

RESEARCH ARTICLE

Different chromosome numbers but slight morphological differentiation and genetic admixture among populations of the *Pulmonaria hirta* complex (Boraginaceae)

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Abstract Hybridization and introgression have a significant impact on the taxonomically controversial genus *Pulmonaria*. Within this genus, the *P. hirta* complex shows puzzling systematic relationships among *P. hirta* s.str. ($2n = [22, 26]$ 28), *P. apennina* ($2n = 22$ [26]), and *P. vallisarsae* ($2n = 22$), showing range overlaps and mixed phenotypes in southern Europe. We carried out morphometric analyses of basal leaves and flower features along with AFLP characterization of 236 plants belonging to 11 populations within the complex and 1 population of *P. officinalis*. We also implemented an already available phylogeny with sequences from our target populations and characterized their karyotype. For all the populations within the complex, we found molecular evidence of a hybrid origin involving species belonging to different clades (angustifolia and officinalis clades). However, there is a certain morphological differentiation between some populations (“hirtoid” morph) and others (“vallarsoid” morph), albeit single individuals or entire populations show intermediate features. According to our results, hybridization and/or backcrossing/introgression have occurred, and gene flow is currently taking place among these “taxa”. Following the hybridization event(s), we can elaborate three possible evolutionary scenarios: (1) one hybrid “vallarsoid” ($2n = 22$) species spread across the Italian peninsula, and from this originated the “hirtoid” morph ($2n = 28$) through dysploidy; (2) two geographically distinct hybridization events produced both “vallarsoid” and “hirtoid” morphs; (3) one “hirtoid” allopolyploid hybrid species originated and backcrossed with *P. officinalis* generating “vallarsoid” plants. Under scenarios 1 and 2, the different morphs met again in central Italy, with massive current gene flow. Under scenario 3, “vallarsoid” plants spread across the Italian peninsula, but further backcrossed with “hirtoid” plants in central Italy, leaving pure lineages of “vallarsoid” plants only in the extreme north and south of their range. This latter scenario is supported by populations with $2n = 22$, 26 chromosomes, having karyotype asymmetry indices intermediate between those of $2n = 16$ and $2n = 28$ cytotypes. Irrespective of the evolutionary dynamics, today, a single lineage showing three cytotypes occurs throughout the Italian peninsula, supporting the circumscription of a single polymorphic species, namely *P. hirta*.

Keywords AFLP; cpDNA; dysploidy; hybridization; introgression; ITS; morphometry

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Species delimitation can be extremely complicated in plant genera that experience weak isolation and reproductive barriers. The genus *Pulmonaria* L. (lungworts) can be of particular interest in this context since geographic and reproductive barriers among species are low (flowering synchrony and same pollinators shared by species), so that several natural hybrids are reported, and artificial ones are easily produced as well (Sauer, 1975; Bennett, 2003; Meeus & al., 2016). *Pulmonaria* is also well known for its peculiar distylous breeding system (Darwin, 1877; Olesen, 1979; Richards & Mitchell, 1990; Champluvier & Jacquemart, 1999; Meeus & al., 2012a,b) and

its controversial infrageneric systematics (Kerner, 1878; Lacaita, 1927; Sauer, 1975; Bolliger, 1982), in which a rather uniform morphological asset is contrasted by a striking karyological variability. The ancestral basic chromosome number of this genus is likely $p = 7$ (Bolliger, 1982; Vosa & Pistolesi, 2004), so that it includes diploids ($2n = 2x = 14$), tetraploids ($2n = 4x = 28$), and a series of dysploids with $2n = 16, 18, 20, 22, 24, 26, 30, 38$ chromosomes (Tarnavski, 1935; Merxmüller & Grau, 1969; Merxmüller & Sauer, 1972; Sauer, 1975, 1979; Bolliger, 1982).

The whole genus represents thus a wide complex of recently diversified and genetically different, tightly connected lineages, spread throughout Europe, and encompassing a wide

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range of karyological and morphological phenotypes. The first phylogenetic study of *Pulmonaria* (Kirchner, 2004) found that species are difficult to discriminate using molecular markers. However, later phylogenetic analyses using different plastid markers and tree reconstruction methods on the nine commonest lungwort species in western Europe (Meeus & al., 2016) showed that hybridization and introgression are common and contributed to the evolution and species diversity in *Pulmonaria*, even though these phenomena did not lead to convergence of lineages, as species recognized on morphological grounds were equally distinct on genetic grounds. Nevertheless, in the same study, it was shown that at least the lineage of *P. collina* W.Sauer and *P. mollis* Hornem. was generated by a hybridization event, and a similar pattern was also found in a recent study on *P. helvetica* Bolliger (Grünig & al., 2021). The northern Mediterranean area hosts a few cases of systematic uncertainties involving hardly distinguishable taxa: the subspecies of *P. longifolia* (Bastard) Boreau in the Iberian peninsula, *P. dacica* (Simonk.) Simonk. and *P. mollis* in the Balkans, and the species complex of *P. hirta* L. (= *P. saccharata* Mill.) in Italy. Notably, mountain areas of southern European peninsulas were claimed as major and deeply structured refugia, where geoclimatic events may interact with plant distribution ranges favouring both speciation and secondary contacts, producing thus a complex system of hybrid zones (Nieto-Feliner, 2011).

The fuzzy assembly of lineages is markedly evident in the group of *Pulmonaria hirta*, which includes *P. hirta* s.str., endemic to the Tyrrhenian area of NW Italy and SE France, *P. vallarsae* A.Kern, restricted to a small area in NE Italy, and *P. apennina* Cristof. & Puppi, another Italian endemic widely distributed along the Apennines. The latter taxon is currently treated as a subspecies of the allopatric *P. vallarsae* because of striking morphological similarity (Cecchi & Selvi, 2015; Bartolucci & al., 2018). All these taxa are putatively different in features of summer basal leaves, such as shape, maculation, and hair pattern (Kerner, 1878; Bolliger, 1982; Puppi & Cristofolini, 1996). In addition, they also show differences in chromosome number: $2n = 28$ in *P. hirta*, although $2n = 22$ and $2n = 26$ cytotypes have also been reported (Puppi & Cristofolini, 1996); $2n = 22$ in *P. apennina*, although $2n = 26$ has also been reported (Astuti & al., 2019); $2n = 22$ in *P. vallarsae* (Puppi & Cristofolini, 1996). *Pulmonaria apennina* overlaps with *P. hirta* in the northern portion of its range (Tuscan-Aemilian Apennine), where a high morphological convergence and intermediate chromosome numbers have been observed (Puppi & Cristofolini, 1996; Vosa & Pistolesi, 2004). It remains unknown whether these intermediate traits represent a continuous variation within a single polymorphic species or result from hybridization (and introgression) between different species. Indeed, *P. apennina* and *P. hirta* are interfertile (Puppi & Cristofolini, 1996), so that these parapatric lungworts were also previously considered as subspecies of the same species (Bernardo & al., 2010). Furthermore, the ranges of all these taxa partially overlap with that of *P. officinalis* L., a species with $2n = 16$ chromosomes that is otherwise widely distributed in Europe.

Since the circumscription of the taxa within this complex is uncertain, we aim at testing whether there are morphological, karyological and genetic differences that allow for safe identification all over their distribution range. To clarify whether this complex represents an independent lineage or is interspersed with other *Pulmonaria* lineages, we also attempted a phylogenetic reconstruction. We expect to confirm large morphological and karyological variability in this group, opening two main alternative systematic scenarios: (1) two/three morphologically and/or karyologically distinct species exist, either closely related to each other or have evolved from different ancestors; (2) a single morphologically and karyologically highly polymorphic species exists. Under the first scenario, we would expect to find a pattern of genetic differentiation congruent with morphological and karyological variation, whereas under the second scenario, genetic interpopulational differentiation would be expected to be low or not congruent with morphological and karyological variation.

■ MATERIALS AND METHODS

Sampling. — We sampled 236 plants belonging to 12 populations, 3 of *Pulmonaria hirta*, 2 of *P. apennina*, 2 of *P. vallarsae*, 4 taxonomically critical (*mixed* hereafter), and 1 of *P. officinalis*. Sampling localities are shown in Fig. 1. For *P. hirta*, we sampled the two topotypical populations available (H1 and H2), corresponding to the locality of the neotype designated for *P. picta* Rouy (a synonym of *P. hirta*; Puppi & Cristofolini, 1996), and to the locality of the epitype designated for *P. hirta* (Selvi in Cafferty & Jarvis, 2004), respectively. While the former occurs in a geographical area where no other *Pulmonaria* has been reported, the latter occurs in an area where *P. hirta* is supposed to overlap with *P. apennina*. Moreover, plants in this latter site display a leaf phenotype not always concordant with that typical of *P. hirta*. For this reason, we sampled an additional population of *P. hirta* (H3), displaying a leaf phenotype typical of this species. Similarly, also the topotypical population of *P. apennina* (A1) occurs in the abovementioned overlapping area. Hence, we sampled an additional population (A2) of this taxon at the southern limit of its range, where no other *Pulmonaria* is recorded. For *P. vallarsae*, we sampled two populations: one in the topotypical area of Vallarsa (V1) and another outside this area (V2). *Mixed* populations were selected among those where co-occurrence of different chromosome numbers ($2n = 22$ and 28 ; D1, D2) or intermediate chromosome numbers ($2n = 22$ and 25 , D3; $2n = 26$, D4) have been reported (Vosa & Pistolesi, 2004; Astuti & al., 2019, 2020). Despite the large sampling gaps, the choice of populations showing all the different leaf phenotypes and occurring in different geographical contexts should provide a good representation of the overall variability in the entire complex. Plants collected in spring 2018 and 2019 (see Table 1 for details on collection sites) were cultivated in pots kept outdoor in the Botanical

Garden of Pisa (WGS84 43.719743N, 10.396097E; 4 m a.s.l., mean annual precipitation: 900 mm; mean annual temperature: 14.2°C), using the same soil and regular watering.

Morphological analysis. — Corolla tube and lobe length, calyx tube and teeth length were measured on one flower chosen randomly among those open in the inflorescence. Based on the same flower, the individual was assigned to L (long-styled) or S (short-styled) flower morph. Morph bias was measured as $(L - S) / (L + S)$ and varied between -1 (only S-morphs) and 1 (only L-morphs), 0 representing the isoplethy. For plants of *Pulmonaria vallisae* from Lago di Cei (V2), we could not evaluate flower features. Table 2 summarizes the morph distribution in the 11 populations available. The Chi-square test for goodness of fit was used to assess statistically significant differences.

Leaf features were measured on the same individual in spring (at the time of collection) and summer (in cultivation) to compare morphological variation. Shape, maculation, and hairs were evaluated on one mature, undamaged basal leaf chosen randomly. The leaf adaxial surface was captured with a scanner and saved as 24-bit bitmap files.

Leaf shape was calculated by means of elliptic Fourier analysis, reducing the outline to the coefficients of elliptic

Fourier descriptors (EFDs) (Kuhl & Giardina, 1982) by means of SHAPE v.1.3 (Iwata & Ukai, 2002). The longest radius was used as normalization method. To reduce dimensionality, symmetric coefficients were subjected to principal component analysis (PCA). Multivariate analysis of variance (MANOVA) was carried out on the effective principal components (PCs) using PAST v.4.03 (Hammer & al., 2001; Hammer, 2020) to test differences among populations.

Maculated and total leaf area were calculated on pictures by means of ImageJ v.1.47 (Rasband, 1997). The number of spots was also counted on the same leaf.

