



Cytotype and morphological study of two widespread taxa within *Pulmonaria officinalis* agg. (Boraginaceae) in Central Europe: the natural and anthropogenic contact zones with confirmation of a new hybrid

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Abstract

The distribution of cytotypes and their contact zones provides insights into the spatial, morphological, and ecological differentiation of individual lineages, as well as their rates of reproductive isolation, gene flow, and/or selection. In this study, we focused on the *Pulmonaria officinalis* group, which comprises two widely distributed and morphologically similar species, *P. obscura* and *P. officinalis*, that differ in chromosome number. Intermediate plants, often of questionable identity, are rarely reported from mixed populations. In addition, several ornamental cultivars exhibit morphological similarities to *P. officinalis*. We employed flow cytometric relative genome size estimation, chromosome counting, morphometric analysis, and pollen staining methods to evaluate cytotype distribution, morphological variation, and to confirm the hybrid origin of intermediate plants and their frequency. We identified two main cytotypes, each associated with specific taxa, i.e. *P. obscura* ($2n = 2x = 14$), predominant in the northern regions, and *P. officinalis* s. str. ($2n = 2x = 16$), more common in southern areas of Central Europe. We also occasionally detected DNA-triploids in natural populations of both taxa and confirmed an intermediate cytotype ($2n = 15$), mainly in mixed populations of *P. obscura* and *P. officinalis* in natural contact zone, although such populations were rare. The intermediate cytotype displayed differences in relative genome size and chromosome number, as well as several morphological characters (e.g. calyx length and calyx indumentum) compared to the parental taxa. These plants also have lower fertility, with pollen viability ranging from 2 to 33%, compared to their parents, which had pollen viability 70–98%. On the basis of these results, including the confirmation of the hybrid status and the ability to distinguish these plants from both parental taxa, we describe a new hybrid, *Pulmonaria* × *superflua* Vejvodová and Koblřová, as a hybrid between *P. obscura* and *P. officinalis* s. str. We also revised the frequency of non-native populations (garden escapes) and explored their potential coexistence with native populations of the *P. officinalis* complex and forming of the anthropogenic contact zone.

Keywords Contact zone · Cytoecography · Flow cytometry · Hybridisation · Morphology · Pollen viability

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Introduction

The distribution and contact zones of species provide insights into their colonisation potential and interactions, including gene flow, genetic innovations, selection, and/or reproductive isolation barriers (Harrison and Rand 1989). When the position of the contact line is unaffected by environmental factors and depends solely on the balance between dispersal and selection against hybrids, it is characterised as a poorly structured line, known as a "tension zone" (Barton and Hewitt 1985). In contrast, when taxa occupy distinct ecological niches, their contact zone typically aligns

with environmental gradients, producing a patchy structure referred to as a "mosaic zone" (Endler 1977; Harrison and Rand 1989).

In any case, a critical outcome of interactions between taxa forming a contact zone can be the process of gene flow, which occurs through hybridisation (e.g. Abbott et al. 2003). However, this process can be limited by various environmental conditions, including population density and size (Endler 1977; Barton and Hewitt 1985), as well as by internal selection pressures related to breeding barriers (Husband et al. 2002; Lexer and van Loo 2006). These barriers include asynchronous flowering, inhibition of interspecific pollination, reduction of odd seedlings, and non-random pollen transfer (summarised in Castro et al. 2011). As a result, hybrid formation is typically rare (e.g. Wolf et al. 1990; Van Dijk et al. 1992; Castro et al. 2011; Wachowiak et al. 2015). In addition, gene flow may also be limited by low pollen viability of hybrid plants within contact zones (Husband et al. 2002; Li et al. 2015). This phenomenon can result from genomic incompatibility or chromosomal mutations between the parental species (Rieseberg and Carney 1998).

Due to the ongoing transfer of genetic material between parents, parts of the genome from one parent can be incorporated into the other (Li et al. 2015; Wachowiak et al. 2015). This gene flow can result in a continuum of traits within the hybrid zone, bridging the traits of the parental lineages. The resulting hybrids can then represent intermediate types (e.g. Lumaret et al. 1987; Van Dijk et al. 1992; Li et al. 2015; Wachowiak et al. 2015). However, distinguishing between parental and hybrid lineages can be challenging due to the plasticity of parental lineages in response to environmental conditions (Meyer 1987).

In this study, the special attention was given to the most common and quite variable group of European species in the genus *Pulmonaria* L. (Boraginaceae), i.e. the *P. officinalis* agg. (e.g. Merxmüller and Sauer 1972; Sauer 1975; Bolliger 1978; Hultén and Fries 1986; Bennett 2003). This species complex predominantly comprises two closely related diploid taxa (Kirchner 2004; Meeus et al. 2016), *P. obscura* Dumort., and *P. officinalis* L., that are morphologically and ecologically quite similar (see below). In contrast, there is a rather striking karyological variability, as *P. obscura* is characterised by $2n = 14$, whereas *P. officinalis* has two extra chromosomes in its karyotype (i.e. $2n = 16$; e.g. Sauer 1975, 1986; Dersch 1994; Kobrlová et al. 2024). The two species also differ in their relative and absolute genome sizes. *P. obscura* exhibits $2C = 2.92$ pg, while *P. officinalis* exhibits $2C = 3.21$ pg (Kobrlová et al. 2024).

Within the genus, the *P. officinalis* agg. is morphologically well characterised by the summer (or basal) leaves that are mostly cordate at the base, covered with long bristles and small aculeoli on the upper surface (Merxmüller and Sauer 1972; Sauer 1975, 1986; Bolliger 1978; Dersch 1994).

However, the two species are often difficult to distinguish, especially in the non-flowering state, as both species are highly variable in vegetative characters (summarised in Vejvodová et al. 2021). This intraspecific morphological variability is reflected in an inconsistent taxonomic concept of this group across European floras (e.g. Damboldt et al. 1975; Hultén and Fries 1986), but the treatment of the taxa as two separate, distinct species is now widely accepted (e.g. Májovský and Hegedüšová 1993; Fischer et al. 2008; Király 2009; Schmeil and Fitschen 2009; Tison and de Foucault 2014; Jäger 2017; Lauber et al. 2018; Hroneš and Kobrlová 2019; POWO 2025). According to the recent study by Vejvodová et al. (2021), the most reliable characters for identifying these two species are the shape of the calyx, the calyx indumentum and the shape and indumentum of the summer leaves. On the other hand, the traditional emphasis on summer leaf spotting (*P. obscura*: unspotted or barely visible light green spots vs. *P. officinalis*: clearly distinct vivid green or whitish spots, summarised in Vejvodová et al. 2021), does not seem to be fully reliable (field observations and the revision of herbarium specimens, Kobrlová and Hroneš, unpubl. data). Besides, two subspecies of *P. officinalis* are currently recognised in the Italian flora (Cecchi and Selvi 2015, 2017; POWO 2025). Apart from the nominal subspecies *P. officinalis* subsp. *officinalis*, there is *P. officinalis* subsp. *marzoliae* G.Astuti, Peruzzi, Cristof. & P.Pupillo ($2n = 16$), which was described from the high-elevation forests of the calcareous-dolomitic mountains of the Trentino region. It is an endemic taxon to the Italian forests in this area (Selvi et al. 2023). The subspecies primarily differs in the shape of the basal leaves, the leaf spotting (sparse, dark, and greenish spots) and hair types on the upper surface of the summer leaves (see Astuti et al. 2014). As it was not part of this study, we will therefore refer to all populations of *P. officinalis* s. str. only as *P. officinalis*.

In terms of distribution, the *P. officinalis* agg. is one of the most widespread species groups of the *Pulmonaria* genus (Merxmüller and Sauer 1972; POWO 2025), occurring mainly in the temperate regions of Europe (Sauer 1975; Hultén and Fries 1986; POWO 2025). Traditionally cultivated as an ornamental or medicinal plant (Bennett 2003; Scott 2008), *P. officinalis* (in particular) was naturalised in some areas, such as the British Isles (Meeus et al. 2013), further contributing to its widespread presence and anthropogenic contact zone with native populations. The native range of this group covers most of Europe, being less common or completely absent in Western (esp. the Iberian Peninsula, west of France) and Northern Europe (i.e. the Scandinavian Peninsula) and the southern parts of the Apennine and Balkan Peninsulas (Merxmüller and Sauer 1972; Sauer 1975; Gracia and Castroviejo 2012; Tison and de Foucault 2014; Cecchi and Selvi 2015, 2017; POWO 2025). The main centre of diversity is Central Europe, where the areas of both

species overlap (Sauer 1975; Hultén and Fries 1986; Kobrlová et al. 2024; POWO 2025) and form a natural contact zone. Comparing the two species, the native range of *P. obscura* is wider and more northern European, including the south-west France, most of Central Europe, extending northwards to the southern part of Scandinavia and eastwards to the Urals. Toward the south, this species is gradually disappearing and being replaced by *P. officinalis* (Merxmüller and Sauer 1972; Sauer 1975; Hultén and Fries 1986). *Pulmonaria officinalis*, on the other hand, appears to be mainly native to Central Europe, particularly in the wider area of the Eastern Alps, and only reaches the north of the Balkan Peninsula (Sauer 1975; Hultén and Fries 1986; Meeus et al. 2016). It is relatively rare in the northern part of Europe, occurring mainly in the area south of the Alps, where *P. obscura* is absent or very rare (Hultén and Fries 1986).

