediately at the cessation of demethylation agent application; (t2) DNA material (leaves) sampled 10 weeks after t1, i.e. 10 weeks after the cessation of the experiment; (black bars) 50µM 5-azacytidine solution applied twice a week i.e the treatment with the lowest level of %mC; (hatched bars) 5µM zebularine solution applied once a week used instead of controls due to insufficient amount of leaf material and subsequently, isolated DNA

Effect of experimental demethylation on germinating seeds

The germination rate was significantly affected by demethylation treatment ($F_{5,12}$=5.72, P=0.006). More specifically, the germination rate in daily refreshed 50µM zebularine solution was lowered by 37.8% when compared to control plants ($F_{5,12}$=5.7, P=0.063, Fig. S2). Similarly, the survival rate of young seedlings in seedling trays was significantly lowered (by 79 %) in those seedlings which were exposed to 50µM zebularine solution compared to other treatments ($F_{5,12}$=213, P<0.001, Fig. 4).

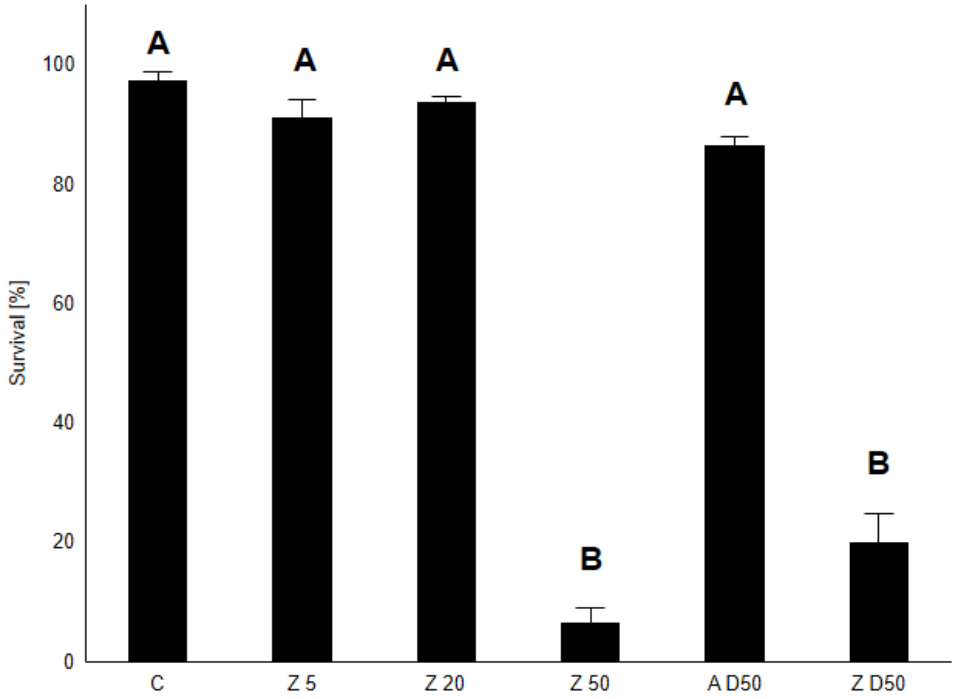

Fig. 4: The effect of different demethylation treatments on the survival rate of *Hieracium alpinum* seedlings in seedling trays after 8 weeks since sowing. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling for the subsequent 4 weeks. Presented are means and standard errors. Different letters indicate significant differences (at P≤0.05) between treatments. (C) control plants; (Z) zebularine; (A) 5-azacytidine; (5) 5 µM; (20) 20 µM; (50) 50 µM concentration of specific demethylation agent; (D50) seedlings from seeds germinated in daily refreshed 50 µM solution.

Effect of experimental demethylation on biomass and leaf N a C contents

Initial biomass assessed after four weeks of cultivation in seedling trays was significantly affected by the type of demethylation treatment ($F_{9,142}$=5.1, P<0.001, Fig. S3). It decreased with increasing concentration and spraying frequency (Fig. S3). Surprisingly, final biomass assessed after 17 weeks was affected by demethylation treatment too, but in the opposite way ($F_{12,166}$=16.24, P<0.001, Fig. 2). Specifically, the amount of final biomass increased with increasing concentration of demethylation agent and spraying frequency except all three treatments with 50μM zebularine which did not differ from the control treatment (Fig. 5 and S4). Amount of final biomass was significantly negatively correlated with %mC (N=65, R^2= 0.17, P<0.001, Fig. S3). In contrast, demethylation treatment did not significantly affect foliar carbon and nitrogen contents (N=30, F=1.65, P=0.18 and N=30, F=0.73, P=0.61, respectively; Figs. S5 and S6) and foliar nitrogen content was not significantly correlated with biomass (N=30, R^2=0.06, P=0.21).

Associations between the efficiency of experimental demethylation and genome size of target plants and their %mC

We found that the efficiency of experimental demethylation across different species is negatively correlated with %mC level of untreated plants and genome size (Fig. 6). In other words, the more methylated and larger genome was, the less efficient was experimental demethylation. In detail, we found that efficiency of experimental demethylation was significantly correlated with the interaction of %mC levels of untreated plants and their genome size (N=11, R^2=0.67, *P*=0.01, Table 2), and with %mC of untreated plants itself (N=11, *P*=0.004, Table 2, Fig. 6), but not with the genome size itself (N=11, *P*=0.18, Table 2). Also, %mC in untreated plants was not correlated with the genome size (N=11, R^2=0.16, *P*=0.21).

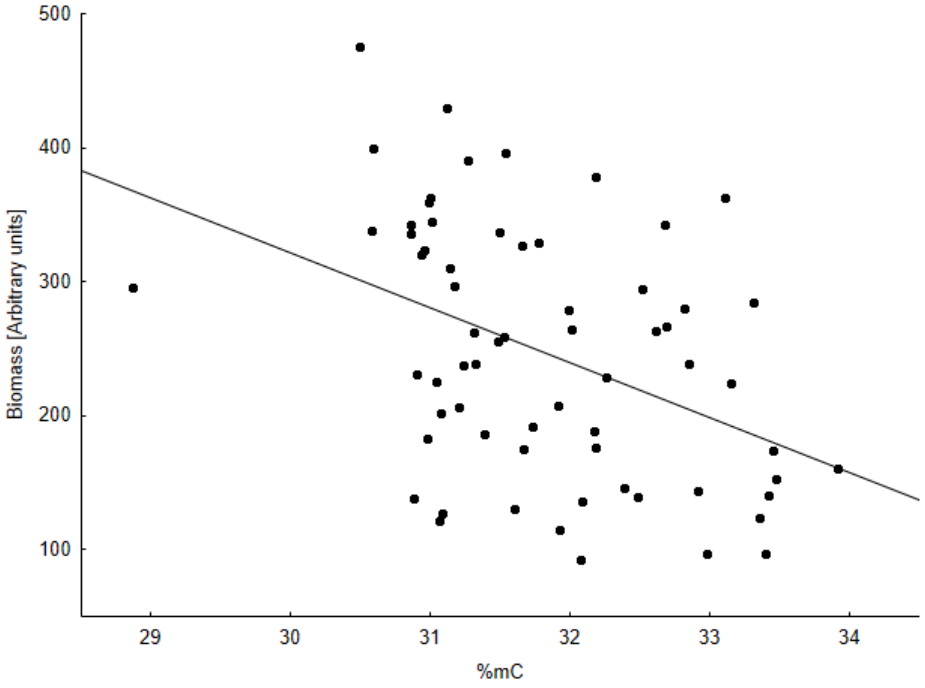

Fig. 5: Relationship between the amount of biomass and global amount of methylated cytidine (%mC) of experimental plants, *Hieracium alpinum.* Included are plants measured at the cessation of demethylation agent application (N=65, R^2= 0.17, P<0.001).