Hair types were categorized as follows (Kerner, 1878; Puppi & Cristofolini, 1996): normal long hairs (or bristles; >0.5 mm), normal short hairs (or puberes; <0.5 mm), glandular hairs (stipitate multicellular glands about as long as bristles), microglands (shortly stipitate, unicellular glands) (suppl. Fig. S1). Hair density was observed under a stereomicroscope in a 3×2 mm area, ca. 1 cm to the left of the middle vein, at the leaf's widest point. In a 1.5×1 mm sub-area at the top left, the number of long normal hairs was counted. Comparison among populations was made through a Kruskal-Wallis test, followed by a post-hoc Mann-Whitney pairwise test with Bonferroni correction. The analyses were carried out using R (R Core

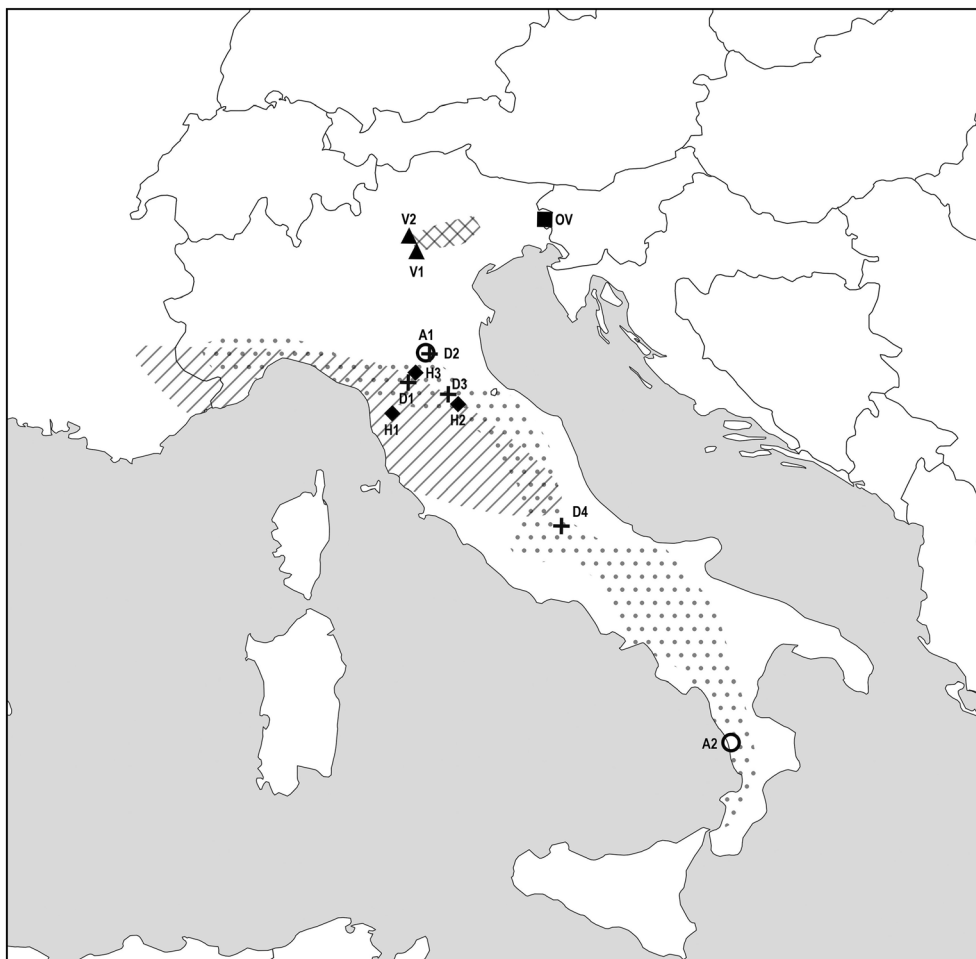


Fig. 1. Geographic distribution of *Pulmonaria apennina* (dotted), *P. hirta* (diagonal lines) and *P. vallisae* (grid). Populations sampled are indicated by circles for *P. apennina*, diamonds for *P. hirta*, triangles for *P. vallisae*, squares for *P. officinalis* and plus for mixed populations.

Team, 2020). Plots were generated with the R package ggplot2 v3.3.5 (Wickham, 2010).

We also performed a linear discriminant analysis (LDA) on the overall dataset, merging data of shape (the first three PCs obtained from the EFD analysis, and the length-to-width ratio), maculation, and hairs. First, we performed LDA without *mixed* populations, a priori grouping the other according to:

(a) a taxonomic hypothesis of four different species, *Pulmonaria hirta* (H1, H2, H3), *P. apennina* (A1, A2), *P. vallarsae* (V1, V2), and *P. officinalis* (OV); (b) a taxonomic hypothesis in which *P. apennina* and *P. vallarsae* belong to the same species. Then, we included the *mixed* populations considering them either (c) as a distinct group or, based on their morphological resemblance, (d) assigning D1, D3, and D4 to *P. apennina* + *P. vallarsae*

Table 1. Sampling localities of the populations of the *Pulmonaria hirta* complex and *P. officinalis* investigated.

Species	Region, municipality (province), locality (code)	No. sampled individuals	Herbarium vouchers	Coordinates
<i>P. apennina</i>	Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon* (A1)	20	PI021283–021302	44.47278N, 11.28416E
<i>P. apennina</i>	Calabria, San Fili (Cosenza), Foresta Luta (A2)	20	PI021323–021342	39.34085N, 16.08722E
<i>P. hirta</i>	Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana* (H1, type locality of the heterotypic synonym <i>P. picta</i> Rouy)	20	PI021343–021357 PI036046–036050	43.71388N, 10.67218E
<i>P. hirta</i>	Tuscany, Poppi (Arezzo), Camaldoli* (H2)	19	PI021358–021376	43.80902N, 11.82518E
<i>P. hirta</i>	Emilia-Romagna, Grizzana Morandi (Bologna), Favari (H3)	20	PI021377–021396	44.22385N, 11.09556E
<i>Mixed</i>	Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone (D1)	20	PI034194–034202 PI035974–035984	44.100658N, 10.962573E
<i>Mixed</i>	Emilia-Romagna, Bologna, Monte Paderno (D2)	17	PI034223–034228 PI035985–035995	44.452272N, 11.320769E
<i>Mixed</i>	Tuscany, San Godenzo (Firenze), Passo del Muraglione (D3)	20**	PI034203–034214 PI035996–036002	43.935302N, 11.658088E
<i>Mixed</i>	Abruzzo, Rocca di Mezzo (L'Aquila) (D4)	20	PI021303–021322	42.2125N, 13.51305E
<i>P. vallarsae</i>	Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze (V1)	20	PI034215–034216 PI034218–034219 PI034230–034234 PI036003–036013	45.760208N, 11.171882E
<i>P. vallarsae</i>	Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei (V2)	20**	PI034220–034222 PI036014–036029	45.96022N, 11.04163E
<i>P. officinalis</i>	Friuli Venezia Giulia, Prepotto (Udine), Castelmonte (OV)	20	PI034229 PI034235–034237 PI036030–036045	46.092828N, 13.516041E

* Topotypical population. ** Only 19 herbarium vouchers are available for the population.

Table 2. The Chi-square test for goodness of fit was used to assess flower morph bias.

	Population code										
	A1	A2	H1	H2	H3	D1	D2	D3	D4	V1	OV
L-morphs	11	18	7	7	11	9	13	10	10	11	8
S-morphs	9	2	9	7	9	6	4	6	10	9	11
d.f.	1	1	1	1	1	1	1	1	1	1	1
χ^2	0.2	12.8	0.25	0	0.2	0.6	4.765	1	0	0.2	0.474
P-value	0.655	<0.001	0.617	1	0.655	0.439	0.029	0.317	1	0.655	0.491

d.f., degrees of freedom. Significant deviation from isopleth is indicated in bold.

and D2 to *P. hirta*. We also tested the hypotheses that *mixed* populations altogether belong either to (e) *P. apennina* + *P. vallarsae* or to (f) *P. hirta*. Lastly, based on AFLP results (see further), which see D4 somehow distinct from D1, D2 and D3, we tested the hypothesis that D4 belongs to *P. apennina* + *P. vallarsae* and D1, D2, and D3 to *P. hirta* (g) and vice versa (h). The different grouping criteria are summarized in suppl. Table S1. A jackknifed a priori correct classification was calculated for each analysis. LDA was carried out by means of PAST v.4.03.

Karyotype asymmetry analysis. — We chose the best metaphasic plate available for each population, already obtained from our previous analyses (Astuti & al., 2019, 2020) (Appendix 1). On these plates, we built monoploid idiograms and calculated karyotype asymmetry indices using Karyo-Type v.3.0 software (Altinordu & al., 2016). We used CV_{CL} (Coefficient of Variation of Chromosome Length) and M_{CA} (Mean Centromeric Asymmetry) for interchromosomal and intrachromosomal asymmetry, respectively (see Peruzzi & Eroğlu, 2013). CV_{CL} measures the variation among chromosome length within a given karyotype (Paszko, 2006): karyotypes showing a large variation of chromosome length are asymmetric (high CV_{CL}), while karyotypes showing chromosomes of the same length are perfectly symmetric ($CV_{CL} = 0$). M_{CA} expresses the contribution to inner asymmetry given by each chromosome, where a metacentric chromosome is perfectly symmetric, while a telocentric one is perfectly asymmetric (Levitsky, 1931). In the calculation of M_{CA} , the centromeric asymmetry of a single chromosome is expressed as $(l - s) / (l + s) \times 100$, where l and s are the long and short arms, respectively: karyotypes constituted by only telocentric chromosomes are asymmetric ($M_{CA} = 100$), while karyotypes constituted by only metacentric chromosomes are perfectly symmetric ($M_{CA} = 0$).

AFLP analysis. — Genomic DNA was extracted from silica dried leaves using a modified version of the CTAB protocol (Doyle & Doyle, 1990).