As the ranges of *P. obscura* and *P. officinalis* partially overlap and the species occupy the same habitats (esp. shaded, often moist and humid woodland habitats; Meeus et al. 2013), records of both species co-occurring were documented in some regions of Central Europe (Merxmüller and Sauer 1972; Bolliger 1978; Dersch 1994; Vejvodová et al. 2021). However, the local coexistence of *P. obscura* and *P. officinalis* in mixed populations was only rarely reported (Bolliger 1978; Dersch 1994; Jarošová 2006; Kobrlová et al. 2024). Also, according to a review of the published chromosome numbers of *P. officinalis* agg. in Europe (Kobrlová et al. 2024), in the vast majority of cases, only a single cytotype per population was evidenced. In fact, the true proportion of mixed populations may be underestimated or not detected at all due to the high morphological variability (summarised in Vejvodová et al. 2021) and/or methodological limitations of classical chromosome counting, which restricts the number of samples that can be analysed. Similarly, the occurrence of potential hybrids could also remain hidden. So far, plants with an intermediate number of chromosomes (i.e. $2n=15$) in the area of co-occurrence of *P. obscura* and *P. officinalis* were only identified in several populations in Germany (Dersch 1994), Switzerland (Bolliger 1978) and in one population in the Czech Republic (Kobrlová et al. 2024). Detailed, large-scale screening in the contact zone may therefore provide a clearer picture of the spatial distribution pattern of *P. obscura* and *P. officinalis* and reveal the extent of hybridisation between these two taxa.

The present study investigates in detail the diversity and distribution patterns of the *P. officinalis* agg. in Central Europe, with the aim of exploring the spatial distribution of cytotypes in their contact zone. Specifically, we address the following questions: (1) What is the native range of *P. obscura* and *P. officinalis*? (2) What are the dominant cytotypes and how are they distributed in the contact zone? (3) How common are mixed populations of *P. obscura* and

P. officinalis? Are there indications that gene-flow might be occurring between the two taxa? Is it possible to detect hybrids in mixed populations? (4) How common are non-native populations (i.e. originally cultivated plants and/or possible garden escapes that morphologically resemble *P. officinalis* or *P. obscura*.)? Do these populations coexist with native populations of the *P. officinalis* agg.?

Materials and methods

Field sampling

Plant material was primarily collected from natural populations in Central Europe, where *P. officinalis* agg. is widely distributed. Areas with reported occurrences of both species, i.e. potential contact zones, were given special attention. In addition, several non-native populations (here listed as "*P. officinalis* agg. cultivated") were also sampled. This category includes originally cultivated plants and/or possible garden escapes morphologically resembling *P. officinalis* s. str., as well as all populations found near human settlements, whose origin may be unclear. Plants with many and intense white spots on the leaves (often erroneously referred to as *P. saccharata*) were not part of this study and will not be discussed further, as they most likely represent a hybrid cultivar of unknown origin (Kobrlová et al. 2024). A total of 1184 plants from 396 populations were examined during the years 2016–2025 (Online Resource 1a). Plants collected in anthropogenic habitats (i.e. ruderal sites in towns, around the gardens, roads, or old pilgrimage sites) were excluded from morphometric measurements and were used only for flow cytometry and pollen viability analyses due to uncertainty about their origin. The herbarium specimens are deposited in the herbarium of the Department of Botany, Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic (CBFS).

Table 1 Morphological characters and their abbreviations used in this study

Quantitative characters:	
GBC	Length of glandular hairs on the base of the calyx
SBC	Length of setae on the base of the calyx
UCW	Width of the unfolded calyx
CL	Calyx length
Ratios:	
RBC	Length of setae / glandular hairs at base of calyx
RC	Length / width of calyx

Flow cytometry and chromosome counts

The DNA ploidy level and relative genome size (RGS; Suda et al. 2006) were estimated using a CyFlowSpace instrument (Sysmex-Partec, Germany), with DAPI (4,6-diamidino-2-phenylindole) staining. Samples were prepared according to the protocol described by Vejvodová et al. (2021). *Bellis perennis* (2C = 3.38 pg; Schönswetter et al. 2007) was used as the internal standard.

RGS differences among the main cytotypes, i.e. *P. obscura*, *P. officinalis*, and their putative hybrids, were also statistically tested in R 4.4.0 (R Development Core Team 2024). Samples were clustered based on RGS using finite mixture Gaussian distribution modelling. The FitGMM function from the MGMM package (McCaw 2021) was used. The number of components (number of groups modelled, i.e. parameter *k*) was set to two, and the maximum number of iterations (parameter *maxit*) was set to 10⁶. All other parameters were left at their default values. Only measurements with the coefficient of variation (CV) of the samples and the standard below 3.5% (i.e. 780 samples) were used to ensure the highest possible accuracy. Boundaries between the two modelled groups were then defined as the 1st and 99th percentiles of the corresponding Gauss curves. Individuals not falling within the defined range were assumed to be hybrids.

Chromosome numbers were counted following the protocol of Koblrová et al. (2024). The preparations were stained with DAPI (Vectashield Mounting Medium with DAPI). Slides were examined with an Axio Imager Zeiss fluorescence microscope (Zeiss, Oberkochen, Germany). A minimum of ten mitotic metaphase plates per slide were captured by the ISIS software 5.4.7 (Metasystems). The chromosome counts were used as reference material for the estimates obtained by flow cytometry.

Pollen viability estimation

Pollen staining using the Alexander's staining method (Peterson et al. 2010) was evaluated as an indirect method to determine pollen viability. In total, pollen viability was estimated for 50 plants. The effort was to select a balanced set of plants belonging to the main native cytotypes, i.e. *P. officinalis*, *P. obscura*, and their putative hybrid. Pollen from mature anthers of one to three crowns of each plant was mechanically extracted on a glass slide and then stained. The proportions of viable and aborted pollen grains from (100–)250(–500) grains per plant were counted under the microscope, with a random selection of the evaluated area of the preparation. The analysis of statistical differences between individual cytotypes was performed using the Kruskal–Wallis ANOVA followed by pairwise t-tests with

the false discovery rate correction for multiple comparisons in R 4.4.0 (R Core Team 2024).

Geographic distribution of cytotypes

All sampled populations were georeferenced and displayed in ArcGIS 3.0.3 Pro software (Esri 2022). The distribution maps were also supplemented with data from the previous studies by Vejvodová et al. (2021) and Koblrová et al. (2024), including published chromosome counts (see Online Resource 1b).

The distribution of cytotypes in *P. obscura*, *P. officinalis*, and their putative hybrids was interpolated on the basis of flow cytometry (new data and data from previous studies: Vejvodová et al. 2021; Koblrová et al. 2024) and karyology (new data and reports from literature, Koblrová et al. 2024), using the Natural Neighbour method in ArcGIS 3.0.3 Pro software. Only native populations were considered. For the analysis, chromosome numbers counted in several individuals of each cytotype were extrapolated to all individuals of that cytotype and used as the input (*z*) value of the resulting grid. The area of occurrence (dominance) of each species was then defined as one-third of the interval of values of the resulting raster grid, i.e. 14.00–14.66 represented *P. obscura*, and 15.33–16.00 represented *P. officinalis*. The intermediate values, i.e. 14.66–15.33, were characterised as the potential contact area in which neither species dominated.

The distribution patterns of "*P. officinalis* agg. cultivated" plants were analysed separately and only as an overview of the populations detected, not as a detailed study of the cytoecography of these plants. The aim of the study was an interest in the co-occurrence with native populations, or possibly some evidence of hybridisation.

Classical morphometric analysis

According to the results of Vejvodová et al. (2021), we focused only on the evaluation of the most reliable morphological characters (calyx shape, calyx indumentum, and summer leaf shape, Table 1). The morphometric analysis was carried out on *P. obscura* and *P. officinalis*, as well as their putative hybrids. The selection of plants for this part of the study was based on the results of the flow cytometry analysis and pollen viability test, in which species identification was clearly defined. The specific characters that showed the strongest correlation with the individual taxa was selected (see Vejvodová et al. 2021), i.e. the length of calyx (CL), the length of glandular hairs and setae at the base of the calyx (GBC, SBC). Apart from them, the width of the unfolded calyx (UCW; Online Resource 3a) was added. Two ratios were also evaluated, i.e. length of setae/glandular hairs at

base of calyx (RBC) and length/width of calyx (unfolded; RC). Three calyces were measured from each plant (where possible) and the values obtained were used to calculate the means. Measurements were performed using a stereo microscope Zeiss Stemi 2000-C in the AxioVision v. 4.9.1 software (Carl Zeiss Microscopy 2013). In order to have a more robust dataset, the results obtained here were combined with those of Vejvodová et al. (2021).

As the unfolded calyx width (UCW) character could not be added to the original dataset, two separate datasets were analysed, i.e. dataset A (53 populations/160 individuals) with the original and newly added populations with only those characters used in Vejvodová et al. (2021), and dataset B (30 populations/92 individuals) with the newly measured populations only, in which all the above characters (including the new one, i.e. UCW) were measured. Both datasets were analysed separately. All variables were transformed using the Box-Cox method to improve normality prior to analyses that assume normally distributed variables (PCA).

General patterns of morphological variation in the datasets were visualised using the principal component analysis (PCA). The power of the morphological characters to discriminate between the two species was tested using the linear discriminant analysis (LDA). The characters that contributed significantly to the discrimination of the cytotypes, selected by forward selection, were then used to perform a classificatory discriminant analysis (CDA). The analyses were performed using Morphotools (Koutecký et al. 2015).

The relationship between the morphological characters and the geographical location of the samples was tested using redundancy analysis (RDA) in the R package vegan (Oksanen et al. 2025). The four characters from dataset A (i.e. CL, GBC, SBC, USW) were used as the explanatory variables and the geographical coordinates of the samples (i.e. latitude and longitude) as response variables. Subsets for each cytotype were tested separately. Response variables were normalised using the Box-Cox transformation and scaled to zero mean and unit variance prior to the analysis.

Table 2 Overview of all cytotypes of *Pulmonaria officinalis* agg. and their relative DNA content (RGS) assessed by flow cytometry, including populations of uncertain origin and/or possible garden escapes (here listed as *P. officinalis* agg. cult.)