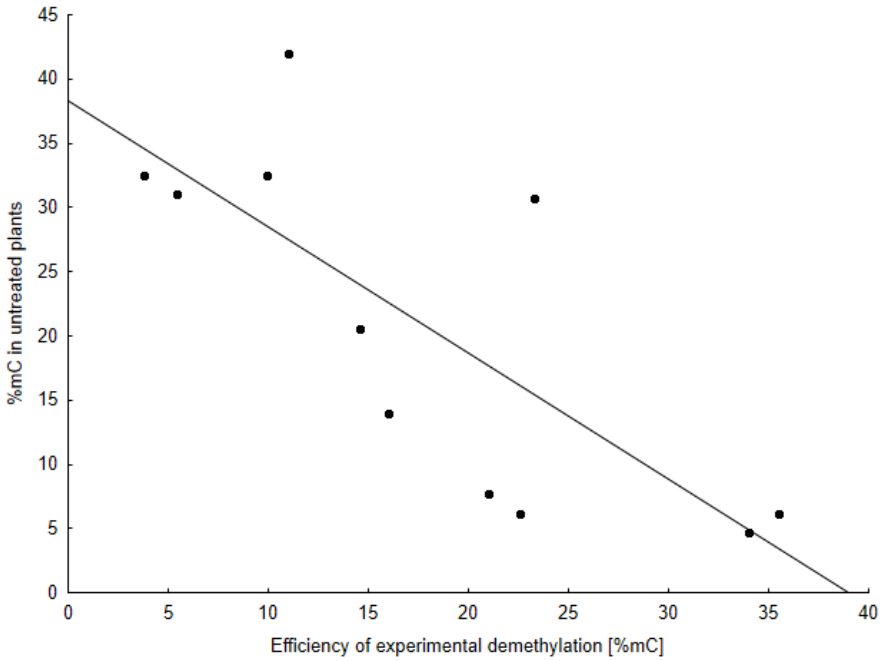

Fig. 6: Linear correlation of experimental demethylation efficiency (decrease of the global amount of methylated cytidine in %) and level of global methylation in untreated vascular plant species listed in Table 2 (N=11, R2=0.58, P=0.006).

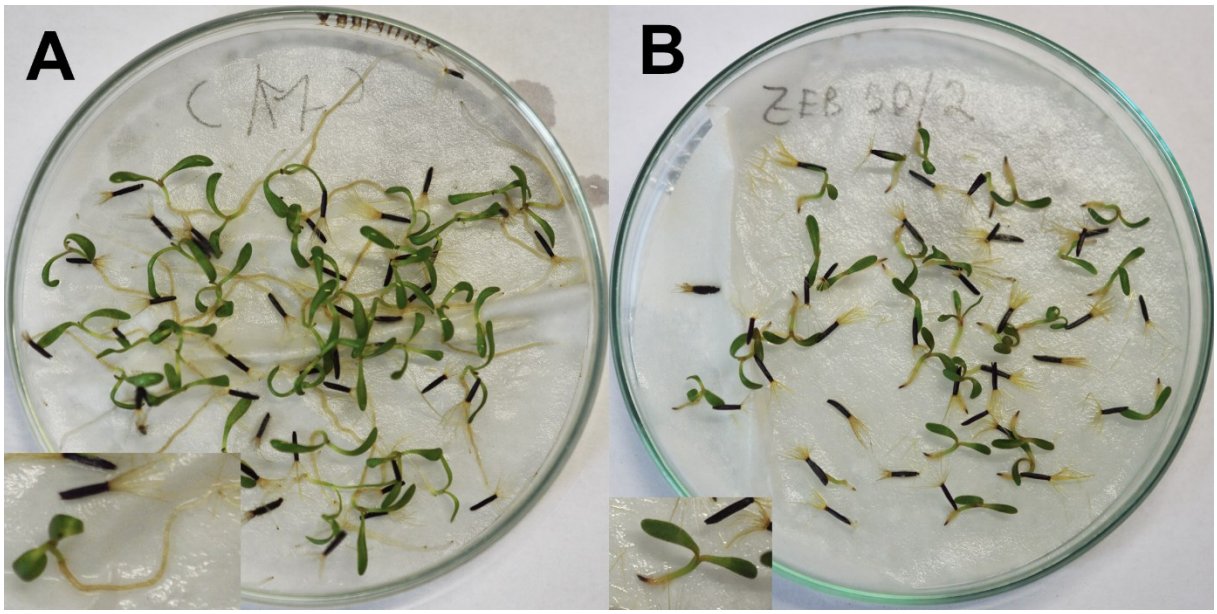

Fig. 7: Four weeks old seedlings of *Hieracium alpinum* germinating in water (control treatment; A) and 50 µM zebularine solution (B). Seedlings in 50 µM zebularine solution are visibly smaller with a poorly developed root system and brownish root tip when compared to the control treatment

Discussion

Our study demonstrated that the efficiency of experimental demethylation depends on the type of demethylation agent and application method (germination vs. spraying with different concentration applied with different frequencies). In detail, we found that 50 µM solution of 5–azacytidine, the most frequently used concentration in experimental studies (Bossdorf et al., 2010; Vergeer and Ouborg 2012; González et al., 2016; Puy et al., 2018) caused the largest decrease of %mC. In addition, the same concentration of zebularine, the analogue of 5 azacytidine, caused developmental aberrations of roots leading to the death of experimental plants in early stages of plants development and thus an actual decrease of %mC in 50 µM 5-azacytidine treatment cannot be estimated. Moreover, our results showed that experimentally lowered level of %mC was not stable in time, but DNA has been remethylated after cessation of demethylation agent application. Analysis of 11 studies using demethylation approach showed that the efficiency of experimental demethylation is significantly negatively correlated with the total amount of methylated cytidine in untreated plants.

