Morphology mirrors ploidy and reproductive modes in *Pilosella officinarum*

Morfologie odráží ploidní úroveň a reprodukční způsoby druhu *Pilosella officinarum*

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Pilosella officinarum is represented predominantly by tetraploid (2n = 36), pentaploid (2n = 45) and hexaploid (2n = 54) cytotypes reproducing, to various degrees, both sexually and apomictically. Its current intraspecific taxonomical treatment is based mainly on selected apomictic lineages, the large number of which makes the treatment confusing and not generally applicable. We therefore tested the breeding modes of a representative set of plants from central Europe, cultivated under experimental conditions. Each ploidy level was associated with a different reproductive pattern (4x – sexual, 5x – prevalently apomictic and 6x – sexual and apomictic). Whereas sexual tetraploids occurred in the western part of the study area (the Czech Massif), apomictic pentaploids and hexaploids were scattered in its eastern part (Western Carpathians and Pannonia). Moreover, the hexaploid cytotype formed a distinct exclusively sexual group restricted to steep river canyons of the Czech Massif. Morphometric analyses were performed to determine the set of characters which distinguish major lineages characterized by different ploidy and reproductive modes. Their results confirm the existence of morphological differences between plants of different ploidy levels and, in the case of the hexaploid cytotype, different modes of breeding. Knowledge of ploidy and reproductive modes is therefore essential for elucidating the reticulate infraspecific structure of *Pilosella officinarum.*

Keywords: morphometrics, *Pilosella officinarum,* ploidy, reproductive mode

Introduction

Apomictic or agamic groups have long posed difficult taxonomic challenges in plant science (Majeský et al. 2017). One glaring example of an extremely complex, reticulate genus is the genus *Pilosella* Vaill. (Krahulcová et al. 2000). Despite its close relationship with the genus *Hieracium*, under which it is sometimes included as a subgenus [*H.* subgen. *Pilosella* (Hill) Gray; see e.g. Gottschlich 1987 vs Bräutigam & Greuter 2007], *Pilosella* differs from *Hieracium* markedly in its patterns of variation and the underlying evolutionary mechanisms. Those differences exist in morphology, absolute genome size, the type of apomixis (agamospermy), ITS ribotypes and chloroplast haplotype diversity (Rosenberg 1907, Pogan & Wcisło 1995, Bräutigam & Bräutigam 1996, Fehrer et al. 2007, Suda et al. 2007, Mráz & Zdvořák 2019). Frequent hybridization and polyploidization are major microevolutionary forces influencing the complexity of *Pilosella* and differentiate it further from the genus *Hieracium* (e.g. Krahulcová et al. 2000).

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The taxonomic concept of the genus *Pilosella* is structured into groups of basic species ('Hauptarten') and intermediate species ('Zwischenarten'). Basic species are taxa characterized by unique morphological traits whereas intermediate species combine specific characters of basic species, indicating their hybridogenous origin. The distinction between basic and intermediate species also corresponds well to patterns of ploidy level and breeding mode differentiation; basic species are frequently diploid/polyploid and sexual whereas intermediate ones are predominantly polyploid and apomictic (Krahulcová et al. 2000).