	N pop	N ind	RGS—mean	RGS—min	RGS—max	RGS—S.D.	2n	2n—N ind
<i>P. officinalis</i> agg.:	343	1023						
<i>P. obscura</i>	239	646	0.76	0.72	0.80	0.02	14	1
<i>P. obscura</i> —DNA triploid	2	5	1.15	1.12	1.17	0.02	—	—
<i>P. officinalis</i>	107	355	0.86	0.82	0.90	0.02	16	2
<i>P. officinalis</i> —DNA triploid	1	2	1.30	1.29	1.31	0.02	—	—
<i>P. officinalis</i> × <i>P. obscura</i>	3	15	0.80	0.79	0.81	0.01	15	3
<i>P. officinalis</i> agg. cult.:	62	161						
- <i>P. obscura</i> -like cytotype	3	10	0.75	0.74	0.77	0.01	—	—
- <i>P. officinalis</i> -like cytotype	54	135	0.86	0.83	0.91	0.02	—	—
- <i>P. officinalis</i> -like cytotype × <i>P. obscura</i>	11	16	0.80	0.80	0.82	0.01	—	—

N pop. number of populations analysed, N ind. number of individuals analysed, RGS—mean mean of the relative genome size (RGS), RGS—min/max/S.D. minimum, maximum, and standard deviation of the RGS, 2n. number of chromosomes, 2n—N ind. number of individuals with counted chromosomes. All RGS values were calculated relative to the internal standard *Bellis perennis*

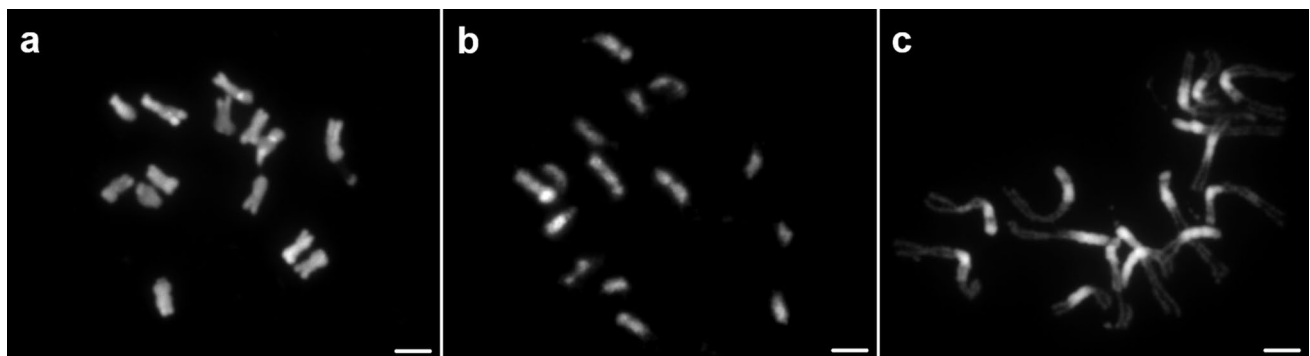


Fig. 1 Mitotic metaphase chromosomes of **a** *Pulmonaria obscura*: 2n=14 (ID B469; CZ), **b** *P. obscura* × *P. officinalis*: 2n=15 (ID K653; CZ), and **c** *P. officinalis*: 2n=16 (ID B470; CZ). For locality ID see Online Resource 1. Chromosomes were counterstained with DAPI. Bars = 5 μm

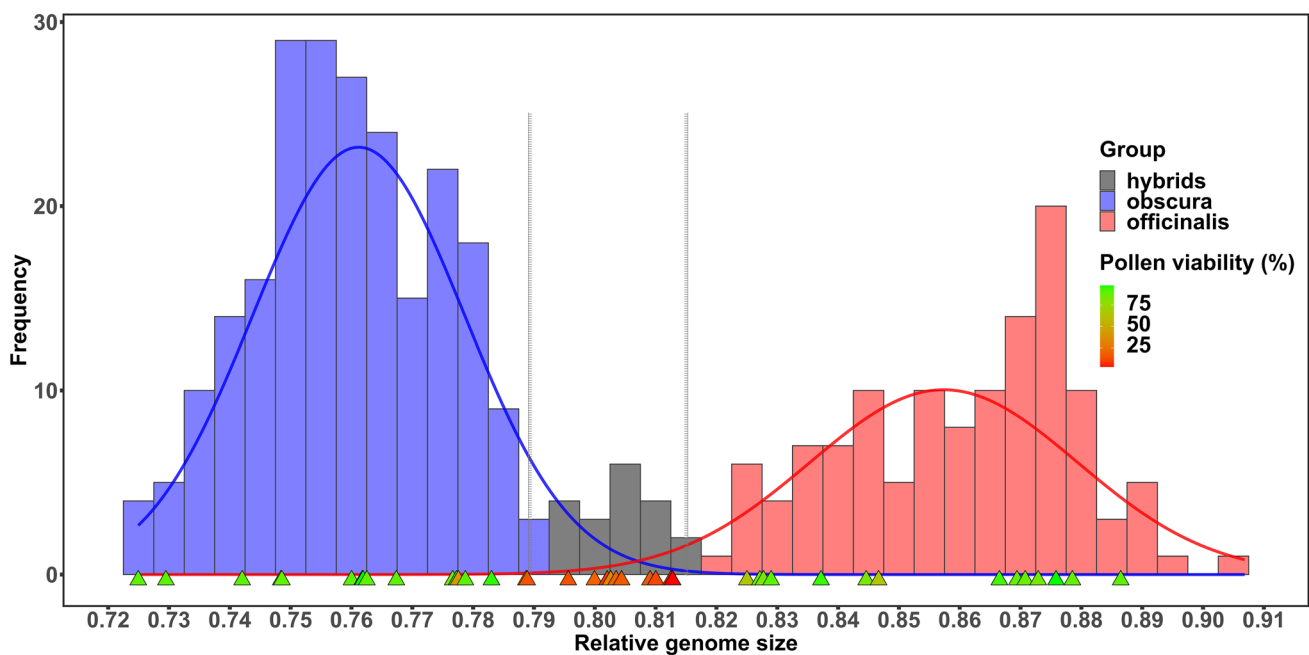


Fig. 2 Range of relative genome sizes (RGS) and their frequencies of the two main cytotypes detected, corresponding to *Pulmonaria obscura* ($2n=14$; BLUE) and *P. officinalis* ($2n=16$; RED), with their hybrids outlined ($2n=15$; GREY). The curves show the fitted finite mixture Gaussian distributions. The grey jagged vertical lines show

the 1st and 99th percentiles of the Gaussian curves. The triangles displayed at the bottom of the histogram show pollen viability, where each triangle represents a single plant for which pollen viability was determined

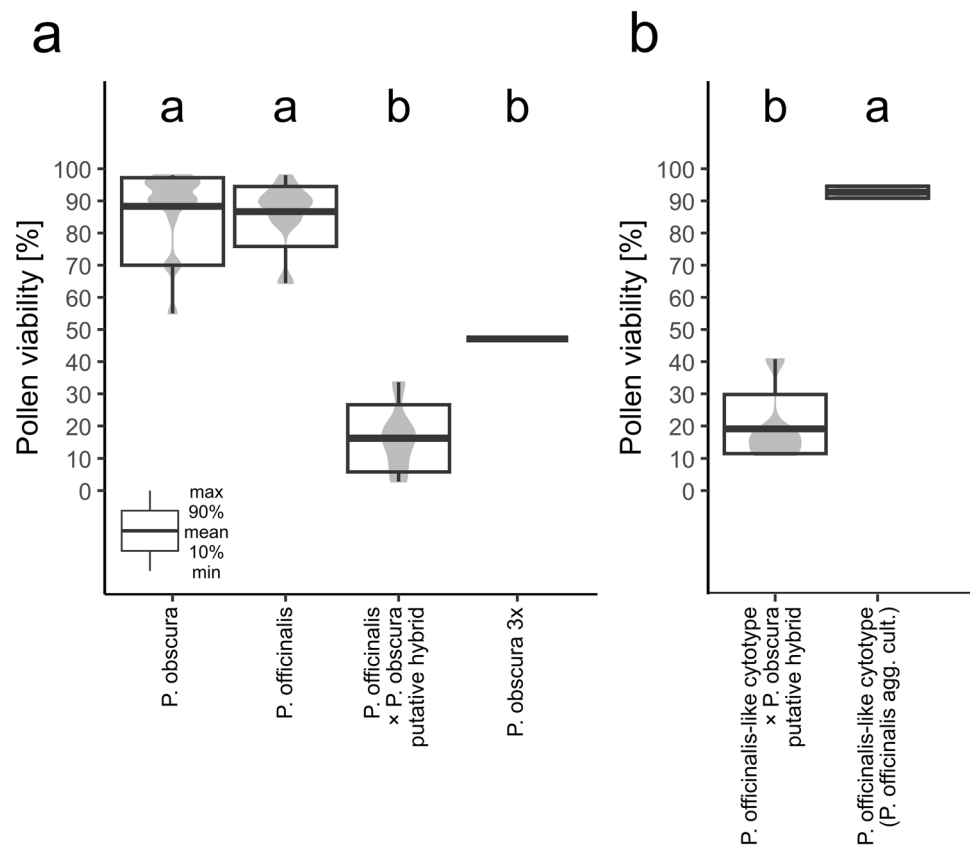
The significance of the model was tested. If statistically significant, the marginal and conditional effects (in a forward selection) of each character were tested in a forward selection. All significance tests were performed using a permutation test with 999 permutations. The values of the significant characters were interpolated for the study area using bicubic spline interpolation and visualised on a map using the R package *akima* (Akima and Gebhardt 2022).