Accuracy of %mC estimation by HPLC

Reproducibility of %mC estimation by HPLC in our experiment expressed as coefficient of variation (relative standard deviation), was in average 0.78 % in the case of two measurements of the same hydrolysis product (i.e. technical replicates) and 1.03% in case of two measurements of the same sample prepared independently (DNA isolation and hydrolysis). Our results are thus comparable to those presented in Rozhon et al. (2008) who showed that reproducibility varied between 0.29 and 4.51% between two HPLC runs of the same sample. However, the latter value was likely caused by an insufficient amount of measured DNA (Rozhon et al., 2008). Based on the 9.9 % maximum difference of measured %mC between treated and untreated treatments, and 1.06% CV of %mC HPLC estimation, we can conclude that HPLC is an appropriate method for %mC estimation in our experiment as reliably detected the difference in %mC. When the difference would be smaller, then another more precise method should be used (Kurdyukov and Bullock 2016). From this, it is obvious that reproducibility of %mC measurements should be consistently assessed in each experiment to know whether decrease %mC or differences between plants in their global level of methylation is real or is an artefact of the measurement. Despite this, information about the reproducibility of measuring %mC level notably in demethylation studies (Sano et al., 1990; Horváth et al., 2003; Vergeer and Ouborg 2012), but also in various studies testing the global level of DNA methylation is often missing (Herringer et al., 2013; Orłowska et al., 2016; Quinga et al., 2017).

The efficiency of experimental demethylation

Our study demonstrated that the efficiency of experimental demethylation increases with increasing concentration of demethylation agent and increasing spraying frequency. We found that the application of 50 μM 5-azacytidine in all three spraying frequencies caused the largest decrease in %mC (Fig. 1). On the other hand, those seedlings, which germinated in 50 μM zebularine solution showed developmental aberrations resulting in high mortality (see below) and thus an actual decrease of %mC in 50 μM 5-azacytidine treatment could not be estimated. The largest decrease of %mC was observed in plants germinated in 50 μM 5-azacytidine and later sprayed with the same solution and concentration once a week. The decrease by ca 9.9 % of %mC (relative value) in these plants when compared to the control plants is comparable to the decrease observed in other species on which demethylation was applied (Table 2).

Furthermore, 50 μM zebularine treated plants, which were only sprayed by demethylation agent in seedling trays and pots, were not demethylated as efficiently as plants treated with 50 μM 5-azacytidine or lower concentrations of zebularine (Fig. S4). Based on these results, we suppose that application of demethylation agents during the germination stage, when seedlings could uptake demethylation agents from their water solution directly by roots, caused the largest decrease of %mC when compared to spraying only. These results are congruent with Puy et al., (2018), who found that decrease of %mC was similar between the plants uptaking demethylation agent by roots during germination stage and between plants sprayed by demethylation agent on the leaf surface during germination. Furthermore, the decrease of %mC in plants sprayed by demethylation agent in later ontogenetic stages was smaller than in plants exposed to demethylation agent during seed germination (Puy et al., 2018). Based on these findings, we hypothesize that experimental demethylation in early ontogenetic stages works more efficiently due to a smaller amount of DNA in germinating seeds or juvenile plants which has to be demethylated with the similar amount of demethylation agent compared to adult plants containing many more cells and thus DNA. Moreover, plants which were exposed to demethylation agent from the beginning of their life cycle are simply treated for a longer period than plants which treatment started later. Finally, uptake of demethylation agents by roots can be more effective than absorption by leaf surface similarly to nutrients uptake (Johnson et al., 2001). Nevertheless, several studies showed that nutrients/pesticides/water uptake by leaves through stomata, trichomes, aqueous pores or microscopic cracks in leaf cuticle is possible (Kerstiens 1996; Schlegel and Schönherr 2001; Schlegel et al., 2005). Nevertheless, the effectivity of chemical uptake by leaves varies greatly with plant species and used chemicals (Yamada et al., 1964; Schreiber 2005; Wang and Liu 2007).

Effects of experimental demethylation

Our results showed that demethylation agent could harm plants' fitness (germination and survival rates) in early ontogenetic stages. However, this has been observed only when 50 μM zebularine solution was used. The high mortality of seedlings was presumably caused by an aberration in root system development manifested already during germination experiment on Petri dishes (Fig. 7). Our results are in agreement with other studies showing the negative effect of demethylation agent on germinating seeds (Baubec et al., 2009; Puy et al., 2018). Interestingly, daily refreshed solution of 50 μM 5-azacytidine, an analogue of zebularine with

supposedly the same mechanism of demethylation as zebularine (Pecinka and Liu 2014), did not appear to have any significant negative impact on plants' performance, similarly to lower concentrations of zebularine. This finding is therefore in disagreement to Baubec et al. (2009) using of 5-azacytidine of the same concentration caused similar aberration of root systems as using of zebularine (Baubec et al., 2009). Based on our findings, we can hypothesize, that high viability of seedlings exposed to 50 μM 5-azacytidine could be explained by low stability of 5-azacytidine in water solution compared to the considerably more stable solution of zebularine (Yoo et al., 2004; Stresemann and Lyko 2008). As a result of rapid degradation, the concentration of 5-azacytidine could drop soon after application of this agent and do not alter root development so much as 50 μM zebularine. Alternatively, we can hypothesize that permeability of cell walls to 5-azacytidine in comparison to zebularine is lower and thus 5-azacytidine of the same concentration cannot cause developmental aberrations. Finally, because both chemicals have an identical mechanism of action but the slightly different chemical structure (Pecinka and Liu 2014), zebularine could have higher cytotoxicity in a plant cell that 5-azacytidine.

Furthermore, we found that initial biomass decreased with increasing concentration and application frequency of demethylation agent (Fig. 2). This pattern was already observed in demethylated seedlings of *Taraxacum brevicorniculatum* (Puy et al., 2018). We hypothesize that this result is a consequence of growth inhibition in the early stages of plants development caused by a demethylation agent. Growth inhibition caused by demethylation agent was manifested already on Petri dishes in 50 μM zebularine treatment which results in the death of treated plants (Fig. 7). In contrast to initial biomass, final biomass of *Hieracium alpinum* increased with increasing concentration and application frequency of demethylation agent, except plants from 50 μM zebularine treatment which were, however, replaced by back up control plants in seedling trays due to high mortality of this treatment. Depending on experimental study and study system, experimental demethylation had either positive (*Festuca rubra* in Münzbergová et al., 2019 and *Trifolium repens* in González et al.., 2016) or negative (*Arabidopsis thaliana* in Bossdorf et al., 2010 and *Oryza sativa* in Akimoto et al., 2007) effect on plant biomass. Different effect of demethylation agent can be caused by different application method. For instance, spraying of demethylation agent was applied on *F. rubra*, *T. repens* and *H. alpinum*, but in the case of *A. thaliana* and *O. sativa,* demethylation agent was applied on germinating seeds only. Alternatively, different effects of experimental demethylation on plants biomass can be also explained by mechanisms of experimental demethylation. In detail, demethylation agents are incorporated into DNA indiscriminately across the whole genome and

lower DNA methylation (Pecinka and Liu 2014). As a result, different genes can be demethylated differently and caused variable outcomes.