Processes increasing the complexity of *Pilosella* were reviewed twice in the past years (Krahulcová et al. 2000, Fehrer et al. 2005). Homoploid and heteroploid hybridization, facultative apomixis with autonomous endosperm formation (apomixis combined with a sexual reproductive mode, often within the same capitulum), polyploidization (cytotypes detected in the field: 2x, 3x, 4x, 5x, 6x, 7x, and 8x; x = 9) and vegetative reproduction via stolons (Gadella 1987, 1991, Krahulcová et al. 2000) have been considered the most significant. These sources of enormous variation have been found to be accompanied with the production of fertile pollen (even in cases of sterile plants such as F1 triploids) and the occurrence of haploid parthenogenesis or autogamy via the mentor effect, i.e. induced selfing via facilitation by foreign pollen (Krahulcová et al. 1999, 2000, Krahulcová & Krahulec 2000, Krahulec et al. 2011). Moreover, all the aforesaid features are usually combined in intricate ways. Apomictic lineages are apomictic only facultatively, so they can hybridize with other cytotypes or taxa, which results in extremely reticulate groups mirrored by enormous numbers of cytotypes and morphological forms (e.g. Urfus et al. 2014, Krahulec et al. 2020). The occurrence of gene flow has been described several times (e.g. Fehrer et al. 2007, Krahulcová et al. 2012, Urfus et al. 2014). The taxonomy of the genus *Pilosella* cannot be dealt with as is usual with the other apomictic groups; in other words, each apomictic lineage is evaluated as a microspecies, as, for example, in the genus *Sorbus* L. or partly in the genus *Rubus* L. (Trávníček & Zázvorka 2005, Lepší et al. 2008, 2015, Majeský et al. 2017). Several substantially different taxonomic approaches have been devised to address the extreme complexity of the genus *Pilosella,* resulting in hundreds or even thousands of described taxa (Zahn 1921, 1922, 1923, Sell & West 1976, Schlyakov 1989, Tyler 2001). The current situation, which is clearly irresolvable by traditional taxonomic approaches, is that authors have given up on any detailed taxonomic concept (Bräutigam & Greuter 2007).

Pilosella officinarum Vaill. (*Hieracium pilosella* L., *Asteraceae*) may be considered a typical species that represents the complexity of the entire genus. The species belongs to the section *Pilosellina*, which is characterized by a single capitulum per stem and dense stellate hairs covering the abaxial side of the leaf (Zahn 1923). This section is distinctly defined within the genus and comprises several species with rather limited variation, mostly diploids [*P. argyrocoma* (Fr.) F. W. Schultz et Sch. Bip., *P. hoppeana* (Schult.) F. W. Schultz et Sch. Bip., *P. leucopsilon* (Arv.-Touv.) Gottschl., *P. peleteriana* (Mérat) F. W. Schultz et Sch. Bip., *P. pseudopilosella* (Ten.) Soják and tetraploid *P. saussuroides* Arv.-Touv.] and one extremely variable polyploid, *P. officinarum* (e.g. Zahn 1923, Sell & West 1976). *Pilosella officinarum* is the most common species of the genus *Pilosella.* It is a phenotypically hugely plastic species with large ploidy level variation (2x, 4x, 5x, 6x, 7x and 8x) and different modes of reproduction (apomictic and sexual; Delcourt 1972, Gadella 1972, 1984, 1991, Mráz et al. 2008). Tetraploid cytotypes

occur in western and central Europe and in the Iberian Peninsula whereas pentaploids and hexaploids prevail in deglaciated areas of Scandinavia and the British Isles (including the Alps and the Carpathians) and in the Mediterranean (Gadella 1984, Mráz et al. 2008). Several sexual tetraploids have been reported from Europe (e.g. Krahulcová et al. 2013); however, apomictic tetraploids have been found in Bulgaria (Krahulcová et al. 2009) and New Zealand (Houliston & Chapman 2001). Pentaploids have mostly been found to be apomictic, with rare sexual individuals, whereas the hexaploid cytotype consists of both sexual and apomictic lineages (Gadella 1984). The heptaploid cytotype is extremely rare, occurring mostly as individual sterile or apomictic plants within populations of other ploidal cytotypes (pentaploid and hexaploid; Turesson & Turesson 1960, Gadella 1984, Mráz et al. 2008, Křišťálová et al. 2010). The octoploid monoclonal apomictic cytotype has so far been recorded once from Slovakia (Krahulcová & Krahulec 2020). In addition, even higher ploidy levels, such as nonaploids and decaploids, were produced by experimental crossing (Gadella 1987); however, these have never been found in the field. A diploid cytotype has been described by Gadella (1972) from the Alps (the Aosta Valley) and by Delcourt (1972) from the Hautes-Alpes. Apparently, *P. officinarum* has a crucial role in the evolution of the entire genus because it participates in the majority of hybridization events (Krahulec et al. 2004, Křišťálová et al. 2010, Krahulcová et al. 2018).