The datasets were also re-evaluated using classification trees, a non-parametric alternative to discriminant analysis. Decision trees use hierarchical classification by sequential univariate partitioning, resulting in easily interpreted tree diagrams (Breiman et al. 1984). Although not widely applied in plant taxonomy, this method was shown to be effective in several taxonomic studies (e.g. Joly and Bruneau 2007; Depypere et al. 2009; Kúr et al. 2012). We utilised the *rpart* function from the *rpart* package within the R software environment (Therneau and Atkinson 2025), setting the minimum number of node observations for a split (minsplit) to 1 and the complexity parameter (cp) to 0.001. A cross-validation procedure was used with individual populations as validation subsets, optimal tree selection was guided by the 1-standard error rule (Venables and Ripley 2002).

Outline analysis of summer leaves

Summer leaf shape was only assessed for *P. obscura* and *P. officinalis*, due to the limited sample size of putative hybrids. A total of 671 plants from 235 populations (1–13 plants per population) were examined. One fully developed summer leaf was selected from each individual. The methodological approach followed the study of Vejvodová et al. (2021), i.e. the elliptic Fourier approximation (Kuhl and Giardina 1982) implemented in the R package *Momocs* (Bonhomme et al. 2014). The elliptic Fourier descriptors (EFDs) were centred and scaled to have zero mean and unit variance. Principal component (PC) scores for each sample were calculated from the standardised EFDs to visualise patterns of leaf shape variation. These PC scores were also used in a linear discriminant analysis (LDA) and a classificatory discriminant analysis (CDA) in *Morphotools* (Koutecký et al. 2015) to test for differences in leaf shape between *P. obscura* and *P. officinalis*. The significance of the model was tested using a permutation test (999 permutations), and a forward selection of the PC was performed to select only those components that significantly contributed to the species discrimination, with significance further tested using permutation

Fig. 3 Estimation of pollen viability in native (a) and non-native (b) populations of *P. officinalis* agg. **a** *P. obscura* (diploid, DNA-triploid), *P. officinalis* (diploid) and *P. officinalis* × *P. obscura* putative hybrid. **b** *P. officinalis*-like cytotype (*P. officinalis* agg. cult.) and *P. officinalis*-like cytotype × *P. obscura* putative hybrid. Groups not sharing any letters differ significantly ($p < 0.05$)



tests (999 permutations). These selected components were then used in CDA.

The relationship between leaf shape and the geographical location of individuals was also tested. The PCs from the PCA of the EFDs were used as the explanatory variables in RDA with the geographical coordinates of the samples as response variables. The significance of the model was tested using a permutation test (999 permutations). Two separate analyses were performed, one for each species, in the *vegan* package in R (Oksanen et al. 2025). The response variables were centred to zero mean and scaled to unit variance prior to the analysis. All analyses used individuals as operational taxonomic units.

The differences in the summer leaf morphology between *P. obscura* and *P. officinalis* were also tested using Automated machine learning (AutoML). Only scans of fully coloured and undamaged leaves were selected for this analysis. In total, 503 individuals from 178 populations (*P. obscura*: 87 populations and *P. officinalis*: 91 populations) were analysed. The samples within each population were divided into training (50%), validation (30%), and testing (20%) subsets. The AutoML analysis was performed using AutoML on the Vertex AI platform (Google LLC).

Results

Flow cytometry and chromosome numbers

The flow cytometric measurements revealed the presence of two main cytotypes in the natural populations of the study area, corresponding to the individual taxa, i.e. *P. obscura* (239 populations/646 plants) and *P. officinalis* (107 populations/355 plants), which were confirmed to have chromosome numbers of $2n = 14$ and $2n = 16$, respectively (Table 2, Fig. 1). In addition, several mixed populations were found to contain the third cytotype, putative hybrid plants (three populations/15 plants), which were confirmed to have $2n = 15$ (Table 2, Fig. 1). The exact range of RGS of the two main cytotypes and their presumed hybrids (Online Resource 3b) was determined by statistical support of chromosome numbers and the proportion of viable pollen grains (see below, Fig. 2). In addition, two other rare cytotypes were also detected by flow cytometry in otherwise pure natural populations of *P. obscura* or *P. officinalis* (Table 2). As the exact chromosome numbers of these cytotypes were not determined, we refer to them as DNA-triploids of *P. obscura* (two

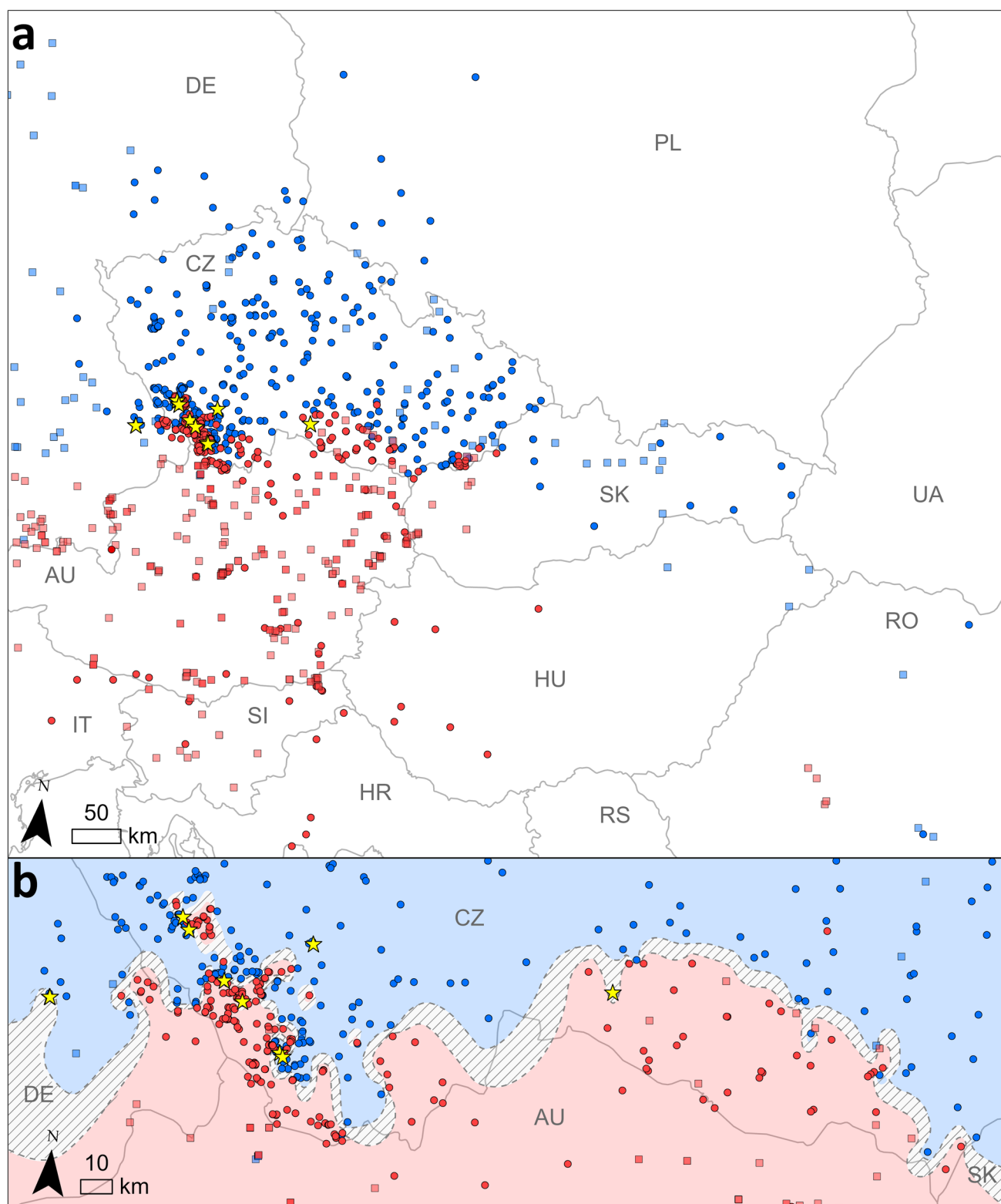


Fig. 4 Cyto geography of natural populations of *Pulmonaria obscura* and *P. officinalis* in the study area (**a**—whole study area, **b**—detail of denser sampling in the area where both species occur together with area of their dominance illustrated by colour). Generalised areas of dominant occurrence of each species are marked in colour (*P. obscura*—blue, *P. officinalis*—red, *P. officinalis* × *P. obscura*—yel-

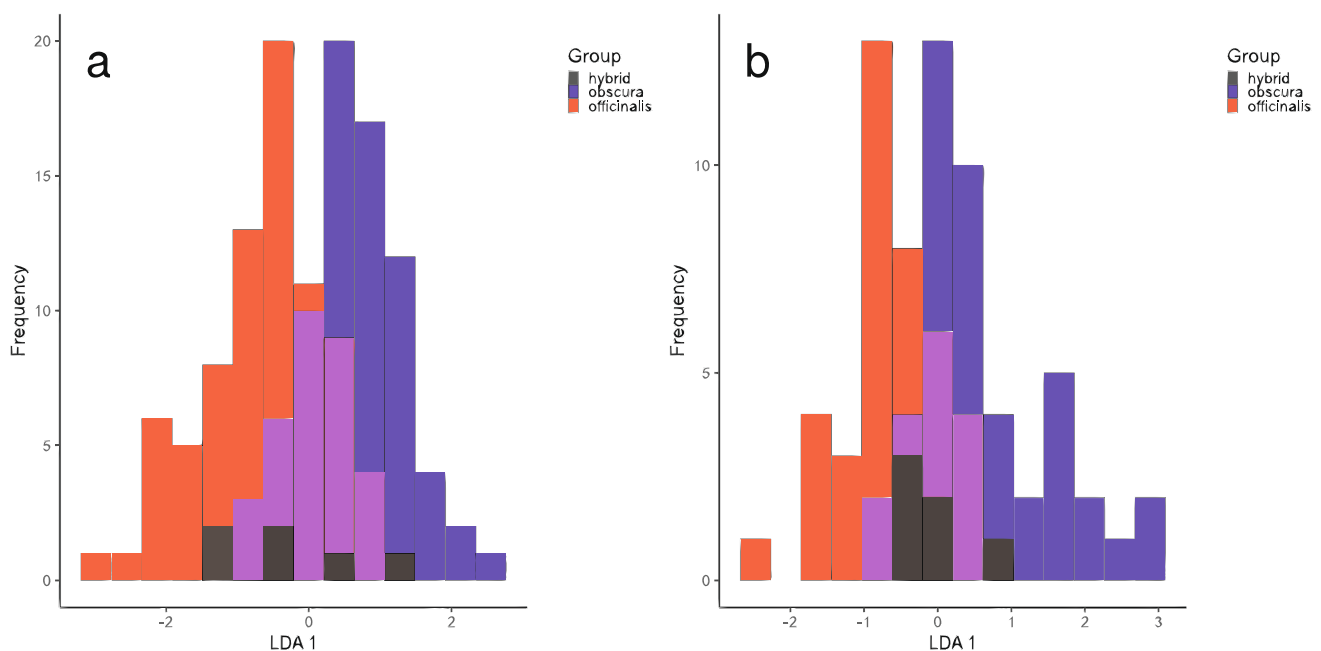
low stars) on the basis of flow cytometry (circles) and karyological data (squares)—new data and reports from literature were used (see Online Resource 1b). The area of dominance of *P. officinalis* is indicated in red, *P. obscura* in blue, and the contact zone where neither species dominates is shown in white with hatching