AFLP analysis was attempted on all 236 individuals, but was successful in only 226 individuals. The quality of AFLP products was preliminarily tested on 16 samples randomly selected from the 12 populations (ca. 7% of the total dataset); these preliminary products were used as replicates in the following steps. DNA restriction, ligation, PCR amplification with selective primers, and selection of amplification products were carried out according to the AFLP protocol (Vos & al., 1995). For each sample, 200 ng of genomic DNA was used for restriction digestion with the endonucleases *EcoRI* and *MseI*. Digested DNA was ligated with 10 pmol of double-strand oligonucleotide adapters for 2 h at 20°C. For amplification, 5 µl of 1 : 2-diluted ligation mixture was added to a final volume of 20 µl of the reaction mixture, which contained 2.5 µl of 10× reaction buffer, 4 µl dNTPs, 0.25 µl Taq DNA polymerase, 10 pmol of (6-carboxyfluorescein)-labelled *EcoRI* TAC primer and 10 pmol *MseI* ATG primer. The PCR programme was 13 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, followed by 24 cycles of 30 s at 94°C, 30 s at 56°C

and 1 min at 72°C, with a final step of 2 min at 72°C. Subsequently, 5 µl samples of PCR products were preliminarily examined by agarose gel (1.2% w/v) electrophoresis in TAE buffer containing ethidium bromide and visualized using a UV-transilluminator. The visual inspection of PCR products allowed to better organize the preparation of samples for the subsequent sizing analysis. The AFLP electropherograms were obtained by capillary electrophoresis using the Applied Biosystems 3130xl Genetic Analyzer. The analysis of molecular profiles was performed with GeneMarker v.1.5 (SoftGenetics LLC, Pennsylvania, U.S.A.). Following the programme manual, we set up the software to remove stutter peaks within 2.5 bp of each detected allele peak. After running, the data with the standard size were analysed along with the replicates, and no mismatch was detected. The presence, absence, and questionable presence of alleles are shown for each sample during this step. In case of a complete mismatch of the peaks, the programme automatically removes the sample to eliminate errors. AFLP analysis yielded 200 unambiguously scorable fragments from 547 profiles of the combined selected primers. Fragment length ranges from 40 to 600 bp. Within-population genetic variation was assessed in terms of polymorphic loci, number of private fragments at single locus and expected heterozygosity (H_E). Analysis of molecular variance (AMOVA; Excoffier & al., 1992) as implemented in Arlequin v.2.000 (Schneider & al., 2000) was used to analyze the partition of total genetic variation at two different hierarchical levels: within populations and among populations. Genetic structure among populations within each species was also investigated using the Bayesian-based clustering as implemented in STRUCTURE v.2.3.3 (Pritchard & al., 2000). Five runs per each K were performed by setting the number of clusters (K) from 1 to 12. Each run consisted of a burn-in period of 200,000 steps, followed by 10^6 MCMC (Markov chain Monte Carlo) replicates, assuming admixture model and correlated allele frequencies. The optimal number of clusters (K) was assessed by calculating an ad hoc statistic ΔK (Evanno & al., 2005) as implemented in STRUCTURE HARVESTER v.0.6.94 (Earl & von Holdt, 2012). To assign samples to clusters, a membership coefficient $q > 0.1$ was used, while coefficients ≤ 0.1 were discarded. In order to test the hypothesis that our populations belong to four (*Pulmonaria apennina*, *P. hirta*, *P. officinalis*, *P. vallarsae*) or two different taxa (*P. hirta* s.l., *P. officinalis*), the population structure was surveyed also at $K = 2$ and $K = 4$.

To test the relationship between morphological and genetic variation at population level, we obtained pairwise distance matrices from both datasets: for morphological distance, the Euclidean method was applied to z-transformed average values per population of each variable; for genetic distance, Slatkin linearized pairwise F_{ST} values were used. Matrices were compared using the multiple regression method (MRM) (Legendre & al., 1994; Lichstein, 2007) as implemented in eco-dist v.2.0.1 (Goslee & Urban, 2007). The statistical significance of the coefficient of regression was evaluated with 10,000 permutations.

Phylogenetic analyses. — Phylogenetic analyses were performed on five individuals for each population, except for two populations (H2, H3) where only four individuals were used, using the *rpl16*, *trnH-psbA*, and *rps16* chloroplast markers, and the nuclear ITS region (Appendix 2). Primers and temperature programmes used for the amplification followed Meeus & al. (2016) for *rpl16*, Sang & al. (1997) for *trnH-psbA*, Oxelman & al. (1997) for *rps16*, and Cheng & al. (2016) and White & al. (1990) for ITS. Excess salts and primers were removed from the PCR reactions with the PCR Purification Kit (Roche, Mannheim, Germany). Automated DNA sequencing was carried out directly from the purified PCR products using BigDye Terminator v.2 chemistry and an ABI310 sequencer (PE-Applied Biosystems, Norwalk, Connecticut, U.S.A.).

Raw sequences were checked for homology with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignments (suppl. Appendix S1, S2) including sequences from our accessions together with sequences published by Meeus & al. (2016), downloaded from NCBI GenBank, were built with MAFFT v.7 (Kato & al., 2002) and then manually edited with BioEdit v.7.0 (Hall, 1999). We avoided the inclusion of sequences from Meeus & al. (2016) that showed incongruences between cpDNA and nrDNA phylogenies, except those corresponding to entire lineages, such as *Pulmonaria affinis* Jord., *P. collina*, and *P. mollis*. Sequences of *Borago officinalis* L. and *Symphytum asperum* Lepech. were chosen as outgroup. Polymorphic sites were coded with ambiguous marks only when the $L / (L + l)$ ratio was between 0.50 and 0.55 (peaks of similar height), where L is the highest peak in the electropherogram and l the lowest one. Gaps were coded as separate characters according to the modified simplex coding and simple coding (Simmons & Ochoterena, 2000) for cpDNA and nrDNA, respectively, using SeqState v.1.4.1 (Müller, 2005). The best-fit nucleotide substitution model for each chloroplast and nuclear dataset was determined using jModelTest v.2.1.4 (Posada, 2008) under the Akaike information criterion (AIC). A Bayesian inference (BI) using 2 runs of 4 chains (3 heated, 1 cold) from a starting random tree and 25% of burn-in was conducted with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) on ITS and a combined cpDNA data matrix (combining all chloroplast markers) separately. Nucleotide sites and gaps were treated as different partitions using GTR + Γ + I and Jukes-Cantor substitution models, respectively. Calculation was stopped when the average standard deviation of split frequencies was below 0.01. Trees below the burn-in (25%) were discarded from the analysis, and a majority-rule consensus cladogram was built with the remaining trees. In addition, we built phylogenetic trees using BI adding one population at a time for both combined cpDNA and nrDNA alignments. Phylogenetic trees were drawn using TreeGraph2 v.2.14 (Stöver & Müller, 2010). A cpDNA haplotype network reconstruction was made using the function haploNet available in pegas package v.1.0-1 in R environment (Paradis & Schliep, 2019), whereas an ITS NeighborNet split network with uncorrected P distances was built using SplitsTree v.5 (Huson & Bryant, 2006). For the survey

of polymorphic sites in the ITS alignments, we only considered sites with $L / (L + l)$ between 0.50 and 0.90.

■ RESULTS

Morphological analysis. — Following the LDA (Fig. 2) performed on the dataset of basal leaf characters – combining features evaluated in both spring and summer leaves – the best jackknifed correct classification (87.93%) is obtained when *mixed* populations are excluded and populations of *Pulmonaria apennina* and *P. vallarsae* are merged into a single group (Fig. 2B). However, when *mixed* populations are included, the best jackknifed correct classification (82%) is obtained by assigning D1, D3, and D4 to *P. apennina*/*P. vallarsae* (“vallarsoid” morph hereafter) and D2 to *P. hirta* s.str. (“hirtoid” morph hereafter) (Fig. 2D). Flower features (suppl. Fig. S2) and leaf maculation (suppl. Fig. S3) show a morphological continuum and differences among populations are only found for few of them and/or for few pairwise comparisons (not shown). We found a balanced flower morph ratio in almost all populations, with the exception of A2 and D2 (Table 2; suppl. Fig. S4). Basal leaf shape is highly polymorphic within the complex (suppl. Fig. S5), albeit significantly different between “hirtoid” and “vallarsoid” morphs in both spring and summer (not shown); nevertheless, significant differences are also found between populations within the same morph (suppl. Tables S2, S3). The number of short and long hairs is significantly different between morphs in both spring and summer (not shown), and, also in this case, a few populations within the same morph are significantly different (suppl. Fig. S6; suppl. Tables S4, S5). In summary, the “hirtoid” morph is characterized by lanceolate leaves, with a lamina extending across a more or less winged petiole and an indumentum showing a higher number of long hairs with respect to short hairs. On the contrary, the “vallarsoid” morph shows rounded leaves with a more or less truncate base, and an indumentum characterized by a higher number of short hairs with respect to long hairs.

Karyotype asymmetry analysis. — The variation of interchromosomal (CV_{CL}) and intrachromosomal (M_{CA}) asymmetry indices across populations is shown in Table 3, while comparisons between values of all populations and monoploid idiograms for each cytotype ($2n = 16, 22, 26, 28$) are shown in Fig. 3. Cytotypes with $2n = 22, 26$ show M_{CA} values intermediate between those shown by cytotypes with $2n = 16$ (lower) and $2n = 28$ chromosomes (higher).

AFLP analysis. — Within-population heterozygosity (H_E) ranges from 0.168 (H2, OV) to 0.305 (D4), and the number of polymorphic loci from 89 (D2) to 167 (D4) (suppl. Table S6). AMOVA (suppl. Table S7) shows that genetic differentiation among populations is relatively high. However, the greatest part of the total variation in our sample (65.74%) is due to intra-population differences. The structure analysis supports the recognition of seven genetic clusters (Fig. 4A), as evidenced by ΔK scores (suppl. Fig. S7). Populations of *Pulmonaria*

vallarsae (V1, V2) and *P. officinalis* (OV) are distinct from all the others in having a homogeneous profile characterized by a peculiar genetic group (in green, blue and orange for V1, V2, and OV, respectively) that are rarely, or not, shared with any other population. Populations of *P. apennina*, *P. hirta* s.str. and those *mixed* are instead characterized by an admixture of different genetic groups. Interestingly, all the *mixed* populations show a prevailing group (in light blue), which is absent in *P. apennina* and *P. hirta* s.str. populations, and some individuals within the *mixed* populations, especially in D1, show the peculiar genetic group of the topotypical *P. vallarsae* (V1). Considering a lower number of clusters, such as $K = 2$ and $K = 4$, we can see that there is still no correspondence between genetic groups and taxonomy, since populations of *P. vallarsae* (V1, V2), *P. officinalis* (OV) and *mixed*

populations D1, D2, and D3 share the same genetic group (Fig. 4B,C, in orange), and other genetic groups are shared among populations of *P. apennina*, *P. hirta*, and *mixed* population D4. Populations of *P. apennina*, *P. hirta*, and the *mixed* population D4 also show admixtures of different genetic groups (Fig. 4B,C).

Morphological and genetic distances are not related, as shown by the statistical test ($R^2 = 0.030$, $P = 0.233$).

Phylogenetic analyses. — The alignment is 1926 bp long (*rpl16* 868 bp, *trnH-psbA* 246 bp, *rps16* 812 bp), with 93 gap positions (indel maximum length of 21), for combined cpDNA, and 675 bp long (indel maximum length of 2), with 12 gap positions, for nrDNA. We found 59 parsimony-informative sites and 93 singletons for the cpDNA alignment, and 50 parsimony-informative sites and 118 singletons for the