Numerous morphotypes of *P. officinarum* have been classified at the subspecific level (e.g. the monographer of the genus, K. H. Zahn, listed 624 subspecies; Zahn 1923). Although such a vast number often precludes any intraspecific evaluation, correlations between some phenotypic characters (e.g. rosette size and the number and length of stolons) and ploidy have been indicated by Gadella (1991). Nevertheless, these indications were never subjected to any rigorous statistical evaluation. During a study on the cytotype distribution within central Europe (see Mráz et al. 2008), we succeeded in obtaining a representative set of material of known ploidy levels. In the present study, we employed a reproductive mode test and multivariate morphometrics to analyse whether the morphological variation is correlated with any of the particular ploidy levels or reproductive modes within *Pilosella officinarum*.

We aimed to address the following questions: (i) Does the pattern of reproductive modes within *Pilosella officinarum* follow major cytotypes? and (ii) Is the morphological variation of *P. officinarum* linked to the ploidy level or reproductive mode?

Materials and methods

Plant material

We examined a subset of plants that were used for ploidy level determination in a previous study (Mráz et al. 2008), deposited in the herbaria PRA and PRC. The herbarium specimens had originated from 152 populations (384 individuals in total, mostly from central-European regions, see Electronic Appendix 1). To exclude the influence of specific environmental conditions, all plants were cultivated in the Experimental Garden of the Institute of Botany of the Czech Academy of Sciences in Průhonice. Because central Europe is the core region of the distribution of *P. officinarum* and the contact zone of all its major cytotypes, it is an excellent source of different *P. officinarum* accessions.

Methods

The DNA ploidy level of all cultivated samples was determined by flow cytometry using a Partec Ploidy Analyser PA-II equipped by a UV mercury arc lamp light source and DAPI as the fluorescent dye. Diploid *P. lactucella* (Wallr.) P. D. Sell et C. West plants with verified chromosome counts were used as the internal standard. A standard two-step procedure (Doležel et al. 2007) was used for the sample preparation. Fresh leaves of both the sample and the standard were chopped together using a sharp razor blade in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid and 0.5% Tween 20; Doležel et al. 2007), filtered through a 42-μm nylon mesh, and incubated at room temperature for twenty minutes. One ml of the staining solution consisted of the Otto II buffer [0.4 M Na2HPO4×12H2O], 2-mercaptoethanol (2 μl/ml) and DAPI (4',6-diamidino-2-phenylindole; 4 μg/ml), and the solution was shaken gently. After ten minutes the solution of stained nuclei was analysed. Symmetric peaks were analysed using Partec FlowMax Operating and Analysing Software (version 2.4d; Partec GmbH, Münster, Germany). For each analysis, the coefficient of variation (CV) was calculated for both the internal standard and the sample. Although aneuploidy is extremely rare in entire genus (Krahulcová & Krahulec 2000, Rotreklová et al. 2002), the ratio between the sample and the standard peak was checked to exclude its potential influence. To calibrate the flow cytometric analyses for each ploidy level, the chromosome numbers of eight plants were determined previously (see Mráz et al. 2008).

The reproductive modes of 35 randomly selected tetraploid plants and of all pentaploid and hexaploid plants were ascertained using a routine emasculation test, as described in Krahulcová & Krahulec (1999). The upper parts of unopened capitula (containing the anthers and the stigma) were sliced off with a sharp razor blade, and the flowerheads were bagged in monofilament fabric sacks. Fully developed achenes were considered a result of an apomictic (or, more precisely, parthenogenetic) mode of reproduction (Gadella 1987, Krahulcová & Krahulec 1999).