Table 3 Descriptive statistics (mean, standard deviation (S.D.), minimum and maximum) for all morphological characters studied, i.e. the dataset A and B together (Table 1), for *Pulmonaria obscura* (n = 75), *P. officinalis* (n = 80) and putative hybrid plants (n = 6)

	GBC				SBC				RBC			
	Mean	S.D.	Min	Max	Mean	S.D.	Min	Max	Mean	S.D.	Min	Max
<i>P. obscura</i>	450.67	210.66	183.11	1595.04	623.00	345.79	253.93	3177.07	1.39	0.78	0.58	4.44
<i>P. officinalis</i>	721.25	222.38	295.60	1224.97	634.44	145.76	323.18	978.06	1.09	0.40	0.41	2.73
<i>P. officinalis</i> × <i>P. obscura</i>	702.18	217.11	449.63	996.23	647.46	168.99	441.95	846.08	1.01	0.44	0.51	1.79

	UCW				CL				RC			
	Mean	S.D.	Min	Max	Mean	S.D.	Min	Max	Mean	S.D.	Min	Max
<i>P. obscura</i>	11,539.88	1559.37	7171.90	15,409.47	9802.11	2051.88	939.31	13,362.67	1.64	2.36	0.87	12.57
<i>P. officinalis</i>	13,563.63	2493.06	9017.30	19,415.02	10,852.38	1674.33	7006.00	16,209.73	1.24	0.20	0.84	1.60
<i>P. officinalis</i> × <i>P. obscura</i>	11,336.28	1093.54	10,132.76	12,592.19	9600.66	1873.85	7948.85	12,252.09	1.20	0.14	1.02	1.33

Six morphological characters were measured: GBC=length of glandular hairs on the base of the calyx in μm , SBC=length of setae on the base of the calyx in μm , UCW=width of the unfolded calyx in μm , RBC=length of setae/glandular hairs at base of calyx, CL=calyx length, RC=length/width of calyx

**Fig. 5** Scores of the discriminant function from LDA on the dataset A and B

populations/five plants) and DNA-triploids of *P. officinalis* (one population/two plants), respectively, as their RGS matched those of hypothetical autotriploids formed from the respective species.

We also collected and analysed several populations of uncertain origin and/or possible garden escapees, classified here as "*P. officinalis* agg. cultivated" plants (62 populations/161 plants). Within this category, we identified plants

falling within the RGS range of the *P. officinalis* ("*P. officinalis*-like" cytotype, 54 populations/135 plants) and plants with lower RGS values corresponding to *P. obscura* ("*P. obscura*-like", three populations/ten plants). In all but one case, both cytotypes occurred singly. In addition, we also detected 16 plants with RGS values intermediate between the *P. obscura* and *P. officinalis* cytotypes, i.e. the interval of the putative hybrid with $2n = 15$ (see Table 2), which

also showed low pollen viability (see below, Fig. 2). This cytotype was rarely observed in separate populations (three populations) or as a mixture within the separate single populations of the "*P. officinalis*-like" cytotype (four populations) or *P. obscura* (three populations) and in one of a mixed population with *P. obscura* and "*P. obscura*-like" cytotype.

Pollen viability estimation

The proportions of viable and aborted (non-viable) pollen grains were evaluated mainly in native populations of *P. officinalis* agg., i.e. for *P. obscura* (diploid: 18 plants and DNA-triploid: one plant), *P. officinalis* (17 plants), and their putative hybrids (six plants). We have also analysed several plants from non-native populations, i.e. *P. officinalis* agg. cult. – *P. officinalis*-like cytotype (two plants) and *P. officinalis*-like cytotype $\times$ *P. obscura* (six plants; Fig. 3). The groups differed significantly in their pollen viability (Kruskal–Wallis ANOVA: $X^2 = 30.1$, $p < 0.001$). The results show a high level of pollen grain viability for *P. obscura* (86.1%), *P. officinalis* (86.7%) and *P. officinalis*-like cytotype (92.7%), whereas it was very low in *P. officinalis* $\times$ *P. obscura* natural putative hybrids (16.3%) and also in *P. officinalis*-like cytotype $\times$ *P. obscura* (19.5%) putative hybrids of cultivated and natural plants. A moderate decrease in the viability of pollen grains (48.1%) was also found in a DNA-triploid plant of *P. obscura* (Fig. 3). Statistically, *P. officinalis* and *P. obscura* differed from all other groups except

P. officinalis-like cytotype (*P. officinalis* agg. cult.), but not from each other. Similarly, there was no significant difference among the other three groups – putative hybrids *P. officinalis* $\times$ *P. obscura* and *P. officinalis*-like cytotype $\times$ *P. obscura*, and *P. obscura* DNA-triploid.

Cytogeography and natural contact zone of *Pulmonaria obscura* and *P. officinalis*

The study of the distribution pattern of the *P. officinalis* agg. revealed a significant geographical differentiation between the native ranges of *P. obscura* and *P. officinalis* in Central Europe (Fig. 4a). *Pulmonaria obscura* was the dominant taxon in the northern part of the study area (i.e. Germany, most of the Czech Republic, Poland, and Northern Slovakia), whereas *P. officinalis* predominated towards the south (i.e. Austria, southern part of the Czech Republic, Southern Slovakia, Hungary, and Slovenia). There is a natural contact zone, where both taxa may co-occur, i.e. the area of the southern part of Germany, the wider area around the border of Austria and the Czech Republic, and most probably the western part of Slovakia and the northern part of Hungary. This contact zone roughly follows the course of the Danube River in Germany, the mountain ranges of the Šumava Mts, Novohradské hory Mts and Bohemian-Moravian Highlands north of the Austrian-Czech border, and the basin of the Dyje River and the White Carpathians on the Czech-Slovak border (Fig. 4b).

Although populations of *P. obscura* and *P. officinalis* were often found in close proximity (within a few kilometres), only three mixed populations of both species were confirmed in the contact zone in our dataset (Online Resource 3c). The intermediate cytotypes (putative hybrids) were detected in all these populations, indicating that hybridisation between the species occur whenever they co-occur. In one of these populations (ID K653), we also detected DNA-triploid plants of *P. obscura*. In addition, in six populations, an intermediate (hybrid) cytotype was found in a population of only one of the two parental species. In each of these cases, the second parent was present in a close proximity to the sampling site. We also found one population consisting entirely of the intermediate cytotype.

The anthropogenic contact zone results from the introduction of cultivated and escaped ornamental plants of the *P. officinalis* agg. into areas inhabited by native populations of the group. As these cultivars are difficult or impossible to identify in *P. officinalis* s. str. populations (at least on the basis of the methods used), we only documented their presence in *P. obscura* populations (Online Resource 1). Even in these mixed populations, we were able to detect the presence of plants with intermediate RGS values, indicating ongoing hybridisation (see Online Resource 3d).

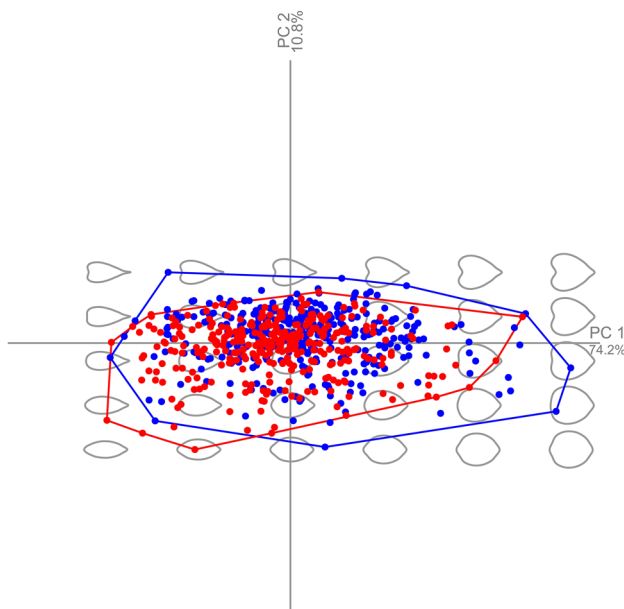


Fig. 6 PCA of the outline analysis of the summer leaves of *Pulmonaria obscura* (blue symbols) and *P. officinalis* (red symbols). Grey figures in the background show reconstructions of leaf shape for each position in the multidimensional space

In order to have the most robust dataset, the results obtained here were combined with those of Vejvodová et al. (2021). Therefore, two datasets (A, B; Table 1) were analysed (See Materials and methods). The full dataset (dataset A) revealed that *P. obscura* and *P. officinalis* individuals formed only partially distinguishable clusters, with most individuals of both taxa overlapping (Table 3, Online Resource 3e). The length of calyx (CL), the length of glandular hairs at the base of the calyx (GBC), and the ratio of the length of setae/glandular hairs at the base of the calyx (RBC) contributed most to the observed pattern (Online Resource 2, 3e, f). A similar pattern was also observed in the reduced dataset (dataset B; Online Resource 3f), where all characters were included (Table 3), with high discriminatory power of the width of the unfolded calyx (UCW, Online Resource 2a, b). The putative hybrids between *P. obscura* and *P. officinalis* (only in full dataset A) showed a predominantly intermediate ranking of morphological characters, falling within the range of overlap of the parental species.