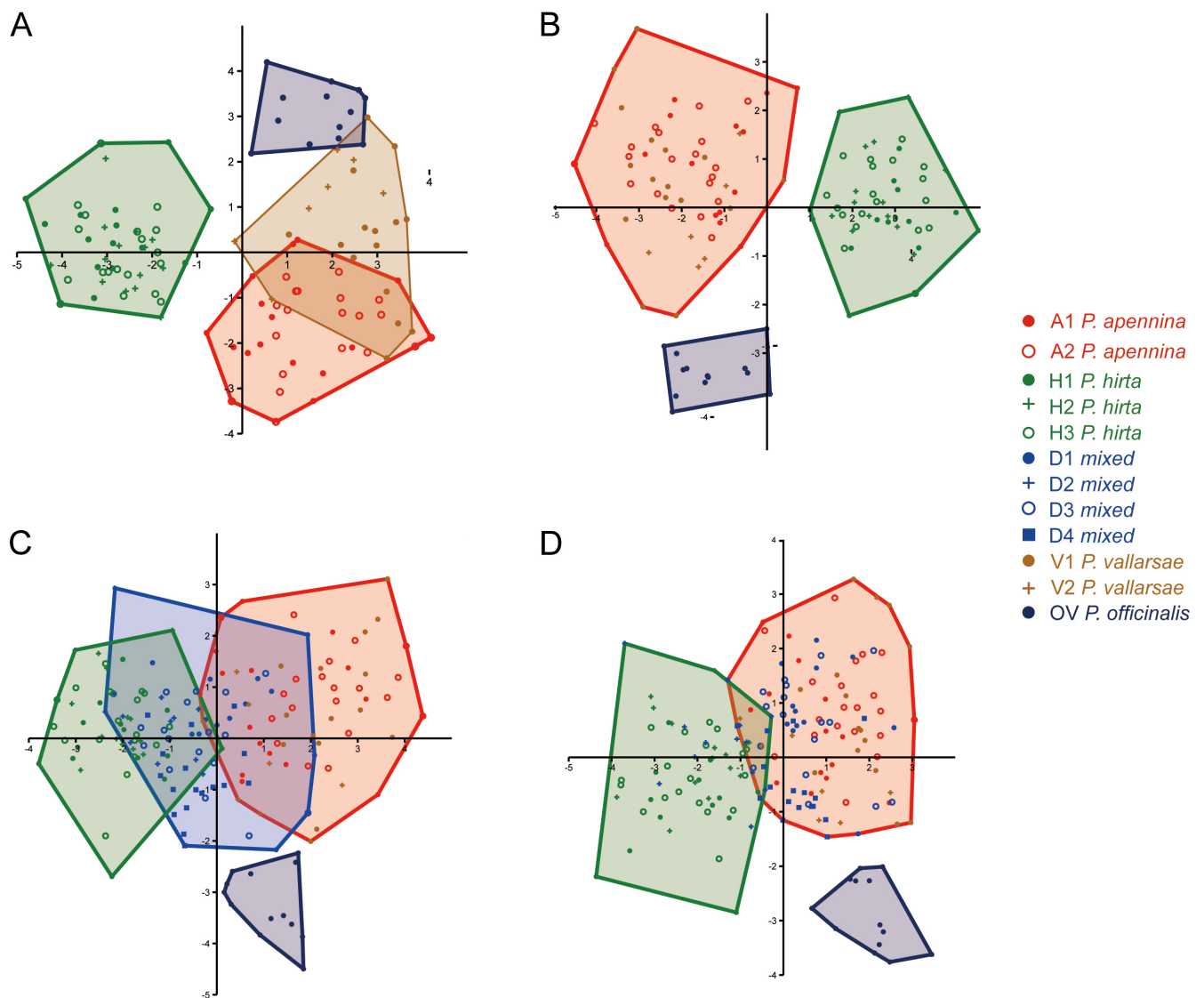


Fig. 2. Linear discriminant analysis of the whole dataset of basal leaf characters (in spring and in summer) of the *Pulmonaria hirta* complex and *P. officinalis* based on different groupings: **A**, No *mixed* populations; **B**, *P. apennina* and *P. vallarsae* merged together; **C**, *Mixed* populations added as a single distinct group; **D**, D1, D3 and D4 along with A1, A2, V1 and V2 assigned to “vallarsoid” morph, and D2 along with H1, H2 and H3 to “hirtoid” morph.

nrDNA alignment. The occurrence of topological differences between cpDNA and nrDNA cladograms prevented us to use a single combined dataset.

In the cpDNA tree (Fig. 5), we found that all the accessions of the *Pulmonaria hirta* complex fall in a clade with *P. affinis*, *P. angustifolia*, *P. collina*, *P. longifolia*, *P. mollis*, *P. montana*, and ‘*P. saccharata*’ accessions (angustifolia clade), except for two populations (A2, V1), which instead fall in the same clade with *P. obscura* and *P. officinalis* (officinalis clade). Within the angustifolia clade, H1 is sister to the rest of the accessions that are grouped together with a low support (PP = 0.60). A strongly supported subclade (PP = 0.99) includes *P. affinis*, *P. angustifolia*, *P. collina*, *P. longifolia*, *P. mollis*, *P. montana*, ‘*P. saccharata*’ accessions, three mixed populations (D1, D3, D4), and one individual of H2. In the cpDNA haplotype network (Fig. 6), 49 haplotypes were detected, differing by 1 to 16 mutation steps. They can be divided into two main clusters: one includes the haplotypes of the officinalis clade, A2 and V1, the other one includes the haplotypes of the angustifolia clade and the rest of the *P. hirta* complex sequences, and all the accessions from central Italy form a unique lineage, separated from V2.

Table 3. Karyotype features of sampled populations of the *Pulmonaria hirta* complex and *P. officinalis*.

Species	Population	Chromosome number	CV _{CL}	M _{CA}
<i>P. apennina</i>	A1	2n = 22 ^a	19.49	20.07
<i>P. apennina</i>	A2	2n = 22 ^a	19.88	17.33
<i>P. hirta</i>	H1	2n = 28 ^a	17.72	23.46
<i>P. hirta</i>	H2	2n = 28 ^a	19.31	23.66
<i>P. hirta</i>	H3	2n = 28 ^a	22.10	26.32
Mixed	D1	2n = 22 ^b	14.16	21.05
Mixed	D1	2n = 28 ^b	19.21	27.30
Mixed	D2	2n = 22 ^b	/	/
Mixed	D2	2n = 28 ^b	13.42	20.01
Mixed	D3	2n = 22 ^b , 25 ^c	15.62	17.35
Mixed	D4	2n = 26 ^a	17.95	17.60
<i>P. vallarsae</i>	V1	2n = 22 ^b	20.07	18.55
<i>P. vallarsae</i>	V2	2n = 22 ^b	23.19	19.82
<i>P. officinalis</i>	OV	2n = 16 ^b	15.22	16.75
<i>P. officinalis</i>	/	2n = 16 ^d	12.42	13.36
<i>P. officinalis</i>	/	2n = 16 ^d	10.71	14.68
<i>P. officinalis</i>	/	2n = 16 ^d	14.09	12.29
<i>P. officinalis</i>	/	2n = 16 ^d	14.96	14.29
<i>P. officinalis</i>	/	2n = 16 ^d	13.64	11.78
<i>P. officinalis</i>	/	2n = 16 ^d	17.58	13.41

Chromosome numbers and metaphasic plates taken from: ^a Astuti & al. (2019); ^b Astuti & al. (2020); ^c Vosa & Pistolesi (2004); ^d Astuti & al. (2013). CV_{CL} = Coefficient of Variation of Chromosome Length; M_{CA} = Mean Centromeric Asymmetry.

In the ITS tree, the inclusion of sequences of the *Pulmonaria hirta* complex heavily destabilizes the phylogenetic reconstruction, yielding numerous polytomies and weakening the support of nodes (Fig. 5). When added one at a time (suppl. Figs. S8–S11), all the sequences cluster in the angustifolia clade, except for few sequences of H3 and D2. Population V2 is sister to *P. montana*. The ITS split network (Fig. 7) shows that all the accessions of the *P. hirta* complex are in between the two extremes of the network, i.e., *P. montana* on one side and the officinalis clade on the other side. Most of the populations within the *P. hirta* complex fall close to the centre of the network, where all edges meet. On the other hand, A2 and V1 diverge distinctly from the centre and occupy tip edges, whereas other populations occupy an intermediate position linking the centre of the net to tip edge. There are 18 polymorphic sites (Table 4) in the ITS sequences of the *P. hirta* complex showing nucleotide additivity if compared to sequences of angustifolia and officinalis clades. The number of polymorphic sites varies among sequences from 2 to 16.

DISCUSSION

Lineages assembly within the *Pulmonaria hirta* complex. — Morphological features of summer basal leaves have been considered diagnostic for discriminating species of the *Pulmonaria hirta* complex (Merxmüller & Sauer, 1972; Bolliger, 1982; Puppi & Cristofolini, 1996). However, from our results, morphological patterns are generally similar in spring and summer leaves, and we did not detect a homogenizing effect of common garden conditions on population features.

Generally, a safe recognition of species is nearly impossible in central Italy, and a patchy distribution of morphological and karyological phenotypes is instead present. Nevertheless, this morphological pattern may be interpreted as either a single lineage in course of differentiation, or as multiple lineages converging after secondary contacts. At first glance, phylogenies built using cpDNA and nrDNA seem to support a multiple origin of taxa within the complex, as sequences are dispersed over the gene trees (Fig. 5). However, in the cpDNA network, we can see that most of the sequences of the *P. hirta* complex form a single cluster, very close to the rest of the species within the angustifolia clade (Fig. 6). Only three populations, located at the northern (V1, V2) and southern (A2) extremes of the distribution range of the complex, fall outside this cluster. From the nrDNA network, however, all the sequences of the *P. hirta* complex occupy the same position in the middle of the network (Fig. 7). The topologies shown by networks are more consistent with a single origin of the *P. hirta* complex, and the patterns shown by phylogenetic trees may be due to reticulate evolution, rather than to multiple lineages branching in different clades, as further discussed below.

Phenotypic and genetic differentiation. — Our results clearly show that phenotypic and genetic features in the investigated *Pulmonaria hirta* complex populations are not related.

Indeed, taken altogether, our results highlight a certain degree of morphological variation occurring within the complex (Fig. 2, suppl. Fig. S5), with two extreme morphs grossly corresponding to the traditional circumscriptions of *Pulmonaria hirta* s.str. and *P. vallisarsae*/*P. apennina*, that is a “hirtoid” morph showing more elongate leaves with decurrent base and few short hairs intermingled between longer hairs, and a “vallarsoid” morph with more rounded leaves with truncate base and more short hairs mixed to longer hairs. These two different morphs usually correspond to different chromosome numbers and karyotypes (Fig. 3), with the “hirtoid” morph showing $2n = 28$ chromosomes and the “vallarsoid” one showing $2n = 22$, 26 chromosomes. However, the morphological variation between these two extremes is rather continuous and could attest for some gene flow between the two morphs. This is also supported by the fact that all the populations showing intermediate features occur in those areas where *P. hirta* s.str. and *P. apennina* are reported to overlap (Puppi & Cristofolini, 1996; Vosa & Pistolesi, 2004; Cecchi & Selvi, 2015). Accordingly, we expected to find a similar pattern in AFLP data, reflecting a genetic differentiation between *P. hirta* s.str. and *P. apennina*/*P. vallisarsae*, and possibly signals of admixture in all populations, but especially in

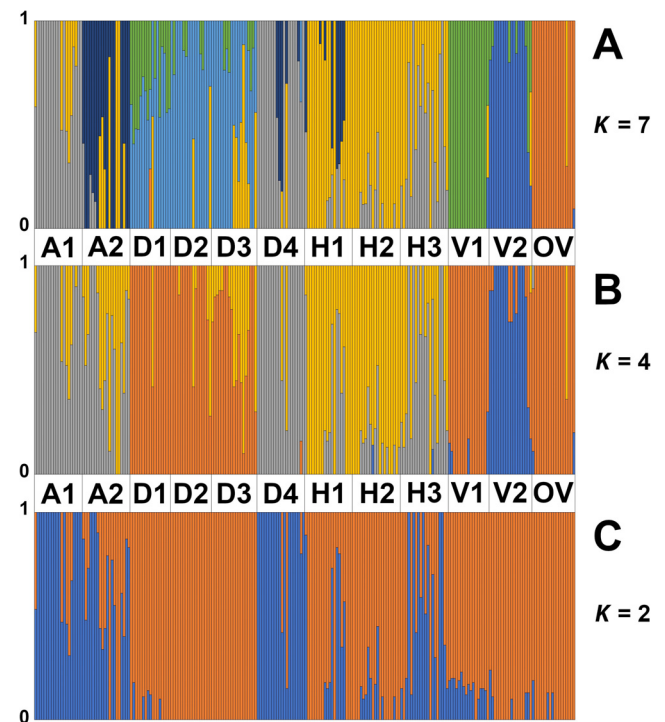


Fig. 4. Population structure analysis of 226 accessions of the *Pulmonaria hirta* complex and *P. officinalis* inferred using STRUCTURE. The different colours distinguish the genetic groups found for each K .