In total, 28 morphological characters of 384 herbarium specimens (from 152 populations) were measured or scored (see Electronic Appendix 2, 3). The characters, measured or scored, included traits used for the determination of subspecies according to the taxonomic concept accepted by Sell & West (1976). Eleven of the characters measured described the proportions and shape of the vegetative and generative parts. Another group of characters (16) consisted of measured or scored features of the indumentum. Finally, the ratio between leaf length and leaf width was included.

Basic statistical parameters, such as the mean, standard deviation, and the 1st, 5th, 95th and 99th percentiles, were computed for each of the characters. Character data were standardized to zero mean and unit standard deviation. The normality of distribution for each character was tested with the Shapiro-Wilks test as implemented in R (version 3.1.2; R Core Team 2013). All of the included variables showed deviations from a normal distribution. Non-parametric Spearman's rank coefficient was used (characters with strong correlations were omitted).

Principal component analysis (PCA; Krzanowski 1990, Marhold 2011) based on a correlation matrix was applied to reduce the multidimensional nature of the character space and reduce the variation pattern to one expressed by the first two component axes. Subsequently, discriminant analyses (canonical discriminant analyses, CDA, and classificatory discriminant analyses; Klecka 1980) were performed. DNA ploidy level and

breeding mode (in the case of the hexaploids) were used as discrimination characters. Finally, non-parametric k-nearest neighbours classificatory discriminant analyses (one leave-out method) were used to determine the percentage of individuals correctly classified into the predetermined groups. The number of correctly classified individuals was calculated for *k* values from 1 to 30, and the value with the highest success rate was used in further analyses. The multivariate analyses of the morphometric dataset were performed with R scripts described in Koutecký (2015) and PAST software (v. 2.17c; Hammer et al. 2001) and the results were visualized in the standard spreadsheet program Excel (ver. 2010, Microsoft).

Results

Ploidy level analysis

The DNA ploidy level was determined for 384 plants from 152 populations. The coefficient of variation (CV) ranged from 1.67% to 3.21% (with a mean of 2.52% and a standard deviation of 0.46). Among ploidy-uniform populations, the tetraploid cytotype prevailed (78 populations; 51%), followed by the hexaploid (34; 22%) and then the pentaploid cytotype (25; 17%). In total, 159 tetraploid, 83 pentaploid and 142 hexaploid plants were analysed. Fifteen mixed-ploidy populations (10%) were included: 4x + 5x – three populations, 4x + 6x – three populations and 5x + 6x – nine populations.

Breeding mode test

All tetraploid plants analysed were sexuals occurring in the western part of the sampling area (i.e. the Czech Massif) whereas pentaploids were mostly (97.6%) apomictic and sampled in the Western Carpathians and Pannonia. The only sexually breeding pentaploids were found in the Vltava river canyon in the Czech Massif (two plants out of two populations mixed with hexaploids). By contrast, hexaploid individuals were either apomictic (50 individuals/36 populations) or sexual (92 individuals/51 populations) with allopatric geographical distribution patterns. Apomictic hexaploid plants occurred in habitats of many types across large areas (predominantly the Western Carpathians and Pannonia) whereas the sexual hexaploid cytotype was strictly connected to steep river canyons of the Czech Massif (see Electronic Appendix 4).

Distance-based morphometrics

No correlation between two characters exceeded the Spearman coefficient value of 0.95. Principal component analysis indicated three partly overlapping groups of individuals. Tetraploid and hexaploid plants were separated along the first component axis (explaining 22.5% of the variation) whereas the second axis (explaining 9.9% of the variation) partly distinguished the pentaploid group (Fig. 1). Characters most tightly correlated with the component axes are presented in Electronic Appendix 5.