The LDA of both datasets showed a high degree of overlap between *P. obscura* and *P. officinalis*, including their hybrids (Fig. 5), with the accuracy of the classificatory discriminant analyses being 77.9% and 75.3% in datasets A and B, respectively (Online Resource 2b). The analysed hybrid individuals had mostly higher posterior probabilities of belonging to *P. officinalis* (Online Resource 2c).

The final classification trees (Online Resource 3g, i) had two terminal nodes for both dataset A and B, selected following the 1-SE rule. The only character that discriminated between the two species was the length of glandular hairs at the base of the calyx (GBC). However, its discriminatory power was clearly asymmetrical, being able to correctly identify *P. obscura* in 82.5% (A) and 92.3% (B) of the cases, whereas *P. officinalis* could only be correctly identified in 74.7% (A) and 64.4% (B) of the cases.

Correlation of geography and morphology

For *Pulmonaria obscura*, no significant association between the morphological characters and geographical coordinates was found by RDA ($F = 1.82$, $p = 0.074$). However, for *P. officinalis*, there was a significant association between the morphological characters and geographic coordinates ($F = 5.85$, $p < 0.001$). The character calyx length (CL) was weakly positively correlated with the longitude, whereas the character the length of setae/glandular hairs length at base of calyx (RBC) was weakly positively correlated with latitude (Online Resource 3i).

Outline analysis of summer leaves

Leave shape analysis was only performed for *P. obscura* and *P. officinalis*. The first 10 harmonics were selected to

approximate the leaf shape. The PCA of the harmonic coefficients showed a strong overlap between the taxa. Only a small proportion of extreme morphotypes of *P. obscura* with an almost rounded leaf base could be distinguished from *P. officinalis* (Fig. 6).

The LDA of the PC scores showed a significant differentiation between *P. obscura* and *P. officinalis* ($F = 2.5$, $p < 0.001$). Only the first two principal components (PC1 and PC2) had significant both the marginal and conditional effects (Online Resource 2d). The overall accuracy of the CDA based on the two significant principal components was only 59.0%, with only 61.4% of the *P. obscura* individuals and 56.5% of the *P. officinalis* individuals being correctly classified (Online Resource 3j).

The differences in summer leaf morphology between *P. obscura* and *P. officinalis* were also confirmed by machine learning. The accuracy of the model was significantly better than what would be expected by random chance, with an overall prediction accuracy of 70% (Online Resource 3e).

Correlation of geography and leave morphology

The RDA analysis of the relationship between the principal components and geographical coordinates was significant for both *P. obscura* ($F = 1.61$, $p = 0.005$) and *P. officinalis* ($F = 1.75$, $p = 0.005$). The patterns of the leaf shape variation across the geographical area studied are difficult to describe, but there appears to be a positive correlation between both latitude and longitude and the cordate shape of the leaf base (Online Resource 3k, l).

Discussion

Cytotype diversity in natural populations of *Pulmonaria officinalis* agg.

The general pattern of the cytotype diversity is consistent with previous chromosome counts and flow cytometric reports, as summarised in Vejvodová et al. (2021) and Kobřlová et al. (2024). Two major cytotypes were confirmed within Central Europe, each associated with a single taxon, i.e. *P. obscura* ($2n = 14$) and *P. officinalis* s. str. ($2n = 16$, see Table 2). In addition, rare cytotypes corresponding to DNA-triploids were detected in natural populations of both species, representing the first such cytotype within *P. officinalis*. To date, the triploid chromosome count of $2n = 21$ was only published once for *P. obscura* from Germany (Sauer and Gruber 1979). The minor DNA-triploid cytotypes were observed almost exclusively in single populations of either *P. obscura* or *P. officinalis*, probably resulting from a fusion of a reduced and an unreduced gamete. This process of polyploidisation via the production of unreduced

gametes appears to be quite common in plants (e.g. Köhler et al. 2010; Kreiner et al. 2017a, b; Ekrt et al. 2021) and has also been documented, for example, in the sister genera *Symphytum* L. (Kaplan et al. 2017; Koblrová et al. 2022) and *Borago* L. (Selvi et al. 2006). However, one population of *P. obscura* (ID K653) with presumably DNA-triploid plants was mixed with *P. officinalis* and even with an intermediate cytotype of *P. obscura* and *P. officinalis* (see below), indicating ongoing hybridisation. Therefore, it is necessary to verify the origin of these unusual cytotypes. Similarly, there are four questionable chromosome reports of $2n=28$ in the literature: three for *P. obscura* from Germany (Sauer 1975; Sauer and Gruber 1979), and one for *P. officinalis* from Finland (Sorsa 1962), all of which require verification. One of the locations under investigation (HMB P-302, Sauer 1975) was situated within the area of focus of the present study, and two populations (K309 and K586, with five and three samples respectively) were collected in close proximity to it. All of the analysed plants were found to be diploids of *P. obscura*. As Albertshofer collected the original material in 1972 (see Sauer 1975), it is important to consider the possibility that the population characteristics may have undergone changes since that time. Moreover, the location in question is in close proximity to a town, situated within an anthropogenic habitat, which raises the possibility that the current population has been influenced by escaped cultivated plants, potentially bearing different ploidy levels.

Finally, we occasionally found an intermediate cytotype with $2n=15$, mainly in mixed populations of *P. obscura* and *P. officinalis* (discussed below), indicating to be a hybrid between these taxa. To date, only a few chromosome counts of $2n=15$ (Bolliger 1978; Dersch 1994) documented the possible existence of this hybrid (see below). More recently, however, clear evidence for the origin of the hybrid was provided by the study of Koblrová et al. (2024) using chromosome-specific cytogenetic markers.

Pollen viability in natural populations of *Pulmonaria officinalis* agg.

In general, pure species often exhibit higher pollen viability when compared to hybrids (Simon et al. 2016), and the pollen fertility reduction or pollen sterility can serve as a supporting character in the identification of spontaneous interspecific hybrids. While *P. obscura* and *P. officinalis* showed high pollen viability (*P. obscura*: 70–98% of pollen viable; *P. officinalis*: 83–98% of pollen viable), the percentage of fertile pollen produced by the intermediate cytotype was substantially lower (only 2–33%). This pollen viability reduction in the intermediate cytotype may provide further evidence of its hybrid origin. When Bolliger (1978) studied a mixed population of *P. obscura* and *P. officinalis*, he

found that the hybrids were apparently sterile, with no fruit formation observed either in nature or in the experimental garden. Only three pure plants (one of *P. obscura* and two of *P. officinalis*) showed a slight decrease in the percentage of viable pollen (i.e. 55% and 64%), which could be attributed to adverse environmental effects (e.g. Morrison 1991; Peet et al. 1998). Within the DNA-triploids, we only had plant material available from one *P. obscura* individual. The percentage of viable pollen produced by this triploid plant was reduced, but still almost 50% of its pollen grains were stained. Therefore, we conclude it was not a hybrid, but putative autotriploid, which can be typical by such viable pollen proportion (Gajdošová et al. 2023).

A possible explanation for the overall greater variability in pollen fertility observed in both species could be related to the presence of two flower morphs. The genus *Pulmonaria* is a typical example of a distylous breeding system (Merxmüller and Sauer 1972; Olesen 1979; Brys et al. 2008), characterised by reciprocal polymorphism in stamen and style length, along with associated pollen grain and stigma characters. Brys et al. (2008) reported morph-specific differences in *P. officinalis*, with significantly longer anthers of long-styled morph (LS-flower) producing almost twice as much pollen as anthers of short-style-morph (SS-flowers). On the other hand, pollen grains of SS-flowers are significantly larger in length and width than those of LS-flowers (Champluvier and Jacquemart 1999; Brys et al. 2008; Meeus et al. 2013). Some studies have reported that average pollen viability may vary between different morphs or even within different years of collection (e.g. Ornduff 1980; Souza et al. 2006), but this needs to be further tested in the genus *Pulmonaria*.

Phenotypic differentiation of natural populations of *Pulmonaria officinalis* agg.

The two species are often difficult to distinguish due to their high phenotypic plasticity and variability (Májovský and Hegedúšová 1993; Dersch 1994; Fischer et al. 2008; Jäger 2017; Vejvodová et al. 2021). Despite the confirmation of the relevance of specific morphological characters in the present study, namely the length of calyx, the length of glandular hairs at the base of the calyx and the ratio of the length of setae/glandular hairs at the base of the calyx (Online Resource 2a) being in correspondence with previous results (Sauer and Gruber 1979; Dersch 1994; Vejvodová et al. 2021), our data also illustrate a strong overlap in the characters studied (Figs. 5 and 6), which complicates species identification (Online Resources 2, 3g, h). Furthermore, hybrids between *P. obscura* and *P. officinalis* showed intermediate values that were completely within the overlap range of the parental species. Consequently, it is essential to emphasise the importance of an integrative approach that

utilises flow cytometry or karyology and pollen grain staining (see above) for reliable plant identification.