Karyotype asymmetry

P. apennina *P. hirta* mixed *P. vallisarsae* *P. officinalis*

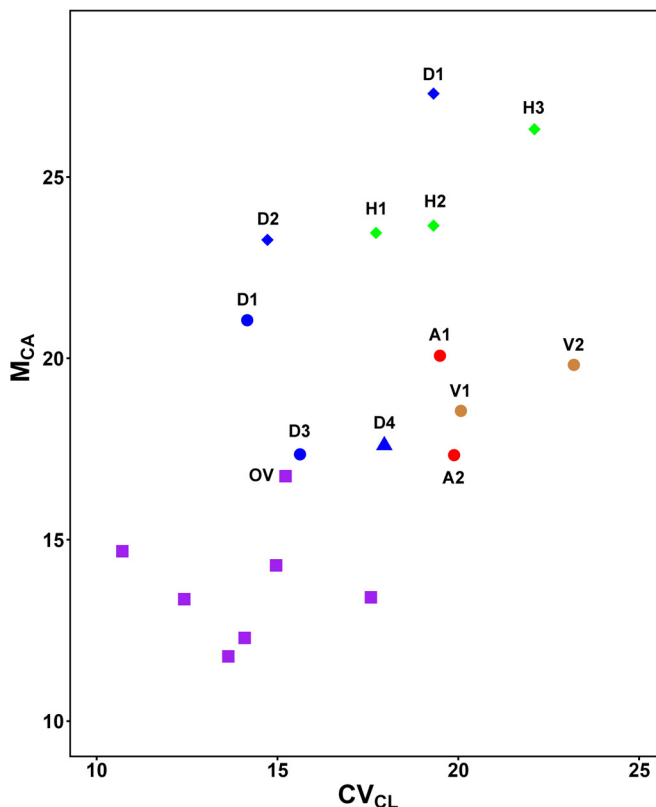


Fig. 3. Karyotype asymmetry indices (CV_{CL} , M_{CA}) of populations within the *Pulmonaria hirta* complex and *P. officinalis* (see Table 3). Values for the latter species (violet squares) were also taken from Astuti & al. (2013). Idiograms of each chromosome number are reported on the right.

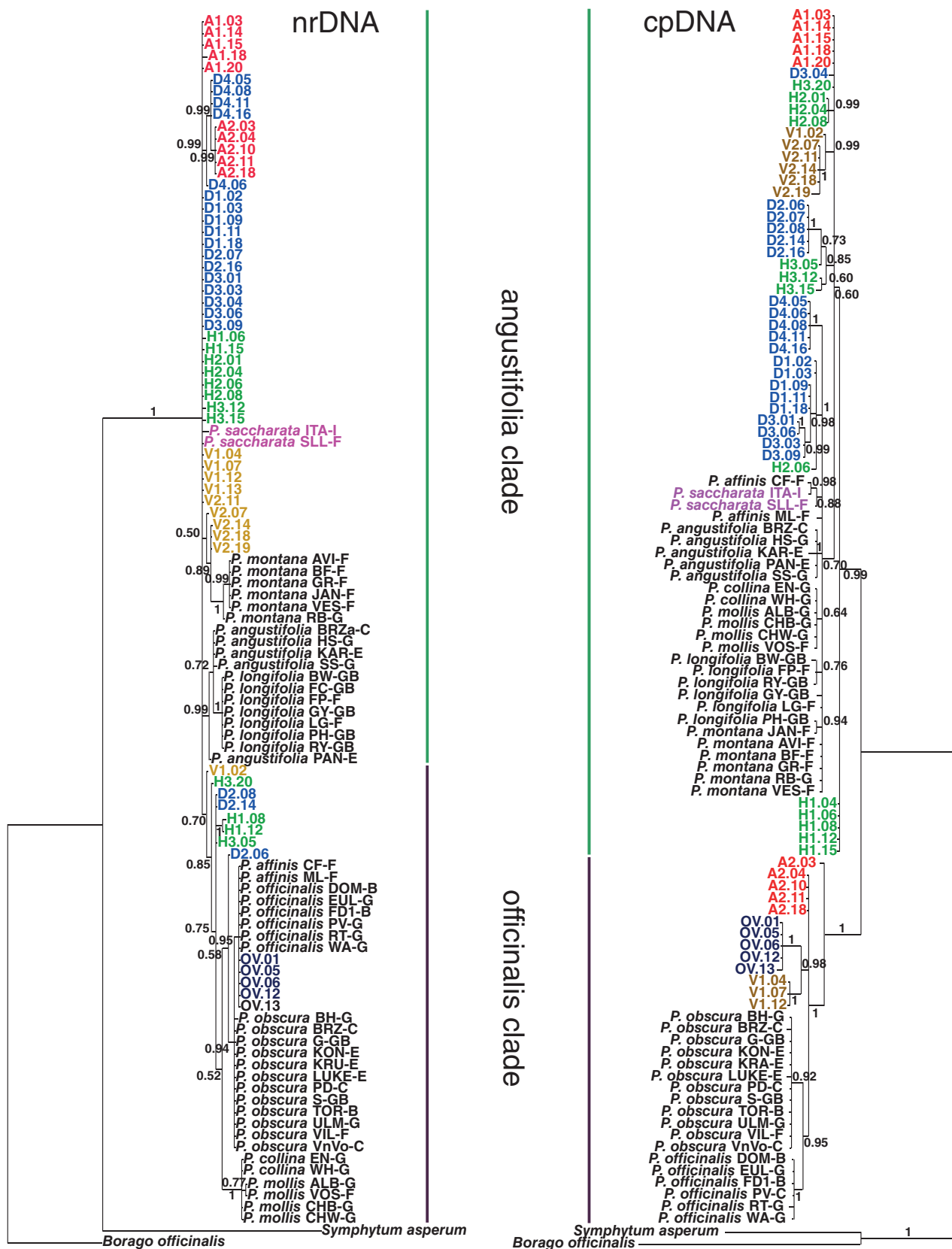


Fig. 5. Bayesian inference (BI) trees of *Pulmonaria*, including accessions published by Meeus & al. (2016), based on a nuclear marker (ITS; left) and three chloroplast markers (*rpl16*, *trnH-psbA*, *rps16*; right). Values on branches are posterior probabilities. For population codes of the *Pulmonaria hirta* complex, see Table 1 and Fig. 1; for population codes of the remaining accessions, see Meeus & al. (2016).

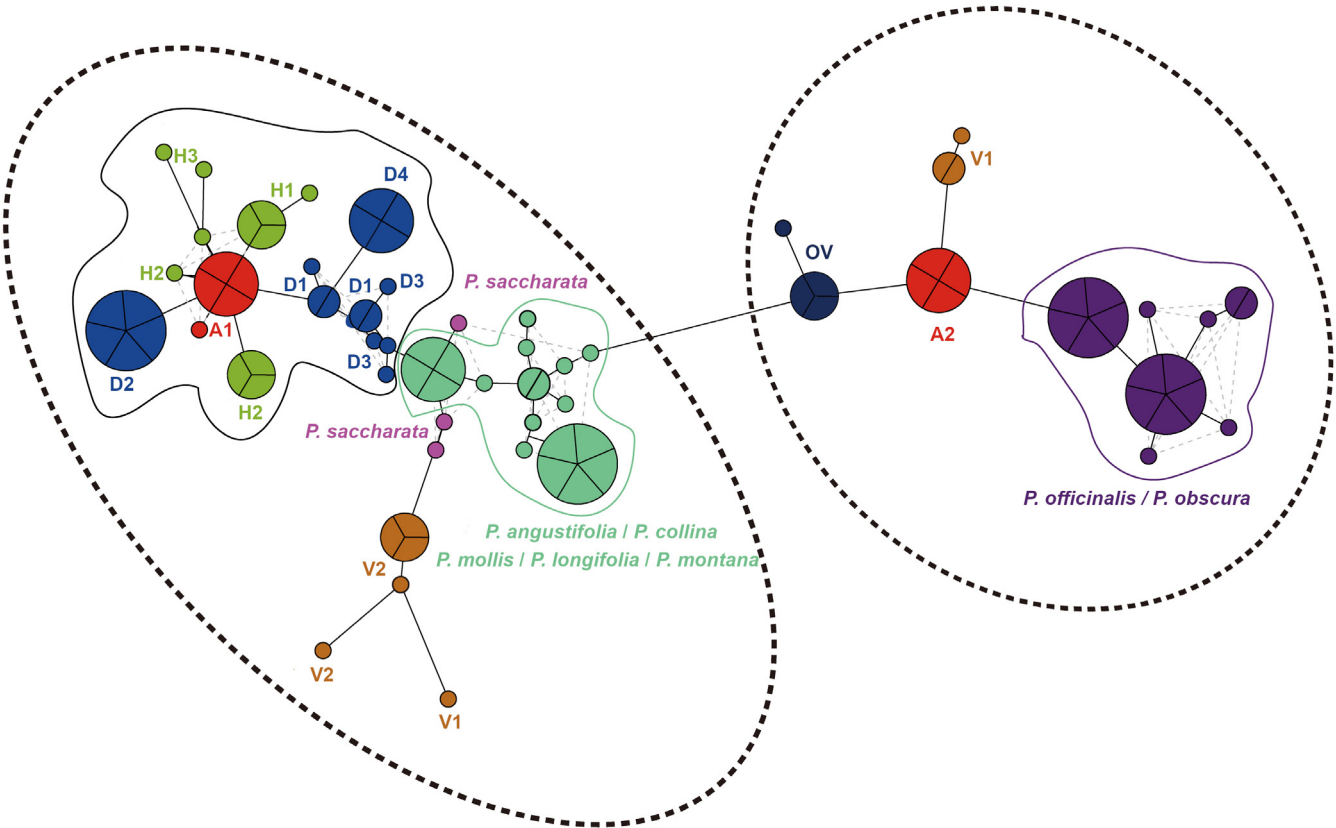


Fig. 6. Network connecting 49 plastid haplotypes, including accessions of the *Pulmonaria hirta* complex studied here and those published by Meeus & al. (2016). For population codes of the *Pulmonaria hirta* complex, see Table 1 and Fig. 1.