Canonical discriminant analysis (CDA) further underlined that tetraploid and hexaploid individuals were morphologically well separated; however, pentaploids were still interconnecting them (Fig. 2). The first canonical axis separated tetraploids and hexaploids whereas the second canonical axis was responsible for the partial separation

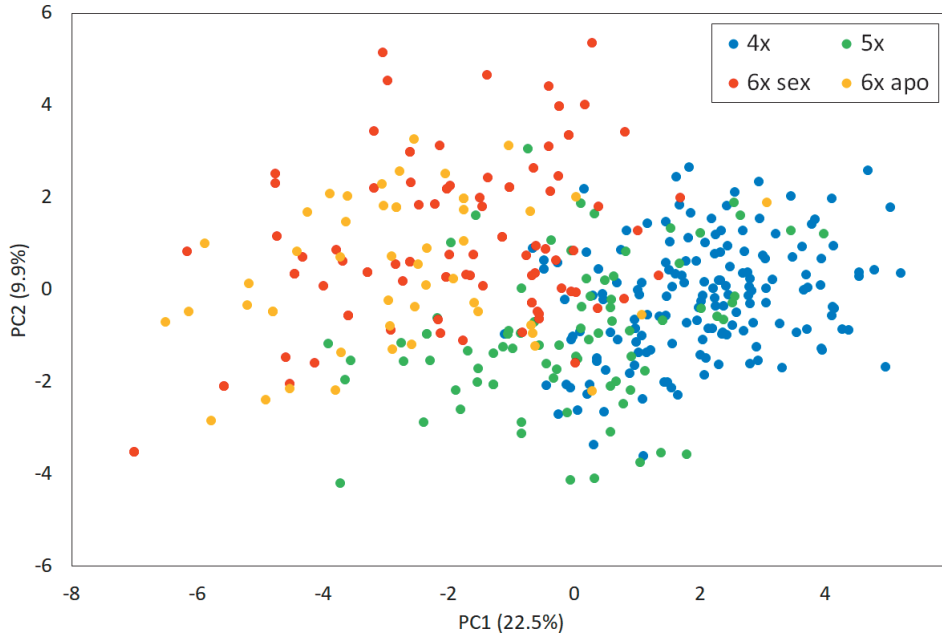

Fig. 1. – Principal component analysis (PCA) ordination diagram (for the entire dataset of 384 *Pilosella officinarum* individuals, based on 28 characters) illustrating the partial separation of *P. officinarum* cytotypes (4x, 5x and 6x); the first and second component axis express 22.5% and 9.9% of the variation, respectively.