Mixed populations and the frequency of hybridisation

Most karyological reports of *P. officinalis* agg. are based on a single plant per population (see Online Resource 1b), limiting insight into local cytotype distribution and coexistence mechanisms. Although our detailed sampling revealed several populations in close proximity, the co-occurrence of both taxa in the same locality is quite rare (see also Jarošová 2006; Vejvodová et al. 2021; Koblířová et al. 2024). Due to the high variability in their vegetative characteristics, less represented taxon may be overlooked in mixed populations. We identified only three natural mixed populations, all of which showed the presence of an intermediate cytotype, indicating ongoing hybridisation. We also recorded putative hybrids in a single population or with only one parent. Such a situation is a common phenomenon in contact zones (Wachowiak et al. 2015; Zozomová-Lihová et al. 2015), due to internal selection on breeding barriers (Husband et al. 2002; Lexer and van Loo 2006) or overwhelming the barriers of the less common species with pollen of the dominant one (Prentis et al. 2007; Lepais et al. 2009; Koutecký et al. 2011). Hybridisation between *P. obscura* and *P. officinalis* has long been discussed, and so far, it has been karyologically documented in Switzerland (Bolliger 1978), Germany (Dersch 1994) and the Czech Republic (Koblířová et al. 2024). In addition, the study by Meeus et al. (2016) discussed the potential presence of hybrids in *P. officinalis* populations from Germany and Belgium.

It appears that when the species meet, they can hybridise despite having different basic chromosome numbers, which was thought to be a barrier to their hybridisation (Májovský and Hegedušová 1993). Sauer (1975) documented homoploid hybrids even from mixed populations of different *Pulmonaria* species groups (e.g. hybrid of *P. officinalis* s. str. ($2n=16$) and *P. mollis* s. str. ($2n=18$; Májovský and Murín 1980) with $2n=17$). Thanks to the clonal propagation by creeping rhizomes with adventitious roots (Meeus et al. 2013), hybrids have a chance of persistence in spite of their lower pollen fertility (see above). This aligns with the observation of Bolliger (1978) of a pure hybrid population of about 50 individuals (apparently sterile) in Switzerland, which he assumed to have originated by vegetative propagation. On the other hand, some authors have suggested that the species have a limited tendency to hybridise, as indicated by karyological analyses of hundreds of plants from regions where both species grow, which found no evidence of intermediate cytotypes (Sauer 1975; Sauer and Gruber 1979; Sauer and Thiv 1995). The lower frequency of hybrids could also be influenced by heterostyly and the likelihood

of legitimate pollination between compatible floral morphs. Most of the distylous species are characterised by mechanisms of self- and intramorph-incompatibility. Consequently, only intermorph pollination (i.e. between plants of opposite morphs) often leads to the production of offspring (Barrett 1990; Barrett and Shore 2008; Cohen 2010, 2019). In *Pulmonaria*, however, the deviations from perfect reciprocal herkogamy are reported to be common and have an impact on legitimate pollen deposition (Godin and Botov 2018; Jacquemyn et al. 2018). Additionally, preference for self-pollination was observed in *P. obscura* (Godin and Botov 2018). Therefore, these aspects of their pollination could prevent hybridisation process. It would be beneficial to investigate whether there are any mechanisms to prevent hybridisation and if so, how strong they are. Notably, plants with RGS values in the range of the intermediate cytotype were also found in populations classified as "*P. officinalis* agg. cultivated". However, we can only speculate about the origin of these plants, as we cannot exclude the contribution of other *Pulmonaria* cultivars or varieties.

Distribution and natural contact zone of *Pulmonaria obscura* and *P. officinalis* s. str.

The large-scale distribution pattern of *P. obscura* and *P. officinalis* in Central Europe revealed a distinct regional division (Fig. 4). *Pulmonaria obscura* clearly predominates in the northern regions (i.e. East Germany, Poland, the Czech Republic, and Slovakia), whereas *P. officinalis* is more common in the southern regions (i.e. Austria, Slovenia, and Hungary). It should be acknowledged that the distribution data presented here are partially geographically biased due to the sparse sampling from the eastern part of Central Europe. However, the data clearly demonstrate the general large-scale distribution patterns of the cytotypes in the *P. officinalis* complex. At the same time, representatives of this group may be less abundant in some parts of this area, such as the Pannonian Basin, due to the limited availability of suitable habitats.

Some previous studies (e.g. Merxmüller and Sauer 1972; Meeus et al. 2016) have reported both species throughout Central Europe, which may be due to a lack of detailed revisions or inconsistent taxonomy within the group (Hultén and Fries 1986), making it difficult to distinguish the species (summarised in Vejvodová et al. 2021). Verification by chromosome counting or flow cytometry is therefore sometimes the most reliable method. In addition, the role of *P. officinalis* in folk medicine and horticulture may have contributed to the confusion in some historical records, making it difficult to assess whether the species is native or not in some regions (esp. in western part of Europe). Conversely, several authors, mostly specialists in the genus (e.g. Sauer 1975, 1976, 1986; Sauer and Gruber 1979; Bolliger 1978, 1982;

Sauer and Thiv 1995; Jarošová 2006; Vejvodová et al. 2021; Koblrová et al. 2024) have partially proposed a similar distribution model, although the line of the contact zone remained unclear. Our results provide the first detailed insight into this contact zone, highlighting the coexistence and the frequency of species co-occurrence.

In general, the observed pattern of vicariance in the distribution of closely related species with a probable common ancestor (Meeus et al. 2016) may provide a suitable explanation for the situation noted, for example, in the lineages of *Rosa pendulina* (Fér et al. 2007), *Lonicera nigra* (Daneck et al. 2011) or *Fraxinus excelsior* (Tollefsrud et al. 2016). It has been hypothesised that the dispersal of individual lineages from separate refugia, which may have been in contact with one another during periods of lower sea level (Frenzel et al. 1992), has led to the observed distribution of lineages. Following the deglaciation, it is believed that the offspring of a common ancestor dispersed independently, resulting in the current diversity observed in the population. Haplotypes from Alpine refugia populated the adjacent Alpine regions, while those from Carpathian and Balkan refugia spread into central and northern Europe. The subsequent emergence of their contact zone has been identified in the Danube Plain or Central Europe (Hewitt 1988, 1999; Taberlet et al. 1998; Fér et al. 2007; Daneck et al. 2011; Tellefsrud et al. 2016). Consequently, it can be hypothesised that *P. officinalis* and *P. obscura* species may have undergone a comparable evolutionary trajectory, potentially representing vicariance species that dispersed from discrete refugia following the last glaciation (*P. officinalis* from the Alpine refugia, and *P. obscura* from the Balkan-Carpathian refugia).

In consideration of the observed distribution of both species and the minimal overlap of their respective ranges, it can be hypothesised that this is a so-called tension contact zone. It is characterised by its independence from environmental selectivity, with its formation being conditioned by colonisation history and maintained by selection (Barton and Hewitt 1985). It is evident that the interaction between the two species is limited in this contact zone. This phenomenon is further substantiated by the notable tendency of the *P. officinalis* complex to engage in self-pollination (Godin & Botov 2018; Jacquemyn et al. 2018). Additionally, the internal selection pressure is found to be minimal, attributable to the disparity in chromosome numbers between the two species. The rationale behind the limited prevalence of mixed populations can be attributed to the constrained dispersal of heavy myrmecochorous seeds (Lengyel et al. 2010).

As evidenced by the findings of the present study, few records were identified that lie outside the main ranges of both taxa (Fig. 4). Similar records were purported in central Germany (see Dersch 1994) and central/south-eastern Moravia (Koblrová and Hroneš, unpubl. data). However, further study using sensitive methods is required to determine their

natural character and precise limits of both taxa. The possibility of other rare occurrences of *P. obscura* and/or *P. officinalis* outside their main ranges cannot be completely excluded.

Frequency and distribution of the non-native populations of *Pulmonaria officinalis* agg.

Traditionally, *P. officinalis* has been widely used and cultivated in horticulture for its medicinal properties and ornamental value (Bennett 2003; Scott 2008; Meeus et al. 2013). As a result of this deliberate cultivation, accidental introduction or naturalisation of cultivated varieties, it can be considered non-native in some regions (e.g. Meeus et al. 2013; Vejvodová et al. 2021). For this reason, all populations found in the vicinity of human settlements, where none of the species occurs naturally, and therefore their origin may be unclear, were analysed separately. The results confirm the presence of a dominant cytotype within these "*P. officinalis* agg. cultivated" populations, corresponding to the RGS values of *P. officinalis* s. str. Most of these plants had morphological characteristics typical of *P. officinalis*, such as white-spotted, cordate leaves, and are most likely naturalised plants of *P. officinalis* or possibly fragments of natural populations in human-influenced habitats. It is likely that a difference in DNA content would be observed if any of these plants were hybrids with *P. officinalis* (Koblrová et al. 2024). Additionally, the pollen staining of those plants was comparable (i.e. 93%) to that of *P. officinalis* from natural populations. Furthermore, three populations with RGS values similar to *P. obscura* and showing *P. officinalis* morphology were identified. It can be hypothesised that these represent the spotted phenotypes of *P. obscura* under-cultured for cultivation purposes; however further research is required to fully comprehend this phenomenon.

Of particular interest is the observation of plants with RGS values in the range of a hybrid between *P. obscura* and *P. officinalis*. We have detected several populations of this intermediate cytotype in mixed "*P. officinalis* agg. cultivated" populations, even within the single populations of natural *P. obscura* in close proximity to anthropogenic cultures. Similar to the intermediate cytotype observed in natural populations, these putative hybrids also had reduced pollen fertility, but at a slightly higher percentage (11–41%).