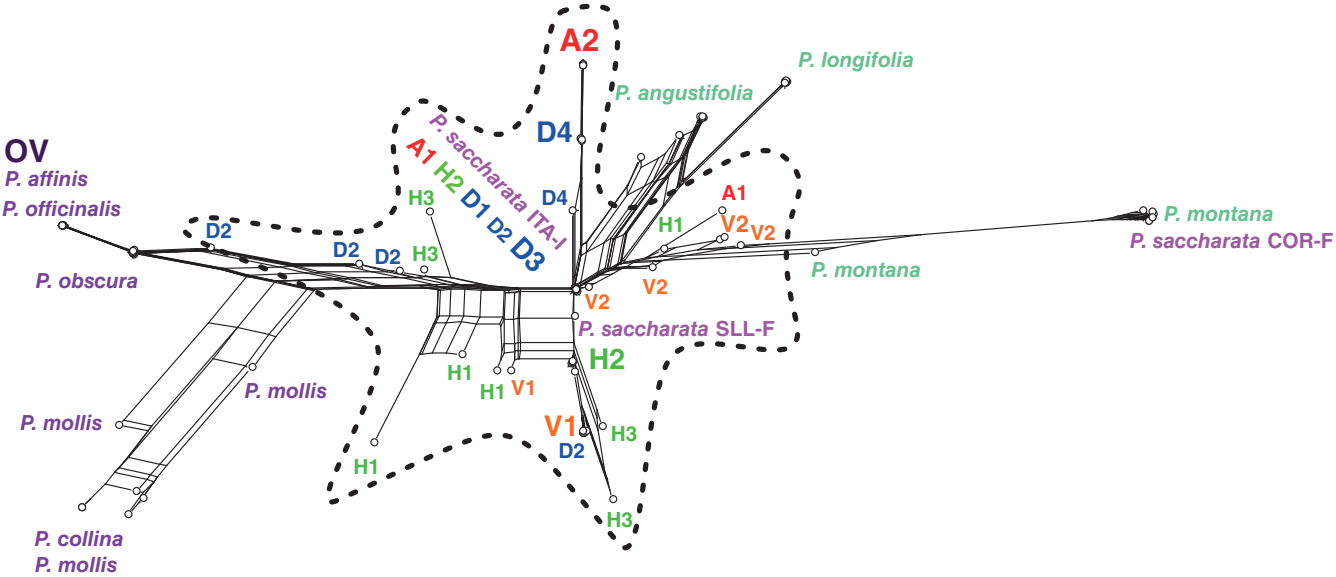


Fig. 7. NeighborNet split network of ITS sequences of all populations of the *Pulmonaria hirta* complex (enclosed in the dashed line) and accessions published by Meeus & al. (2016). Population code size in the *Pulmonaria hirta* complex proportional to the number of haplotypes represented. For population codes of the *Pulmonaria hirta* complex, see Table 1 and Fig. 1.

Table 4. ITS polymorphic nucleotide sites of populations of the *Pulmonaria hirta* complex and comparison with *Pulmonaria* sequences taken from Meeus & al. (2016).

	74	77	91	97	106	107	115	139	143	210	211	255	488	489	490	499	622	648
A1	R	G	G	W(A) ¹	M	R(A) ¹	Y	W	Y	Y	W	W	K	M	Y	Y	R	S
A2	A	K	R+	A	C	A	C	A	T	T	A	T	G	C	C	C	A	G
H1	R	K*(G) ²	G	W	M	R	Y	W	Y	Y	W	W	K	M	Y	Y	A	G
H2	A	G	G	A	C(M) ¹	A	C(Y) ²	W(A) ¹	Y ^{oo}	T(Y) ²	A	W	K(G) ¹	C	C	Y	R	S
H3	A	G	G	A	C	A	Y	W	Y ^{ooo}	T	A	W&	K	C	C(Y) ¹	Y	R	S
D1	R	G	G	W(A) ²	M	R	Y	W	Y	Y	W	W	K	M	Y	Y(C) ¹	R	S
D2	R	G	G	W	M(C) ¹	R(A) ²	Y	W	Y ^o	Y	W	W&	K	M(C) ²	Y	Y	R	S
D3	R(A) ²	G	G	A	M(C) ²	A	Y	W	Y	Y(T) ²	W(A) ²	W	K(G) ¹	M(C) ²	Y(C) ²	Y(C) ¹	R(A) ²	G
D4	A	K	R	A	C	A	Y	W	T	T	A	T	K	C	C	Y	R	G
V1	R(A) ²	G	G	A	C	A	Y	W	Y ^{oooo}	T	A	W&&	K	C	C	Y	R	G(S) ¹
V2	R	G	G	W	M	R	Y	W	Y	Y	W	W	K	M	Y	Y	R	S(G) ²
angustifolia clade																		
<i>P. affinis</i>	A	G	G	A	C	A	T	T	T	T	A	T	T	C	C	T	G	C
<i>P. angustifolia</i>	G	A	G	A	C	A	C	A	T	T	A	T	G	C	C	C	A	G
<i>P. collina</i>	A	G	G	A	C	A	C	T	T	T	A	T	T	C	C	T	A	C
<i>P. longifolia</i>	G	G	G	A	C	A	C	A	T	T	A	T	G	C	C	C	A	G
<i>P. mollis</i>	A	G	G	A	C	A	Y(C) ⁴	A(T) ² (W) ¹	T	T	A	T	T	C	C	T	A	C
<i>P. montana</i>	G	G	G	T(W) ¹	A(C) ¹	G	C	A	T	C(Y) ¹	T	T	G	A(M) ¹	T(-) ¹	C	A	G
<i>P. saccharata</i>	A	G	G	A	C	A	C	A	T	T	A	T	G	C	C	C	A	G
officinalis clade																		
<i>P. obscura</i>	A	G	G	A	C	A	T	T	T	T	A	T	T	C	C	T	G	C
<i>P. officinalis</i>	A	G	G	A	C	A	T	T	T	T	A	T	T	C	C	T	G	C

The number of accessions showing the nucleotide code in brackets is indicated in superscript.

* = T peak higher than G peak; + = A peak higher than G peak; ° = one sequence with C peak higher than T peak; °° = two sequences with C peak higher than T peak; °°° = three sequences with C peak higher than T peak; °°°° = all sequences with C peak higher than T peak; & = one sequence with A peak higher than T peak; && = all sequences with A peak higher than T peak.

the *mixed* ones. On the contrary, *mixed* populations D1, D2, and D3 show only limited admixture, and involving only genetic groups of *P. hirta* s.str. and *P. vallisae* (i.e., yellow and green groups in Fig. 4A), without any trace of *P. apennina*. Furthermore, these *mixed* populations are all characterized by a peculiar genetic group (in light blue; Fig. 4A), not shared with any other population. We could interpret this unexpected pattern as the consequence of retention of alleles otherwise lost in other populations, a pattern that has been claimed to involve hybrid zones as reservoir of genetic diversity (Brennan & al., 2012).

Moreover, *Pulmonaria apennina* and *P. vallisae* are morphologically very similar, but do not share any genetic group, whereas *P. apennina* and *P. hirta* s.str. do (grey, yellow, and dark blue groups in Fig. 4A). This is of course consistent with geographical proximity, albeit geography had a limited effect on the genetic pattern when considering *mixed* populations. Actually, the only *mixed* population that shares genetic groups (grey and dark blue groups in Fig. 4A) with *P. apennina* and *P. hirta* s.str. is D4, which is quite geographically distant from all the others. On the other hand, the peculiar group largely occurring in the topotypical population of *P. vallisae* (V1) may have been lost in populations of *P. hirta* s.str. and *P. apennina*, whereas traces in *mixed* populations may be seen as remnants of the past presence of this genetic group in central Italy. It may even have originated in central Italy, assuming that plants with $2n = 22$ chromosomes spread from the Apennines to the Alps as hypothesized by Bolliger (1982). Despite the smallest distribution range among these taxa, *P. vallisae* is quite variable as shown by molecular markers. Indeed, its two populations show different genetic groups in the STRUCTURE analysis, where V2 is characterized by a peculiar group not shared with any other population. This differentiation may be congruent with the phylogenetic data, where V2 is closely related to *P. montana* in the nrDNA tree and to other species of the angustifolia clade in the cpDNA network. Although being morphologically and karyologically differentiated from the *P. hirta* complex, *P. officinalis* (OV) shows a strong genetic affinity with many populations of this complex (V1, D1, D2, D3) at lower Ks (Fig. 4B,C), and this could be due to hybridization events at different evolutionary scales, as discussed later.

Finally, populations showing an excess of long-styled morphs show either low (D2) or high (A2) genetic diversity, so that, in our case, deviation from isoplethy had a negligible effect on genetic diversity. On the contrary, Meeus & al. (2012b) showed that genetic diversity decreased in populations of *Pulmonaria officinalis* showing an excess of either short-styled or long-styled individuals.

Systematic relationships of the *Pulmonaria hirta* complex. — Considering the relationships with other species within the genus, we found hybridization signals in our phylogenetic reconstruction for all the populations of the *Pulmonaria hirta* complex, and particularly in ITS polymorphic sites (Table 4). As cpDNA and nrDNA data may support a single origin of

the *P. hirta* complex, a unique hybridization event may have occurred through crosses between species of the officinalis clade and species of the angustifolia clade. The in-between position of the *P. hirta* complex accessions in the split network is similar to that found by Grünig & al. (2021) – using ddRAD loci – for *P. helvetica*, another hybridogenic species of *Pulmonaria*, originated from a cross between *P. officinalis* and a species among *P. collina*, *P. mollis*, and *P. montana*. Hypothesizing a hybrid origin for the whole *P. hirta* complex lineage, we should assume that a parental species from the angustifolia clade acted as the ovule donor and a parental species from the officinalis clade as the pollen donor, since most of the accessions cluster within the angustifolia clade in cpDNA phylogeny. However, two “vallarsoid” populations (A2, V1) cluster in the officinalis clade in the cpDNA. The topological incongruence between cpDNA and ITS phylogenies shown by these two “vallarsoid” populations may be due to their distance from the core area of the complex (central Italy) that prevents them from crossing with “hirtoid” plants. However, some AFLP genetic groups are shared between A2 and *P. hirta* s.str. (Fig. 4), probably because of the connection ensured by the rather continuous range occupied by the *P. hirta* complex along the Apennines. Nevertheless, the close phylogenetic relationship of the “vallarsoid” populations A2 and V1 with species in the officinalis clade, and particularly with the only population of *P. officinalis* sampled in Italy (OV), may be the result of a backcross (see further under “Evolutionary hypotheses”) or simple introgression at population level, even though this seems more plausible for V1 than for A2, since *P. officinalis* populations occur close to V1, while no species in the officinalis clade is reported from southern Italy (Bartolucci & al., 2018). AFLP supports the close relationship between *P. officinalis* and V1 at both $K = 2$ and $K = 4$, whereas the affinity between *P. officinalis* and A2 is found only at $K = 2$.

As discussed above, in the ITS phylogeny (Figs. 5A, 7), a single population (V2) shows a particular affinity with *Pulmonaria montana* Lej., another morphologically polymorphic species, showing an equally large variation of chromosome numbers ($2n = 22$ [20, 24, 25, 26, 27, 28]) and occurring in Belgium, S Germany, E France, and W Switzerland (Merxmüller & Sauer, 1972; Bolliger, 1982). Although this species has never been reported from the Italian peninsula, there are records very close to the Italian border in the database of Swiss flora (<https://www.infoflora.ch/it/flora/pulmonaria-montana.html#map>). The affinity of *P. hirta* (under the name *P. saccharata*) with *P. montana* in the ITS phylogeny was already documented by Meeus & al. (2016), who interpreted it as an introgressive relationship at population level. On the other hand, it is possible that the affinity of the *P. hirta* complex with *P. montana* may reside in the putative hybrid origin of the former, possibly involving the latter as one of the parents.

In their phylogenetic reconstruction, Meeus & al. (2016) included a total of three accessions belonging to the *Pulmonaria hirta* complex (under the name *P. saccharata*). Although nodes are not highly supported, our accessions

cluster close to these '*P. saccharata*' populations in nrDNA tree, confirming the synonymy of *P. saccharata* with *P. hirta* (Puppi & Cristofolini, 1991; Selvi & Cristofolini in Cafferty & Jarvis, 2004).