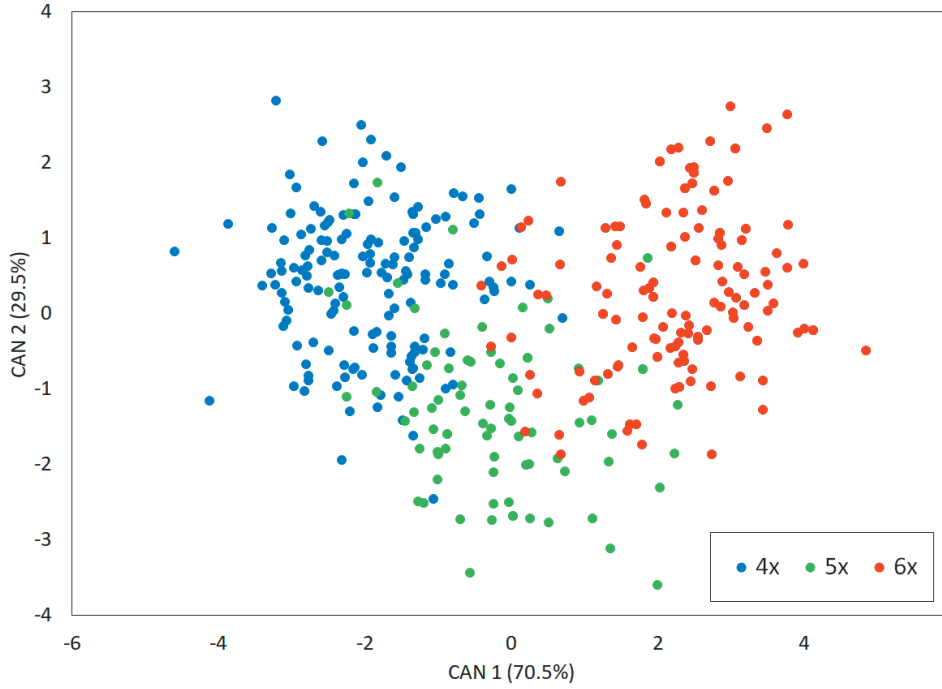

Fig. 2. – Canonical discriminant analysis diagram (for the entire dataset of 384 *Pilosella officinarum* individuals, based on 28 characters); the first and second canonical axis express 70.5% and 29.5% of the intergroup variation, respectively.

of pentaploid individuals. Thus, the discriminant analysis confirmed the intermediate character of the pentaploid cytotype. The first and second canonical axis expressed 70.5% and 29.5% of the intergroup variation, respectively.

Stolon curve shape (SC), stellate hair density on the stem (SES), average of three stolon diameters (SD) and average lengths of five involucral bracts (BL) were the most strongly correlated with the first canonical axis whereas the characters based on glandular trichomes (glandular trichome density rate on the stem, ES, and darkness of the involucrum, ID) were the best correlates of the second canonical axis (Electronic Appendix 5).

Classificatory discriminant analysis of three ploidy levels resulted in the correct classification of 82.0% of all individuals (k = 22); however, the rate of correct classification differed markedly between the cytotypes (Table 1). Tetraploids and hexaploids were, for the most part, classified correctly (for 4x 98.1% and for 6x 82.3% of correct classifications) whereas the classification of pentaploids failed in more than half of the cases (42.2% of correct classifications). In a separate classificatory discriminant analysis of tetraploids and hexaploids, 100% of tetraploids and 90.9% of hexaploids were classified successfully.

The reproductive mode was included in the analysis to distinguish plants belonging to the sexual or the apomictic hexaploid cytotype. Canonical discriminant analysis of all four groups (4x, 5x, 6x sex and 6x apo) indicated a slightly different pattern of sexual vs apomictic hexaploids. Therefore, a separate CDA was performed with the dataset reduced to hexaploids only. Both of the groups were markedly separated with minimum overlap (Fig. 3). The characters rate of dark trichome density on the involucrum, density rate of stellate trichomes on the involucrum and rate of stolon curve shape were most closely correlated with the first canonical axis. Subsequently, non-parametric classificatory discriminant analysis indicated the correct classification of 82.0% of individuals (Table 1).

Table 1. – Results of non-parametric classificatory discriminant analysis expressed by rates of correctly classified individuals (k = 22).

Taxon	4x	5x	6x	n	Correct (%)
4x	156	3	0	159	98.1
5x	34	35	14	83	42.2
6x	11	7	124	142	87.3
Total	201	45	138	384	82.0

Discussion

Our study has revealed that the three major ploidy levels (4x, 5x and 6x) of *Pilosella officinarum* exhibit partly distinct reproductive and morphological patterns. Each of the ploidal cytotypes has a specific reproductive strategy, hexaploids being divided into two distinct groups based on the mode of reproduction. Knowledge of reproductive modes thus explains some important aspects of the cytogeographic pattern.