Increased final biomass in plants treated with higher concentrations of demethylation agents could be explained by several mutually non-exclusive explanations. Firstly, the application of the demethylation agent could provide the treated plant with an additional nitrogen source and serve as a putative fertilizer. However, given regular fertilization of all plants by commercial fertilizer (cumulative estimate is 0.32 g of nitrogen per single plant for the whole experiment), we suppose that plants were not limited by nitrogen and therefore, adding a very small amount of demethylation agent per plant during the experiment (cumulative estimate is ca 8.6×10^{-7} g of 5-azacytidine or 1.97×10^{-7} g of nitrogen per single plant for the whole experiment considering spraying 1ml of 50 μM solution twice a week) would not contribute much to the plant fertilization. Moreover, final biomass was not correlated with leaf nitrogen content (Fig. 5). Secondly, since the final biomass was negatively correlated with %mC, we can hypothesize that larger biomass could be caused by a lower level of DNA methylation itself, probably by higher transcription level certain genes involved in photosynthesis as has been already demonstrated in *Brassica rapa* (Liu et al., 2017).

Temporal stability of experimental demethylation

Although our study demonstrated a significant decrease of %mC after exposure of demethylation agent, our results also showed that artificial demethylation, i.e. decrease of the amount of methylated cytidine in the genome, is not stable in time. In detail, we found that levels of %mC in demethylated plants using 50 μM 5-azacytidine was equal to plants used as control (5 μM zebularine treatment) 10 weeks after cessation of demethylation agent application (Fig. 3). Similar results were found in *Arabidopsis thaliana* (Baubec et al., 2009), where %mC significantly increase 8 weeks after cessation of 20 and 40 μM zebularine treatment. Based on these results, we suppose that without a continuous supply of demethylation agent, the original level of DNA methylation could be restored to ensure natural plant growth. In addition, we suppose that treating only germinating seeds by demethylation agent probably does not effectively lower %mC for a long time. Contrary to our and Baubec's results, long-term stability of %mC after germination treatment only was found in adult *Scabiosa columbaria* (Vergeer and Ouborg 2012), *Oryza sativa* (Sano et al., 1990), *Zea mays* (Sano et al., 1989) and other plants (Table 2).

Patterns in the efficiency of experimental demethylation

Based on the results from other studies listed in Table 2 and dealing with experimental demethylation, we conclude that total amount of methylated cytidine of untreated plants and genome size plays a crucial role in the efficiency of experimental demethylation (Fig. 6, Table 2). Our results suggests that species with higher amount of methylated cytidine and the same time larger genome would require a higher amount of demethylation agent either via higher concentration/application frequency or longer exposure time to achieve a similar decrease of %mC when compared to species with a lower level of the methylated genome and smaller genome size. However, increasing the quantity of demethylation agent should be done with caution, considering the negative effect of higher concentration mainly on root system development (Baubec et al., 2009; Puy et al., 2018).

Originally, we also hypothesized that genome size itself could affect the efficiency of experimental demethylation in a similar way. Alonso et al. (2015) suggested that larger genome should be proportionally more methylated because of higher occurrence of transposons in a larger genome which are usually more densely methylated. As a result, theoretically, a larger genome would require a higher amount of demethylation agent to achieve a similar decrease of %mC than in species with the smaller genome. In contrary to this hypothesis, our results do not support this assumption. Based on the fact that %mC in untreated plants is not correlated with genome size itself, we suppose that the amount of overall methylation is a taxon-specific trait which probably does not necessarily depend on the size of its genome. However, given a rather small number of observations, this conclusion should be taken in cation.

Conclusions

Our results are the first ones showing temporal instability in the amount of methylome caused by demethylation agents in the non-model plant in vivo. It seems that without a constant supply of demethylation agent, DNA can be methylated back i.e. remethylated. Moreover, we found that 5-azacytidine is the best demethylation agent concerning plants' viability in case of *H. alpinum* and that application of demethylation agent on germination seeds followed by spraying provides the highest drop of the global amount of methylated cytidine. Furthermore, we pointed out that the efficiency of experimental demethylation is correlated with the global amount of methylated cytidine in untreated plants. In summary, because other studies dealing with experimental demethylation often brings variable results in different plant species, we must point out that a pilot study is needed to set up an efficient approach of experimental demethylation to answer particular questions. Otherwise, the unsuitable approach can result in the death of experimental plants caused by the well-known negative effect of demethylation agent or simply insufficient decrease of global methylation level.

Acknowledgments

We thank Romana Bartošová, Lenka Flašková, Štěpán Mareš and Viera Mrázová for their help with experiments and lab work. This project was funded by Grant Agency of Charles University (GAUK 907218).

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Supplementary material

Table S1: Temporal stability of 50 μM zebularine water solution in Petri dish (12-hour photoperiod and 24/12°C day/night temperature). Presented results show that zebularine is stable in water solution. Increasing concentration of zebularine is presumably caused by water evaporation.

Hour	Zebularine concentration [μM]
12	54.3
24	55.9
48	55.2
72	58.5
96	60.4

Table S2: Reproducibility of the %mC estimation using HPLC. Differences between two HPLC measurements of the same product of hydrolysis (R1) or of the two independent DNA isolations and hydrolysis of the same sample (R2); (M1) the first measurement of %mC by HPLC; (M2) the second measurement of %mC by HPLC; (Z5, Z20 and Z50) 5, 20 and 50 μM zebularine treatment; (AZA50) 50 μM 5-azacytidine treatment; (f1, f2 or f3) solution of particular demethylation agent applied once in two weeks, once a week or twice a week.

Sample ID	Comparison type	%mC at M1	%mC at M2	Absolute deviation / coefficient of variation %
A50 f1 9	R1	31.32	31.24	0.08 / 0.18
A50 f2 5	R1	30.59	30.56	0.03 / 0.07
Z50 f1 2	R1	31.17	30.94	0.23 / 0.52
Z50 f2 1	R1	31.60	31.96	0.36 / 0.8
Z50 f3 6	R1	31.09	30.83	0.26 / 0.59
Z20 f1 2	R1	32.19	31.70	0.48 / 1.08
Z20 f1 8	R1	31.39	30.84	0.55 / 1.25
Z20 f1 9	R1	31.48	31.37	0.11 / 0.25
Z20 f2 6	R1	32.52	32.03	0.49 / 1.07
Z20 f2 9	R1	31.66	31.23	0.43 / 0.97
Z20 f3 10	R1	33.11	32.42	0.69 / 1.49
Z20 f3 7	R1	32.68	32.27	0.41 / 0.89
Z20 f3 8	R1	31.78	31.59	0.18 / 0.42
Z5 f1 4	R1	32.85	32.32	0.53 / 1.15
Z5 f2 1	R1	30.98	30.80	0.17 / 0.41
Z5 f2 3	R1	32.69	32.23	0.46 / 1
Z5 f2 8	R1	31.74	30.41	1.33 / 3.03
Mean		31.81	31.46	0.40 / 0.78
Z20 f2 1	R2	31.84	32.26	0.41 / 0.93
Z20 f2 2	R2	32.51	32.82	0.31 / 0.67
Z20 f2 6	R2	31.96	32.52	0.55 / 1.23
Z20 f2 7	R2	31.49	32.02	0.53 / 1.18
Z20 f2 9	R2	31.04	31.66	0.62 / 1.40
Mean		31.77	32.25	0.48 / 1.06

Table S3: The amount of methylated cytidine (%mC) in leaves of *Hieracium alpinum* measured at the cessation of demethylation agent application (t1) and after subsequent 10 weeks without application of demethylation agent (t2). (Z5) plants treated with 5 μM zebularine was used instead of controls due to insufficient amount of DNA material, %mC levels of Z5 f1 did not significantly differ from controls (Fig. 3); (A50) plants treated with 50 μM 5-azacytidine; (f3) solution of demethylation agent applied twice a week; (f1) solution of demethylation agent applied once in two weeks.