Evolutionary hypotheses. — Because of its widespread distribution in the northern part of the Italian peninsula and the relationships found in the STRUCTURE analysis, *Pulmonaria officinalis* may be the best candidate as one of the parental species involved in the origin of the *P. hirta* complex. We can hence elaborate three possible scenarios starting from a hybridization event between species of the angustifolia clade and *P. officinalis*: (1) a unique resulting hybrid species, morphologically "vallarsoid" with $2n = 22$ chromosomes, spread across the Italian peninsula and gave rise to the "hirtoid" morph ($2n = 28$) through ascending dysploidy; (2) two geographically distinct hybridization events produced both "vallarsoid" ($2n = 22$) and "hirtoid" ($2n = 28$) morphs; (3) a unique "hirtoid" ($2n = 28$) allopolyploid hybrid species originated, which then produced the "vallarsoid" ($2n = 22$) plants through descending dysploidy or backcrossing with *P. officinalis*. In all scenarios, dysploidy played an important role in producing new cytotypes. Dysploidy is an important driver for speciation, capable of originating species-rich lineages at macroevolutionary level (Escudero & al., 2014). This role has been deeply documented in Brassicaceae (e.g., Mandáková & al., 2015, 2018, 2020), but chromosome number changes through fission and fusion have been claimed as a major cause for species differentiation in *Pulmonaria* as well (Merxmüller & Grau, 1969; Sauer, 1975, 1987; Sauer & Gruber, 1979; Bolliger, 1982; Vosa & Pistolesi, 2004). Nevertheless, hybridization event(s) between species with different chromosome numbers may equally induce speciation through the production of different cytotypes, as hypothesized here concerning the origin of the *P. hirta* complex, and as similarly evidenced by Grünig & al. (2021) for the origin of *P. helvetica*.

Under the first scenario, "vallarsoid" plants originated "hirtoid" morphs, maintaining connections in central Italy, where a massive gene flow caused morphological and molecular convergence, as well as co-occurrence of different chromosome numbers, sometimes within the same population (e.g., mixed populations). However, ascending dysploidy seems less common than descending dysploidy in angiosperms (Carta & al., 2020), and the latter played a major role for speciation in *Nonea* Medik. (Selvi & al., 2002), a genus closely related to *Pulmonaria*.

Under the second scenario, the two independently originated morphs established a gene flow in central Italy, where they co-occur. In this case, the plants with $2n = 28$ chromosomes would have originated through a cross between an unreduced gamete of *Pulmonaria officinalis* ($n = 16$) and a cytotype with $2n = 24$ chromosomes, or through a cross between normal gametes of *P. officinalis* and unreduced gametes of a cytotype with $2n = 20$ chromosomes. The cross between unreduced gametes and normally reduced gametes has been reported by Bolliger (1982) for *P. officinalis* and *P. helvetica*.

Nevertheless, the hypothesis of two different hybrid events is not fully supported by our data, given that polymorphisms in the ITS are mostly found in the same position of all the sequences analyzed.

Under the third scenario, the "vallarsoid" morph originated through descending dysploidy from "hirtoid" plants or through backcross of these latter plants with *Pulmonaria officinalis*. Once originated, "vallarsoid" plants spread across the Italian peninsula, maintaining connection (gene flow) with "hirtoid" plants in central Italy, so that pure "vallarsoid" morphs remained only at the southern and northern extremes of the range, where "hirtoid" plants have never been reported (Cecchi & Selvi, 2015; Bartolucci & al., 2018). The STRUCTURE analysis shows that the genetic group dominant in the populations of *P. hirta* s.str. (Fig. 4, in yellow) is also found in almost all the other populations, so that it may represent the most ancient genetic group among those found. The hypothesis of descending dysploid origin of plants with $2n = 22$ chromosomes from plants with $2n = 28$ chromosomes has been already postulated by Bolliger (1982). In addition, the same author interpreted plants with $2n = 28$ chromosomes from the Apennines as tetraploid relic populations overwhelmed by dysploid swarms with $2n = 22$ chromosomes, that subsequently spread towards the NW and NE Alps. This scenario may be consistent with the overrepresentation of some genetic groups prevailing in plants with $2n = 22$ (e.g., grey group in A1; Fig. 4) in other populations with $2n = 26$, 28 chromosomes (H3, D4). A possible origin of "vallarsoid" plants from a backcross of "hirtoid" plants with *P. officinalis* is instead supported by phylogenetic data, especially by cpDNA: the original "hirtoid" lineage inherited its plastid genome from a species in the angustifolia clade, whereas "vallarsoid" morphs (A2, V1) inherited it from *P. officinalis*. All other "vallarsoid" accessions may have inherited a cpDNA genome through introgression with "hirtoid" plants (A1, D1, D3, D4) or with other species of the angustifolia clade (V2). It is possible that in some cases (D4) introgression produced a stable intermediate chromosome number ($2n = 26$, Astuti & al., 2019). In addition, populations with $2n = 22$, 26 chromosomes show karyotype asymmetry values intermediate between those of *P. hirta* s.str. and *P. officinalis*, corroborating the hypothesis that "vallarsoid" morphs originated from crossing events between these latter taxa (Fig. 3).

Irrespective of the exact evolutionary dynamics, today, a unique lineage showing three cytotypes ($2n = 22$, 26, 28) is widely distributed throughout the Italian peninsula. On taxonomic grounds, we propose to consider these plants as belonging to a single polymorphic species, namely *Pulmonaria hirta*.

In order to fully understand the possible evolutionary trajectories of this group, further studies on mechanisms of reproductive isolation are needed. More detailed cytogenetic analyses may clarify the role of chromosome rearrangements occurred and their contribution to karyotype differentiation. Karyotype differences may indeed cause reproductive isolation, but Bolliger (1982) provided evidence of extreme

versatility displayed by lungwort species at meiotic stage, where normalization of pairings in otherwise unbalanced situations has been found in artificially produced intraspecific (crossing different cytotypes) and interspecific hybrids.

Description and diagnosis. — In order to clarify the circumscription of *Pulmonaria hirta*, we provide here an updated diagnosis and description. *Pulmonaria hirta* is distinct from *P. officinalis* by never cordate basal leaves, without *aculeoli* (conical hairs less than 0.1 mm long) on the upper surface; also, chromosome number is $2n = 22, 26, 28$ and not $2n = 16$. It is distinguished from *P. montana* by basal leaves with short hairs on the upper surface (not showing only long bristles). It is also distinct from the allopatric, but morphologically very similar, *P. affinis* (from central and SW France and Spain) by short hairs showing a narrower base and by the indumentum being generally softer, albeit these subtle differences should be investigated more in detail.

Pulmonaria hirta has lanceolate basal leaves with base decurrent on indistinct petiole (“hirtoid” morph) to ovate-rounded with truncate-attenuate base distinct from petiole (“vallarsoid” morph); leaves (including petiole) up to 33 cm long and 13 cm wide, 1.5–5 times longer than wide; upper surface with evident pale blotches, or with numerous to sparse, white to greenish, spots, or no spots at all (in “vallarsoid” morph); trichomes on upper surface consisting of long bristles mixed with short hairs (up to 40 per mm² in summer leaves), glandular hairs, and sessile microglands; short hairs ranging from ≤ 10 per mm² (in “hirtoid” morph) to 50 per mm² (in “vallarsoid” morph), long hairs 3–4 per mm² up to 12 per mm² (in “hirtoid” morph); inflorescence a terminal scorpioid cyme; flowers actinomorphic, bisexual, distylous; calyx lobe 2–7 mm, tube 5–20 mm; corolla colour varying from purple, or blue to shades of pink and red, petals 5, connate, lobe 3–20 mm, tube 2–8 mm.

■ CONCLUSION

Populations within the *Pulmonaria hirta* complex show a variety of morphological and karyological phenotypes with a certain degree of intrapopulational variability. A continuous morphological variation between two extreme morphs, here called “hirtoid” and “vallarsoid” and grossly corresponding to *P. hirta* s.str. and *P. apennina*/*P. vallarsae*, is highlighted by our analyses. The chromosome number $2n = 28$ is only found in “hirtoid” plants, which occasionally may also show $2n = 22$ cytotypes. On the contrary, “vallarsoid” plants are usually characterized by $2n = 22$ chromosomes, although also $2n = 26$ can be found.

Morphological similarities are not paralleled by genetic data: the reconstruction of genetic structure revealed that morphologically and/or karyologically ambiguous populations, occurring in areas where the ranges of *Pulmonaria hirta* s.str. and *P. apennina* overlap, show a distinctive genetic group and only limited admixture signals. Conversely, *P. apennina* and *P. hirta* s.str. populations show extensive admixture

signals, attesting for a massive gene flow between the two taxa. The two studied populations of *P. vallarsae* are quite distinct from each other and from all the other populations in the complex, without any relationship with *P. apennina*, which is otherwise morphologically very similar.

Phylogenetic data suggest a hybrid origin for the whole complex: a cross between species belonging to the angustifolia clade and officinalis clade may have produced a novel lineage with a novel karyotype. This lineage spread throughout the Italian peninsula, originating other cytotypes through dysploidy and/or backcrosses with parental species. Introgression at population level may also have contributed in shaping the evolution of this complex.

■ AUTHOR CONTRIBUTIONS

GA and LP conceived the idea and designed the experiments. LL and GA conducted the field sampling and collected the plant material. LL and GA performed the morphometric and karyological analyses. LL and AC performed the molecular experiments. LL, GA, AC, and LP analyzed the data and drafted this manuscript. — GA, <https://orcid.org/0000-0001-5790-3516>; AC, <https://orcid.org/0000-0003-4760-8403>; LP, <https://orcid.org/0000-0001-9008-273X>

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Appendix 1. Chromosome counts for the *Pulmonaria hirta* complex and *P. officinalis*.

Taxon, locality, voucher numbers (herbarium), chromosome number. Vouchers with asterisk (*) are those used in this study to reconstruct the karyotype.

Pulmonaria apennina Cristof. & Puppi, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, G. Astuti & L. Liu s.n., PI021283–021302 (PI), PI021295*, 2n = 22; *P. apennina* Cristof. & Puppi, Italy, Calabria, San Fili (Cosenza), Foresta Luta, G. Astuti, L. Liu, F. Roma-Marzio s.n., PI021323–021342 (PI), PI021342*, 2n = 22; *Pulmonaria hirta* L. s.l., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, L. Liu & G. Astuti s.n., PI034194–034202 (PI), PI034194*, 2n = 22; *P. hirta* L. s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, L. Liu & G. Astuti s.n., PI034223–034226 (PI), PI034224*, 2n = 28; *P. hirta* L. s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, L. Liu & G. Astuti s.n., PI034227–034228 (PI), 2n = 22; *P. hirta* L. s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, L. Liu & G. Astuti s.n., PI034203–034214 (PI), PI034214*, 2n = 22; *P. hirta* L. s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), G. Astuti, L. Liu, F. Bartolucci s.n., PI021303–021322 (PI), PI021307*, 2n = 26; *Pulmonaria hirta* L. s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, G. Astuti & L. Liu s.n., PI021343–021357 (PI), PI021345*, 2n = 28; *P. hirta* L. s.str., Italy, Tuscany, Poppi (Arezzo), Camaldoli, G. Astuti & L. Liu s.n., PI021358–021376 (PI), PI021359*, 2n = 28; *P. hirta* L. s.str., Italy, Emilia-Romagna, Grizzana Morandi (Bologna), Favari, G. Astuti & L. Liu s.n., PI021377–021396 (PI), PI021387*, 2n = 28; *Pulmonaria officinalis* L., Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, L. Liu &

Appendix 1. Continued.