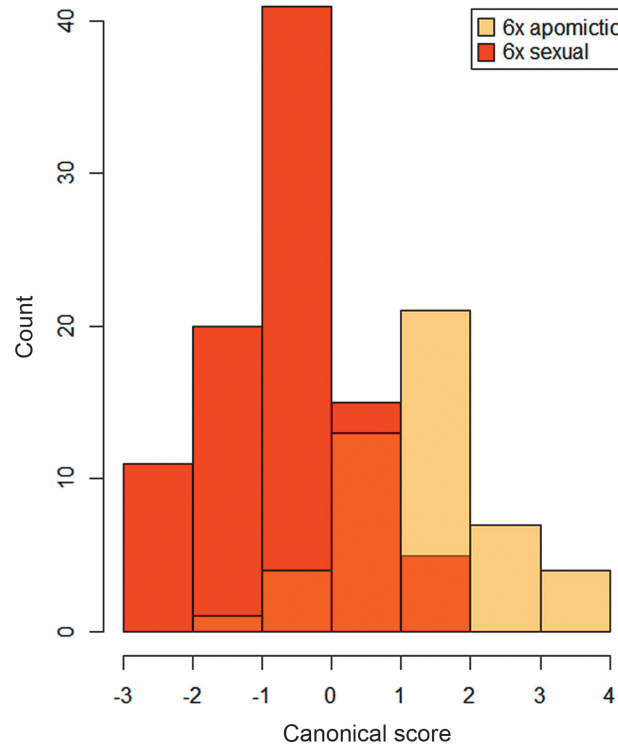

Fig. 3. – Canonical discriminant analysis histogram, based on 28 characters, for 92 sexual (red) and 50 apomictic (yellow) hexaploid *Pilosella officinarum* individuals.

Mráz et al. (2008) found the distribution of *P. officinarum* cytotypes to be markedly structured. Whereas tetraploids are nested in a central part of the species area, pentaploids and hexaploids occur in deglaciated areas of Scandinavia and the British Isles, and in South-eastern Europe. The core area of the species' distribution, occupied by sexual tetraploids, is surrounded by an area with the distribution of higher polyploid apomicts, which perfectly matches the frequently studied phenomenon of geographic parthenogenesis (e.g. Hörandl 2006, Mráz et al. 2019). In addition to already known major ploidal cytotypes we identified a sexual hexaploid cytotype, which occurs in habitats of a specific type, namely on rocky outcrops of river canyons in Czechia. Canyon sexual hexaploids could be related to other already known sexual hexaploid populations growing in the subalpine zone of the central and western Alps, described as *Pilosella officinarum* subsp. *velutina* (Hegetschw.) H. P. Fuchs (Gadella 1984, 1991). On the other hand, the habitus of *P. o.* subsp. *velutina* is distinct (especially in the indumentum; Zahn 1923). Nevertheless, both kinds of hexaploids occur in primary forest-free habitats and may therefore be suspected of being glacial relicts. Our reproductive mode test revealed two sexual pentaploid plants, which originated in the contact zone between sexual hexaploids and tetraploids. They could therefore have resulted from heteroploid hybridization between the aforesaid ploidal cytotypes, but the direction of such crossing remains unclear (similarly as reported by Krahulcová & Krahulec 2000). Breeding strategies are

frequently linked to particular ploidal cytotypes of agamic groups. However, in contrast to our results, the diploid cytotype is usually sexual whereas polyploids reproduce predominantly via apomixis (e.g. *Hieracium* – Mráz et al. 2019, *Sorbus* L. – Lepší et al. 2019, *Potentilla* L. – Dobeš et al. 2013). A comparably complex reproductive pattern (involving a variable degree of facultative apomixis and rare reproductive pathways) has been revealed in the genus *Rubus* L. (Šarhanová et al. 2012).

Our multivariate statistical analyses prove the utility of a combination of morphological characters for the identification of *P. officinarum* cytotypes. Our classificatory discriminant analysis revealed a relatively high rate of correctly classified individuals, even though the intermediate pentaploid group markedly overlapped with tetraploids and hexaploids. Discriminating between the pairs of ploidal groups may be effective even in practice, but the overlapping pentaploid group frequently would allow only partial determination of cytotypes (i.e. not tetraploid and not hexaploid). Nevertheless, the different habitus of the cytotypes may be attributable not only to polyploidy, but also to hybridization with some other *Pilosella* species, followed by introgression. This has been documented by several studies focused on hybrid swarms of *Pilosella* species (Krahulcová et al. 2012, Urfus et al. 2014).