Sample ID	t1	t2	Absolute deviation/ coefficient of variation %
A50 f3 4	30.58	32.30	1.72 / 3.87
A50 f3 7	30.86	33.51	2.65 / 5.82
A50 f3 13	31.12	33.36	2.24 / 4.91
A50 f3 8	31.20	32.27	1.07 / 2.38
A50 f3 9	31.02	32.71	1.70 / 3.75
Mean	30.95	32.83	1.88 / 4.17
Z5 f1 5	31.67	33.23	1.56 / 3.40
Z5 f1 11	32.39	31.02	1.37 / 3.06
Z5 f1 3	33.31	32.67	0.65 / 1.37
Z5 f 4	32.59	31.86	0.72 / 1.6
Z5 f1 13	33.91	34.00	0.08 / 0.24
Mean	32.77	32.56	0.22 / 0.67

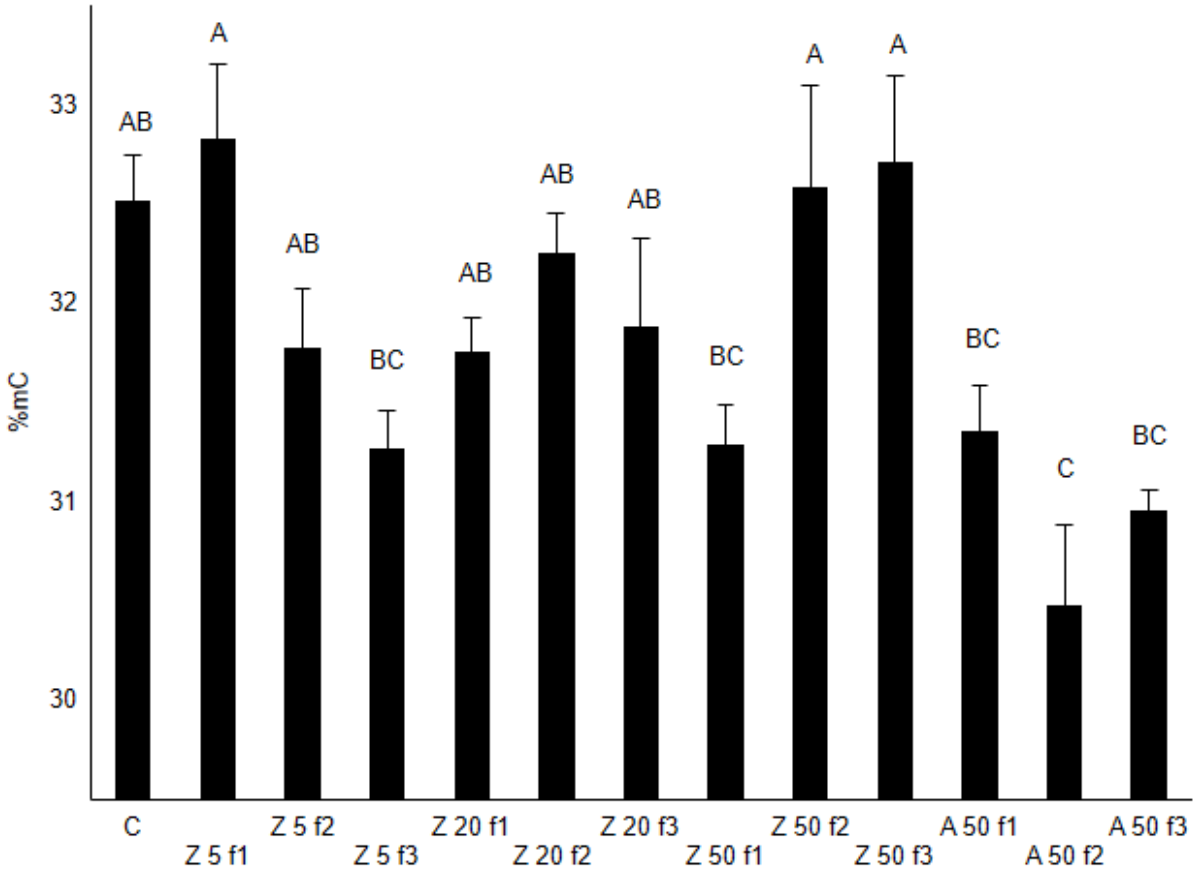

Figure S1: The effect of different demethylation treatments on the global amount of methylated cytidine (%mC) in leaves of *Hieracium alpinum* collected at the cessation of demethylation treatment, after 25 weeks from sowing. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling and young plants for subsequent 21 weeks, but Z50 treatments where only spraying of 50 μM zebularine solution for 17 weeks of back up control plants, which replaced seedlings from Z50 germination experiment due to their high mortality (see Materials and Methods), was applied. Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level $P \leq 0.05$). (C) control plants; (Z) zebularine; (A) 5-azacytidine; (5) 5 μM; (20) 20 μM; (50) 50 μM concentration of specific demethylation agent; (f1) spraying once in two weeks; (f2) spraying once a week; (f3) spraying twice a week.