Conclusions

The focus of this study is examination of the morphological, cytological and cytogeographical differences between diploid *Pulmonaria officinalis* and *P. obscura* species in Central Europe. The present study revealed common cytologically different diploids and rare triploids. Despite the chromosomal differences between the two diploid species,

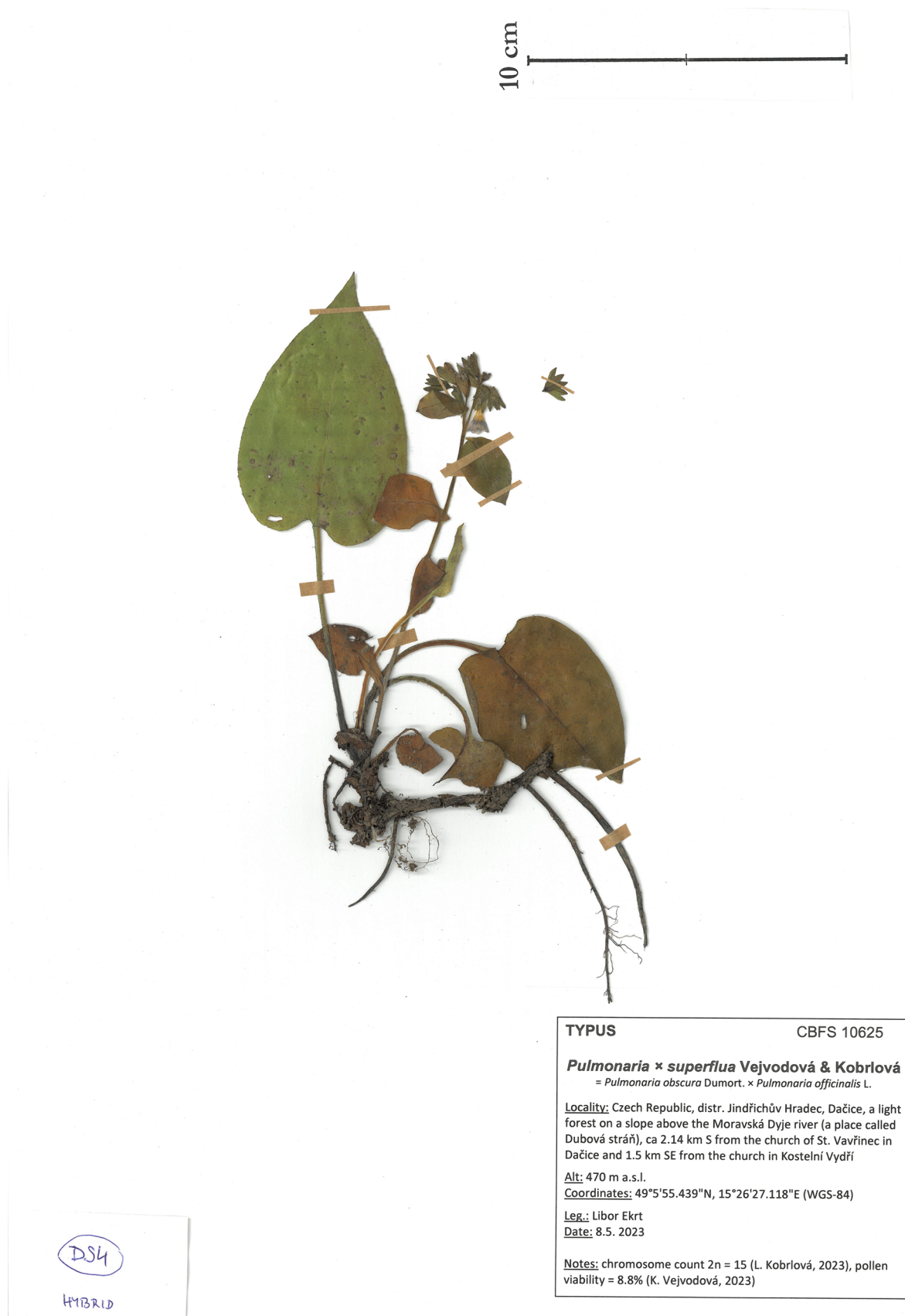


Fig. 7 Type of *Pulmonaria* × *superflua* Vejvodová & Koblrová, hybr. nov. (CBFS 10625)

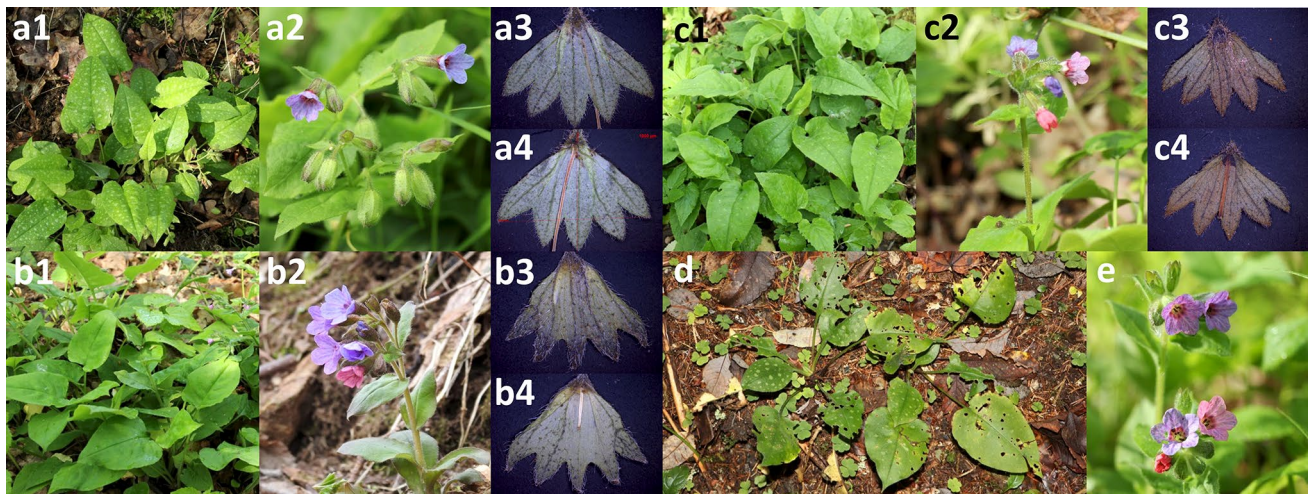


Fig. 8 Cytotypes of *Pulmonaria officinalis* agg. from natural populations: **a** *P. officinalis* (1,2,3,4—ID K653), **b** *P. obscura* (1—ID K653, 2—ID K100, 3,4—ID K485), **c** *P. × superflua* (1,2,3,4—ID K653), **d**

P. officinalis DNA-triploid (ID K626), **e** *P. obscura* DNA-triploid (ID K653). Authors of photographs: a1, a2, b1, c1, c2, e—L. Ekrt; a3, a4, b3, b4, c3, c4—K. Vejvodová; b2—R. Roučková; d—M. Štech

there is a considerable degree of morphological similarities and overlaps of particular morphological characters. This particularly contrasts with their natural distribution areas that exhibit only a slight overlap.

Furthermore, a natural contact zone was identified at the boundary of their respective ranges. Nevertheless, the boundary in itself seems to be determined by the dispersal potential, selfing preference and selection against hybridisation of the species in question. Furthermore, there is indication to suggest that gene flow is also weak, as there was no strong evidence in the conducted analyses that morphological traits were changing towards the contact zone. Additionally, the occurrence of mixed populations of both species was found to be rare in this area.

A newly described hybrid *Pulmonaria × superflua* hybr. nov. was identified in a limited number of populations within the natural contact zone of the above-mentioned parental species. The hybrid was confirmed by means of low pollen viability and an intermediate chromosome count, falling between those of the parental species. Furthermore, the existence of an anthropogenic contact zone comprising natural populations of *P. obscura* and *P. officinalis* in contact with escaped types from cultivation was also identified. These anthropogenic zones were found to also contain hybrid plants of an intermediate cytotype, characterised by the presence of aborted pollen.

Taxonomic treatment

Pulmonaria × superflua Vejvodová & Koblrová, **hybr. nov.**
Pulmonaria obscura Dumort. $\times$ *Pulmonaria officinalis* L.
 subsp. *officinalis*—HOLOTYPE: Czech Republic, distr.

Jindřichův Hradec, Dačice, a light forest on a slope above the Moravská Dyje river (a place called Dubová stráň), ca 2.14 km S from the church of St. Vavřinec in Dačice and 1.5 km SE from the church in Kostelní Vydří, alt. 470 m; 49°5'55.439"N, 15°26'27.118"E; 8 May 2023; Ekrt L. (CBFS CBFS10625) (Fig. 7).

Diagnosis: Spontaneous hybrid between *P. obscura* and *P. officinalis*, in several characters more similar to *P. officinalis*. The length of the calyx is approximately 0.8 to 1.2 cm, the length of the glandular hairs on the calyx base is 450–1000 μ m, the setae on the calyx base are comparable in length or slightly shorter, 440–850 μ m (Fig. 8c). Compared to the two parents, *P. × superflua* has very low pollen viability (2–33%). (Online Resource 3, Fig. 3).

Karyology: *Pulmonaria × superflua* is a diploid taxon with a chromosome complement $2n = 15$ (Fig. 1).

Distribution, habitat, phenology: It can be expected to occur in the area where both parent species are present. Flowering occurs from April to May.

Etymology: The specific epithet indicates that it is often difficult to identify members of the *P. officinalis* agg., with the determination of the parental taxa being more crucial.

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Romana, M. Soukup, J. Zavadilová, J. Vrtková, M. Štech, Z. Štechová, A. Štechová, L. Štechová, T. Kalistová, M. Vejvodová, M. Toncarová. We also thank R. Roučková and M. Štech for kindly providing their photographs. Finally, we especially thank M. Štech for his guidance and recommendations.

Author contributions All authors provided material/samples essential for the study, helped with fieldwork, and reviewed and commented on the final version of the manuscript. Specifically, KV designed the study, KV, PK, and LE performed the fieldwork and sample collection, KV and LE performed the flow cytometry analyses, LK performed the chromosome counting, KV performed the pollen viability analysis, KV managed the data, PK performed the statistical data analyses, KV and PK prepared the figures and tables, KV and LK prepared the first version of the draft, and all authors contributed to and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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