G. Astuti s.n., PI034229–034237 (PI), PI034235*, *2n* = 16, *Pulmonaria vallisae* A.Kern., Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *L. Liu & G. Astuti s.n.*, PI034215–034219 (PI), PI034215*, *2n* = 22; *P. vallisae* A.Kern., Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei, *L. Liu & G. Astuti s.n.*, PI034220–034222 (PI), PI034220*, *2n* = 22.

Appendix 2. GenBank accession numbers of the *Pulmonaria hirta* complex, *P. officinalis* and outgroup sequences. Sequences taken from Meeus & al. (2016) are available on GenBank (KT737495–KT737721).

Taxon, locality, voucher number (herbarium), accession numbers for ITS, *trnH-psbA*, *rpl16*, *rps16*. “*” represents sequences newly generated in this study and “–” indicates missing sequences.

Pulmonaria apennina Cristof. & Puppi, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, *G. Astuti & L. Liu s.n.*, PI021285 (PI), MW344074*, MW346640*, MW346634*, MW346637*; *P. apennina*, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, *G. Astuti & L. Liu s.n.*, PI021296 (PI), MW164985*, MW165999*, MW165892*, MW165946*; *P. apennina*, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, *G. Astuti & L. Liu s.n.*, PI021297 (PI), MW164986*, MW166000*, MW165893*, MW165947*; *P. apennina*, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, *G. Astuti & L. Liu s.n.*, PI021300 (PI), MW164987*, MW166001*, MW165894*, MW165948*; *P. apennina*, Italy, Emilia-Romagna, Casalecchio di Reno (Bologna), Parco Talon, *G. Astuti & L. Liu s.n.*, PI021302 (PI), MW164988*, MW166002*, MW165895*, MW165949*; *P. apennina*, Italy, Calabria, San Fili (Cosenza), Foresta Luta, *G. Astuti, L. Liu, F. Roma-Marzio s.n.*, PI021325 (PI), MW164994*, MW166008*, MW165901*, MW165955*; *P. apennina*, Italy, Calabria, San Fili (Cosenza), Foresta Luta, *G. Astuti, L. Liu, F. Roma-Marzio s.n.*, PI021326 (PI), MW164995*, MW166009*, MW165902*, MW165956*; *P. apennina*, Italy, Calabria, San Fili (Cosenza), Foresta Luta, *G. Astuti, L. Liu, F. Roma-Marzio s.n.*, PI021332 (PI), MW164996*, MW166010*, MW165903*, MW165957*; *P. apennina*, Italy, Calabria, San Fili (Cosenza), Foresta Luta, *G. Astuti, L. Liu, F. Roma-Marzio s.n.*, PI021333 (PI), MW164997*, MW166011*, MW165904*, MW165958*; *P. apennina*, Italy, Calabria, San Fili (Cosenza), Foresta Luta, *G. Astuti, L. Liu, F. Roma-Marzio s.n.*, PI021340 (PI), MW164998*, MW166012*, MW165905*, MW165959*; *P. hirta* L., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, *L. Liu & G. Astuti s.n.*, PI035975 (PI), MW164999*, MW166013*, MW165906*, MW165960*; *P. hirta* L., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, *L. Liu & G. Astuti s.n.*, PI035976 (PI), MW165000*, MW166014*, MW165907*, MW165961*; *P. hirta* s.l., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, *L. Liu & G. Astuti s.n.*, PI034198 (PI), MW165001*, MW166015*, MW165908*, MW165962*; *P. hirta* s.l., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, *L. Liu & G. Astuti s.n.*, PI035979 (PI), MW165002*, MW166016*, MW165909*, MW165963*; *P. hirta* s.l., Italy, Tuscany, Sambuca Pistoiese (Pistoia), Molino del Pallone, *L. Liu & G. Astuti s.n.*, PI035984 (PI), MW165003*, MW166017*, MW165910*, MW165964*; *P. hirta* s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, *L. Liu & G. Astuti s.n.*, PI035987 (PI), MW165004*, MW166018*, MW165911*, MW165965*; *P. hirta* s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, *L. Liu & G. Astuti s.n.*, PI035988 (PI), MW165005*, MW166019*, MW165912*, MW165966*; *P. hirta* s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, *L. Liu & G. Astuti s.n.*, PI035989 (PI), MW165006*, MW166020*, MW165913*, MW165967*; *P. hirta* s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, *L. Liu & G. Astuti s.n.*, PI034227 (PI), MW165007*, MW166021*, MW165914*, MW165968*; *P. hirta* s.l., Italy, Emilia-Romagna, Bologna, Monte Paderno, *L. Liu & G. Astuti s.n.*, PI034228 (PI), MW165008*, MW166022*, MW165915*, MW165969*; *P. hirta* s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, *L. Liu & G. Astuti s.n.*, PI035996 (PI), MW344074*, MW346640*, MW346634*, MW346637*; *P. hirta* s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, *L. Liu & G. Astuti s.n.*, PI034203 (PI), MW165009*, MW166023*, MW165916*, MW165970*; *P. hirta* s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, *L. Liu & G. Astuti s.n.*, PI034204 (PI), MW165010*, MW166024*, MW165917*, MW165971*; *P. hirta* s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, *L. Liu & G. Astuti s.n.*, PI035997 (PI), MW165011*, MW166025*, MW165918*, MW165972*; *P. hirta* s.l., Italy, Tuscany, San Godenzo (Firenze), Passo del Muraglione, *L. Liu & G. Astuti s.n.*, PI034206 (PI), MW165012*, MW166026*, MW165919*, MW165973*; *P. hirta* s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), PI021307 (PI), *G. Astuti, L. Liu, F. Bartolucci s.n.*, MW164989*, MW166003*, MW165896*, MW165950*; *P. hirta* s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), *G. Astuti, L. Liu, F. Bartolucci s.n.*, PI021308 (PI), MW164990*, MW166004*, MW165897*, MW165951*; *P. hirta* s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), *G. Astuti, L. Liu, F. Bartolucci s.n.*, PI021310 (PI), MW164991*, MW166005*, MW165898*, MW165952*; *P. hirta* s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), *G. Astuti, L. Liu, F. Bartolucci s.n.*, PI021313 (PI), MW164992*, MW166006*, MW165899*, MW165953*; *P. hirta* s.l., Italy, Abruzzo, Rocca di Mezzo (L'Aquila), *G. Astuti, L. Liu, F. Bartolucci s.n.*, PI021318 (PI), MW164993*, MW166007*, MW165900*, MW165954*; *Pulmonaria hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021346 (PI), –, MW346642*, MW346636*, MW346639*; *P. hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021348 (PI), MW165013*, MW166027*, MW165920*, MW165974*; *P. hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021350 (PI), MW165014*, MW166028*, MW165921*, MW165975*; *P. hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021354 (PI), MW165015*, MW166029*, MW165922*, MW165976*; *P. hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021357 (PI), MW165016*, MW166030*, MW165923*, MW165977*; *P. hirta* s.str., Italy, Tuscany, Santa Maria a Monte (Pisa), Valle Lupitana, *G. Astuti & L. Liu s.n.*, PI021358 (PI), MW344075*, MW346641*, MW346635*, MW346638*; *P. hirta* s.str., Italy, Tuscany, Poppi (Arezzo), Camaldoli, *G. Astuti & L. Liu s.n.*, PI021361 (PI), MW165017*, MW166031*, MW165924*, MW165978*; *P. hirta* s.str., Italy, Tuscany, Poppi (Arezzo), Camaldoli, *L. Liu & G. Astuti s.n.*, PI021363 (PI), MW165018*, MW166032*, MW165925*, MW165979*; *P. hirta* s.str., Italy, Tuscany, Poppi (Arezzo), Camaldoli, *G. Astuti & L. Liu s.n.*, PI021365 (PI), MW165019*, MW166033*, MW165926*, MW165980*; *P. hirta* s.str., Italy, Emilia-Romagna, Grizzana Morandi (Bologna), Favari, *G. Astuti & L. Liu s.n.*, PI021381 (PI), MW165020*, MW166034*, MW165927*, MW165981*; *P. hirta* s.str., Italy, Emilia-Romagna, Grizzana Morandi (Bologna), Favari, *G. Astuti & L. Liu s.n.*, PI021388 (PI), MW165021*, MW166035*, MW165928*, MW165982*; *P. hirta* s.str., Italy, Emilia-Romagna, Grizzana Morandi (Bologna), Favari, *G. Astuti & L. Liu s.n.*, PI021391 (PI), MW165022*, MW166036*, MW165929*, MW165983*; *P. hirta* L. s.str., Italy, Emilia-Romagna, Grizzana Morandi (Bologna), Favari, *G. Astuti & L. Liu s.n.*, PI021396 (PI), MW165023*, MW166037*, MW165930*, MW165984*; *Pulmonaria officinalis* L., Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, *Liu & G. Astuti s.n.*, PI034229 (PI), MW165034*, MW166047*, MW165940*, –, *P. officinalis*, Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, *Liu & G. Astuti s.n.*, PI036033 (PI), MW165035*, MW166048*, MW165941*, MW165994*; *P. officinalis*, Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, *Liu & G. Astuti s.n.*, PI036034 (PI), MW165036*, MW166049*, MW165942*, MW165995*; *P. officinalis*, Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, *Liu & G. Astuti s.n.*, PI036039 (PI), MW165037*, MW166050*, MW165943*, MW165996*; *P. officinalis*, Italy, Friuli Venezia Giulia, Prepotto (Udine), Castelmonte, *Liu & G. Astuti s.n.*, PI036236 (PI), MW165038*, MW166051*, MW165944*, MW165997*; *Pulmonaria vallisae* A.Kern., Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *L. Liu & G. Astuti s.n.*, PI036003 (PI), MW165024*, MW166038*, MW165931*, MW165985*; *P. vallisae*, Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *L. Liu & G. Astuti s.n.*, PI034231 (PI), MW165025*, MW166039*, MW165932*, MW165986*; *P. vallisae*, Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *Liu & G. Astuti s.n.*, PI034216 (PI), MW165026*, MW166040*, MW165933*, MW165987*; *P. vallisae*, Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *Liu & G. Astuti s.n.*, PI036006 (PI), MW165027*, MW166041*, MW165934*, MW165988*; *P. vallisae*, Italy, Trentino-Alto Adige, Vallarsa (Trento), Pian delle Fugazze, *Liu & G. Astuti s.n.*, PI036007 (PI), MW165028*, –, –, *P. vallisae*, Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei, *Liu & G. Astuti s.n.*, PI036020 (PI), MW165029*, MW166042*, MW165935*, MW165989*; *P. vallisae*, Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei, *Liu & G. Astuti s.n.*, PI036023 (PI), MW165030*, MW166043*, MW165936*, MW165990*; *P. vallisae*, Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago

Appendix 2. Continued.

di Cei, *Liu & G. Astuti s.n.*, PI036026 (PI), MW165031*, MW166044*, MW165937*, MW165991*; *P. vallarsae*, Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei, *Liu & G. Astuti s.n.*, PI036028 (PI), MW165032*, MW166045*, MW165938*, MW165992*; *P. vallarsae*, Italy, Trentino-Alto Adige, Villa Lagarina (Trento), Lago di Cei, *Liu & G. Astuti s.n.*, PI036029 (PI), MW165033*, MW166046*, MW165939*, MW165993*. — **Outgroup:** *Borago officinalis* L., FJ763248, NC_046796 (for all the three chloroplast markers); *Symphytum asperum* Lepech., GQ285238, JF488928, JF488982, —.