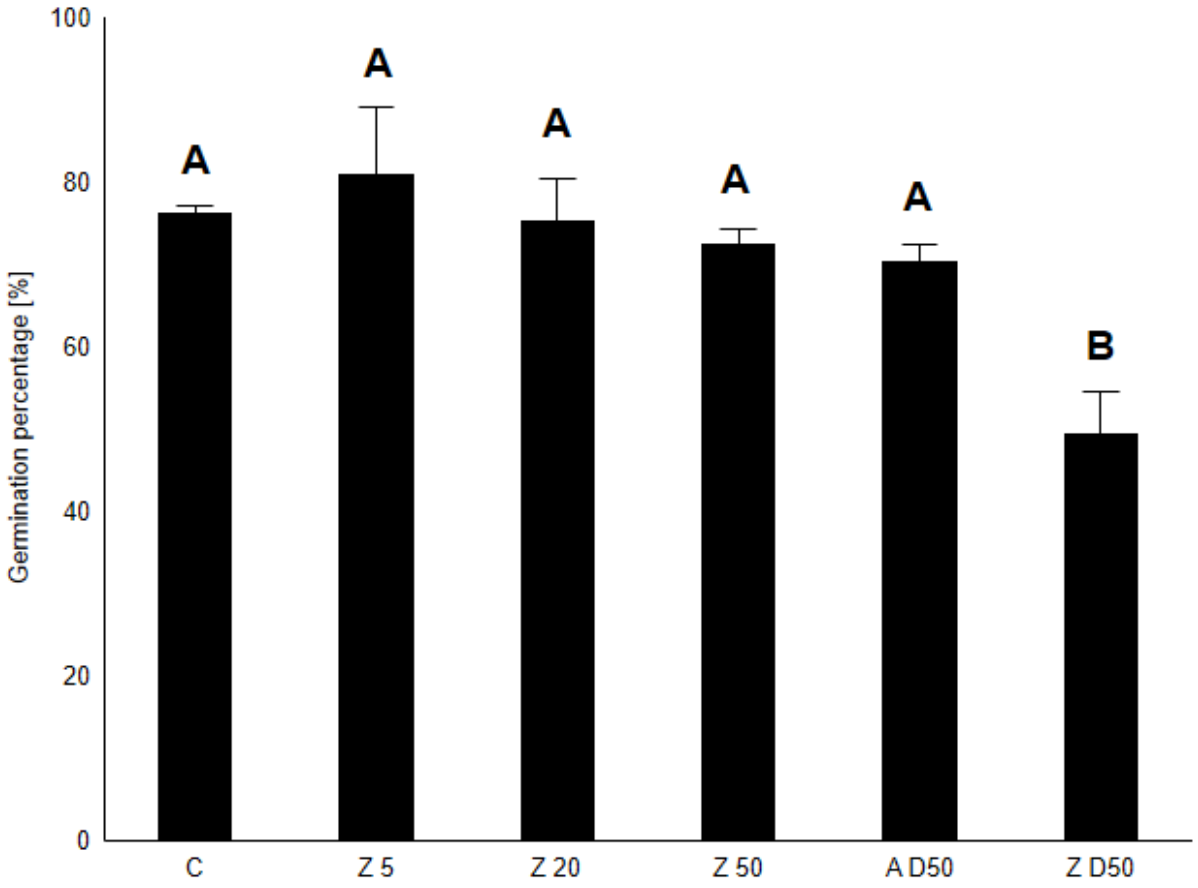

Figure S2: The effect of two demethylation agents and their concentration on the germination rate of *Hieracium alpinum* seeds germinated on Petri dishes for 4 weeks. Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level P≤0.05). (C) control plants; (Z) zebularine; (A) 5-azacytidine; (5, 20 and 50) concentration 5, 20 and 50 µM; (D50) seedlings from seeds germinated in daily refreshed 50 µM solution.

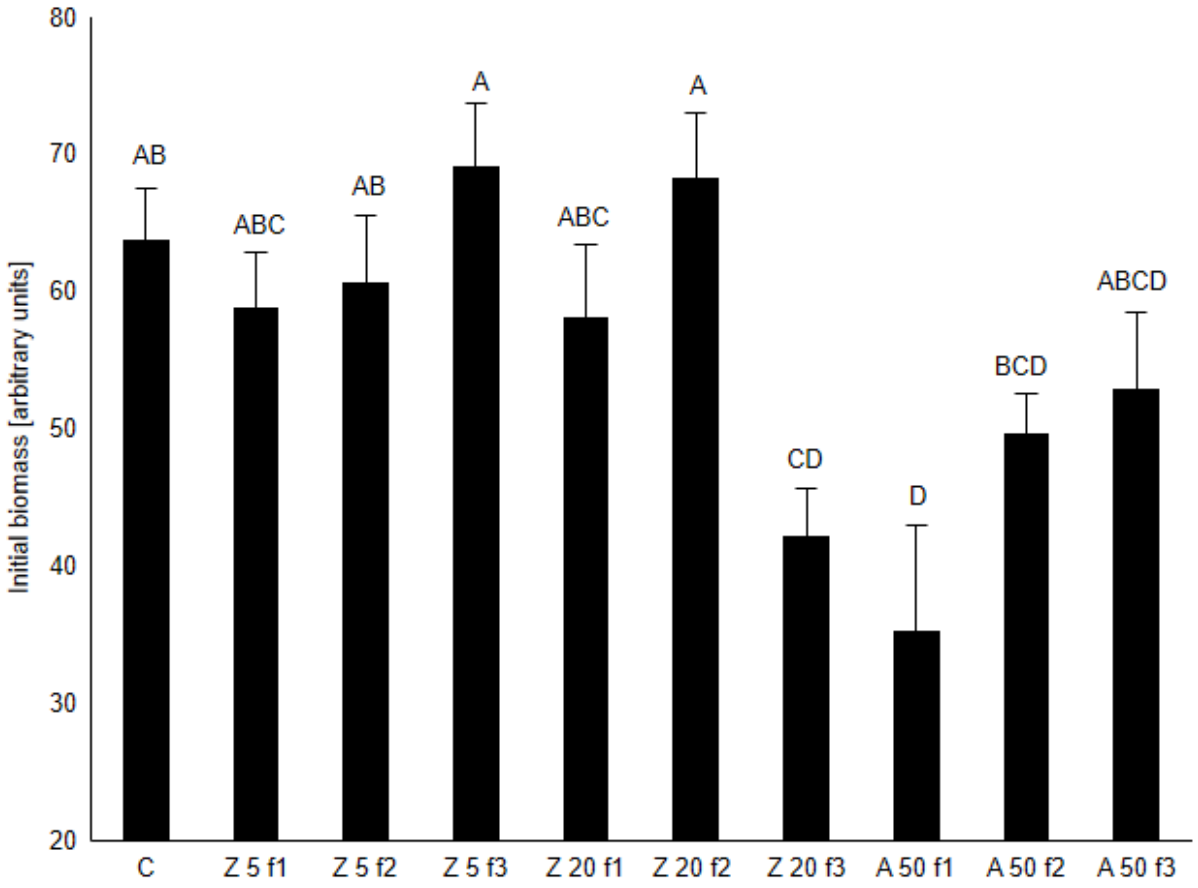

Figure S3: The effect of different treatments on the initial biomass of juvenile, 8 weeks old seedlings of *Hieracium alpinum* cultivated in seedlings trays. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling for subsequent 4 weeks, but Z50 (50 μM zebularine solution) treatments are excluded because of replacement treated seedlings by back up control plants in the initial phase of the experiment due to their high mortality (see materials and methods). Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level $P \leq 0.05$). (C) controls; (Z) zebularine; (A) 5-azacytidine; (5) 5 μM; (20) 20 μM; (50) 50 μM concentration of specific demethylation agent; (f1) spraying once in 2 weeks; (f2) spraying once a week; (f3) spraying twice a week.