The partially distinct morphological, reproductive and cytotypic pattern of central European *P. officinarum* may constitute a basis for an alternative intraspecific concept. The only hitherto used taxonomic concept is based on combinations of morphological characters (glandular indumentum of involuclar bracts; e.g. Zahn 1921, 1922, 1923, Sell & West 1976). By contrast, the most important characters indicated by our PCA and CDA analyses are vegetative (leaf shape, stolon shape and diameter) and the only generative character (density of glandular trichomes on the stem) correlates with the second canonical axis (contributing to the separation of pentaploids; see Fig. 2).

Moreover, traditional infraspecific concepts consider neither reproductive modes nor ploidy. The results of our morphometric analyses indicate that ploidal and reproductive cytotypes are natural units (based on their distinct distribution and morphology). Knowledge of the reproductive mode is important because the approach to evaluating and classifying agamic and sexual groups should differ (see e.g. Lepší et al. 2015). Unfortunately, a critical biosystematic re-evaluation of Zahn's original infraspecific concept (Zahn 1921, 1922, 1923) is unfeasible because living accessions for each of the 624 described subspecies of *P. officinarum* would be difficult or impossible to obtain, as they were described almost a century ago). Moreover, a significant part of type specimens of Zahn's concept got lost, probably for good, during the Second World War, in which several major herbaria were destroyed (see e.g. Merrill 1943). We hope that a comprehensive revision of *Pilosella officinarum* could finally propose a new and more practical intraspecific concept.

Conclusions

The three predominant ploidal cytotypes (4x, 5x and 6x) of *Pilosella officinarum* follow contrasting reproductive strategies. Whereas tetraploids are exclusively sexual, pentaploids reproduce predominantly via apomixis. The hexaploid cytotype is shared by sexual and apomictic plants with distinct allopatric distribution.

Distance-based morphometrics revealed marked differences between tetraploids and hexaploids, both of which are partially overlapped by pentaploids. In addition, apomictic hexaploids can be distinguished from sexual hexaploids. Variation in ploidy and reproductive mode may therefore constitute a basis for a revised intraspecific concept of *P. officinarum.*

See www.preslia.cz for Electronic Appendices 1–5.

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Souhrn

Druh *Pilosella officinarum* zahrnuje převážně tetraploidní (2n = 36), pentaploidní (2n = 45) a hexaploidní (2n = 54) cytotypy, které se mohou rozmnožovat jak sexuálně, tak apomikticky. Jeho dosavadní taxonomické vnitrodruhové členění bylo postaveno na jednotlivých apomiktických liniích. Z důvodu velkého množství popsaných taxonů je však současné pojetí matoucí a nelze jej použít. Testovali jsme reprodukční způsoby reprezentativního souboru rostlin ze střední Evropy, které byly pěstovány v jednotných experimentálních podmínkách. Každá ploidní úroveň měla specifickou vazbu na reprodukční způsob (4x – sexuální, 5x – převážně apomiktické a 6x – sexuální a apomiktické). Zatímco sexuální tetraploidi se vyskytovali v západní části vybraného areálu (Český masiv), apomiktičtí pentaploidi a hexaploidi převažovali ve východní části (Západní Karpaty a Panonie). Vedle toho byla rozeznána odlišná skupina hexaploidů, vázaných na strmé kaňony řek Českého masivu). Pomocí morfometrické analýzy jsme hledali odlišné znaky studovaných cytotypů. Výsledky potvrdily morfologické odlišnosti mezi rostlinami různých ploidií a v případě hexaploidů i dle reprodukčního způsobu. Znalost ploidní úrovně a reprodukčního způsobu je tedy zásadní pro osvětlení spletité vnitrodruhové struktury *Pilosella officinarum.*

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