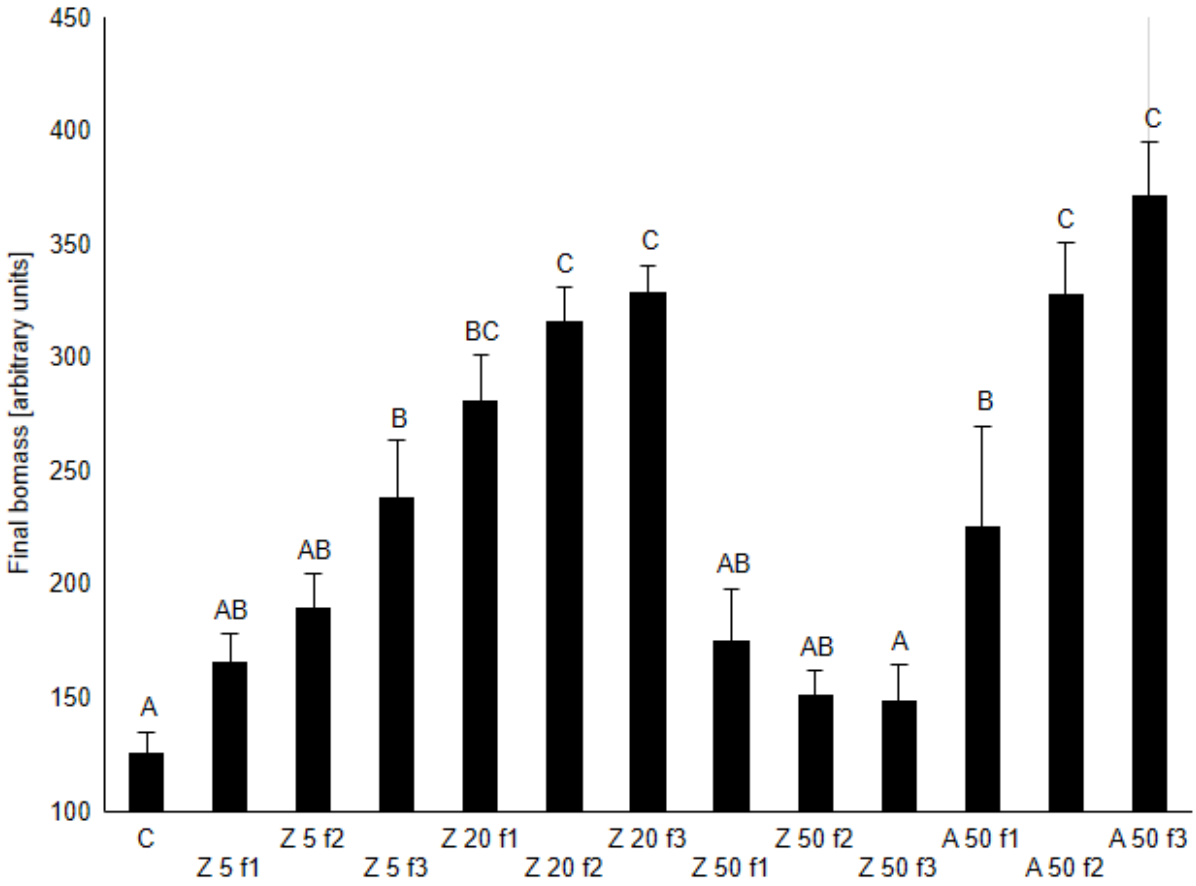

Figure S4: The effect of different demethylation treatments on the final biomass of *Hieracium alpinum* at the cessation of demethylation treatment, after 25 weeks from sowing. Demethylation treatment included germination in demethylation agents for 4 weeks and spraying of seedling and young plants for subsequent 21 weeks, but Z50 treatments where only spraying of 50 μM zebularine solution for 17 weeks of back up control plants, which replaced seedlings from Z50 germination experiment due to their high mortality (see Materials and Methods), was applied. Presented are means and standard errors. Different letters indicate significant differences between categories (at significance level $P \leq 0.05$). (C) control plants; (Z) zebularine; (A) 5-azacytidine; (5) 5 μM; (20) 20 μM; (50) 50 μM concentration of specific demethylation agent; (f1) spraying once in 2 weeks; (f2) spraying once a week; (f3) spraying twice a week.

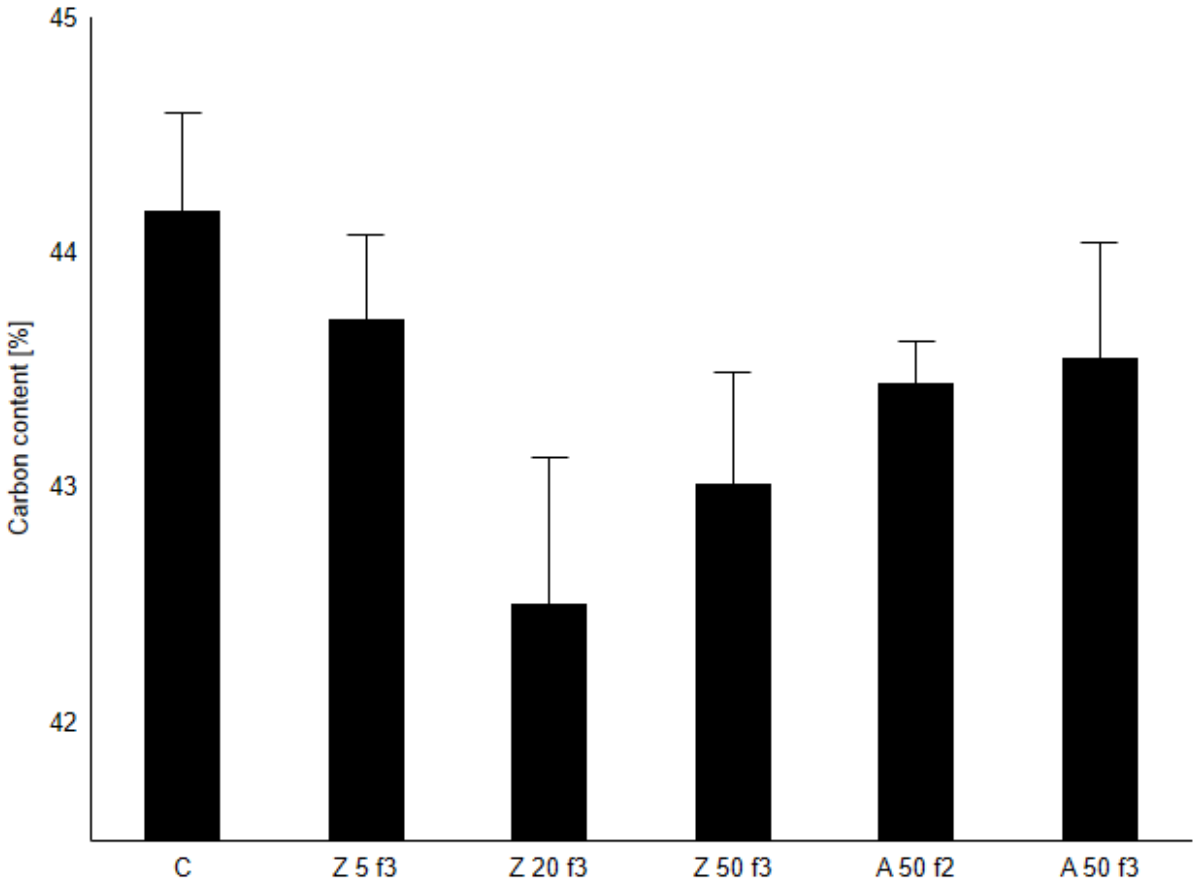

Figure S5: The effect of different treatments on carbon content in leaves of *Hieracium alpinum* collected at the cessation of demethylation treatment, after 25 weeks from sowing. Presented are means and standard errors. Only treatments including plants which DNA was previously isolated and we still had sufficient amount of leaf material to analyse foliar nitrogen and carbon are presented. Differences are not significant (at significance level $P \leq 0.05$). (C) controls; (Z) zebularine; (A) 5-azacytidine; (5) 5 μM; (20) 20 μM; (50) 50 μM concentration of specific demethylation agent; (f2) spraying once a week; (f3) spraying twice a week.

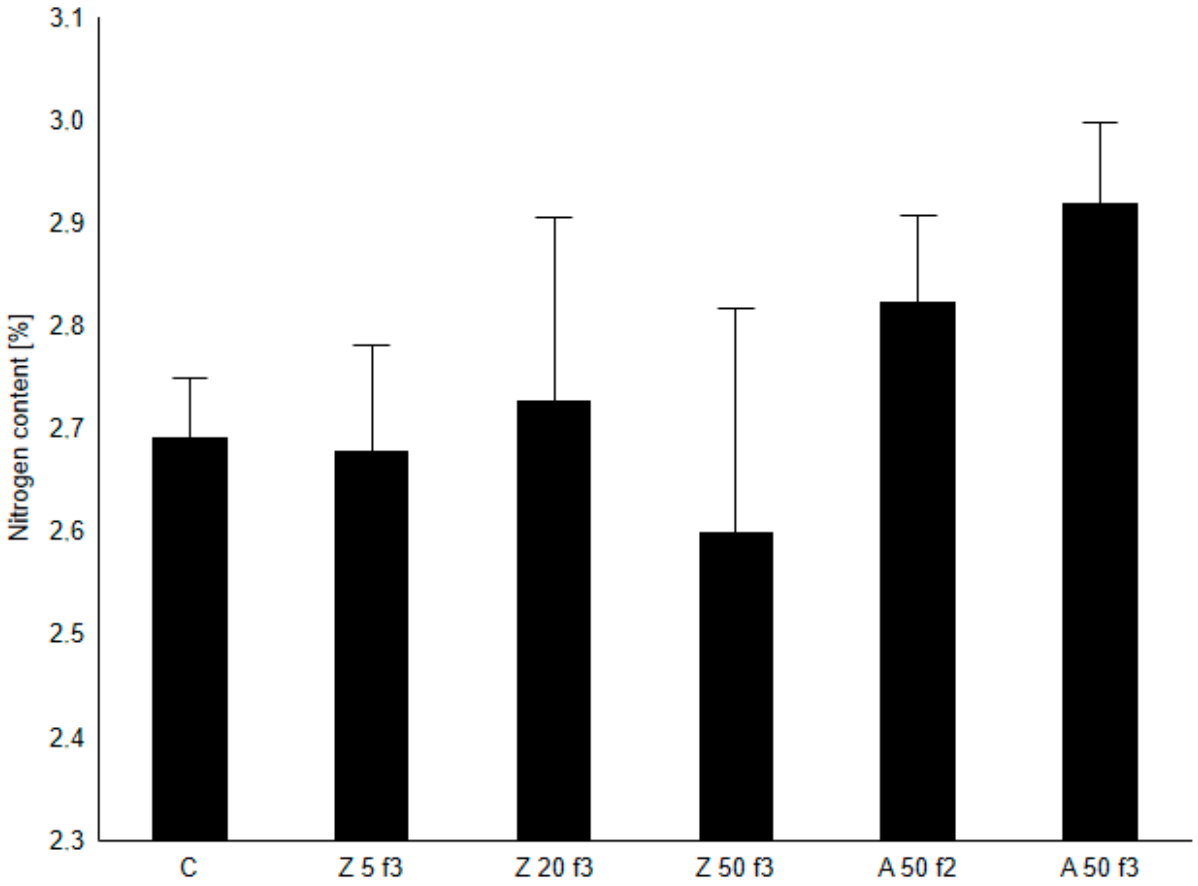

Figure S6: The effect of different treatments on nitrogen content in leaves of *Hieracium alpinum* collected at the cessation of demethylation treatment, after 25 weeks from sowing. Presented are means and standard errors. Differences are not significant (at significance level $P \leq 0.05$). Only treatments including plants which DNA was previously isolated and we had still sufficient amount of leaf material to analyse foliar nitrogen and carbon are presented. (C) controls; (Z) zebularine; (A) 5-azacytidine; (5) 5 μM; (20) 20 μM; (50) 50 μM concentration of specific demethylation agent; (f2) spraying once a week; (f3) spraying twice a week.

Curriculum Vitae

Personal data:

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Education:

2015 – present: Ph.D. study: Department of Botany, Faculty of Science, Charles University. Thesis: Origin, inheritance and ecological significance of apomixis in the genus *Hieracium* s.str. The role of genetic and epigenetic mechanisms, Supervisor: doc. Mgr. Patrik Mráz, Ph.D.

2013 – 2015: Master study: Department of Botany, Faculty of Science, Charles University. Thesis: Pollination of *Clusia blattophila*: quality and quantity components of the plant-pollinator systém. Supervisor: Mgr. Blanka Vlasáková Ph.D.

2010 – 2013: Bachelor study: Faculty of Science, Charles University. Thesis: Plant-pollinator relationships in isolated ecosystems and ecosystems with extreme fluctuations in climatic conditions. Supervisor: Mgr. Blanka Vlasáková Ph.D.

Stays abroad:

2017 – Internship in Dr. Anna Koltunow's lab (CSIRO, Adelaide, Southern Australia) – Learning in the plant embryology techniques

Field experience abroad:

2019: Argentina
2018: Colorado
2012, 2013, 2014: French Guiana

Working experience:

2017 – 2019: Department of Botany, Faculty of Science, Charles University, Prague: Research Associate

2014 – 2016: Institute of Experimental Botany of the Czech Academy of Sciences, Prague: Research assistant

2012 –2014: Institute of Botany of the Czech Academy of Sciences, Průhonice: Research assistant

Teaching experience:

2018 – 2019: Supervision of bachelor thesis:" Importance of epigenetic variability in evolution of clonal plants" by Štěpán Mareš

2017 – 2018: Recapitulation of biology II, Faculty of Science, Charles University

2016 – 2018: Practical courses in botany, Faculty of Science, Charles University

Research grants:

2018-2020: Grant Agency of Charles University, GAUK no. 907218: Importance of epigenetic variability in the evolution of clonal plants

Conferences:

2019: International Conference on Polyploidy in Ghent, Belgium: „Did interspecific hybridisation trigger neopolyploidisation in predominantly polyploid genus Hieracium s.str?“, Poster

2017: Plant Population Biology international Conference in Halle, Germany: „Does biparental inbreeding explain the geographical parthenogenesis pattern in an arcto-alpine species Hieracium alpinum (Asteraceae)?“: Poster

Publications (published and submitted):

Pinc J., J. Chrtek, V. Latzel and P. Mráz: Negative effect of inbreeding on fitness of an arcto-alpine *Hieracium alpinum* (Asteraceae), a species with geographical parthenogenesis (in press, *Plant Systematic and Evolution*, DOI: 10.1007/s00606-020-01692-6)

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