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Intricate variation patterns in the diploid-polyploid complex of *Alyssum montanum-A. repens* (Brassicaceae) in the Apennine Peninsula: Evidence for long-term persistence and diversification[1]

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- *Premise of the study:* The Apennine Peninsula, one of the three peninsulas of the European Mediterranean, is an important hotspot of genetic and species diversity, but studies devoted to plant evolution are still very scarce in this region. We studied the diploid-polyploid complex of *Alyssum montanum-A. repens*, focusing on Apennine and adjacent southwestern Alpine populations from southeastern France, with the aim of examining their taxonomic position and evolutionary patterns.
- *Methods:* We employed AFLP markers and cpDNA sequences, along with cytotype determination using flow cytometry, and a multivariate morphometric approach.
- *Key results:* The Italian and French populations formed two well-delimited groupings within the studied complex that were, in contrast to previous taxonomic treatments, clearly distinct from *A. montanum*. Populations from southeastern France represent *A. orophilum*, a previously described but abandoned species. Those from central and southern Italy correspond to *A. diffusum*, exhibiting high, geographically structured variation (central Apennines, Gargano, and southern Apennines/Calabria). This pattern coincides with hotspot refugial regions, in congruence with the "refugia-within-refugia" hypothesis, and is reflected here in the recognition of three subspecies within *A. diffusum*.
- *Conclusions:* We provide evidence for the presence of Mediterranean refugia for the studied *Alyssum montanum-A. repens* complex located in central and southern Italy, which, however, did not contribute to the postglacial colonization of Central Europe. Past extinctions, genetic bottlenecks, and recent expansion were inferred in Central Europe, while long-term accumulation of diversity as well as polyploidization occurred in the Apennines.

Key words: Abruzzo; AFLPs; *Alyssum diffusum*; Apennines; Brassicaceae; Calabria; Gargano; morphometrics; refugia; southwestern Alps.

The three Mediterranean peninsulas of the Balkans, Italy, and Iberia have been recognized as important hotspots of genetic diversity (Petit et al., 2003) and areas of high endemism (Bilton et al., 1998; Thompson, 2005). By providing more favorable habitats during the Pleistocene glaciations, these peninsulas served as major glacial refugia for many temperate species (Bilton et al., 1998; Hewitt, 1999). As a result of the long-term persistence of populations and their demographic stability in refugia, compared to the populations in more northern areas that underwent a series of bottlenecks, variation may have accumulated and been retained in this region (Hewitt, 1996; Canestrelli and Nascetti, 2008). During warm periods, many of these species expanded northward to Central and northern Europe, but sometimes, natural barriers (e.g., mountain chains, such as the Alps and Pyrenees) or limitations in dispersal have prevented expansions (Taberlet et al., 1998; Hewitt, 1999; Magri et al., 2006; Dapporto, 2010). Consequently, long-term isolation in conjunction with the geological and topographical complexity of the Mediterranean regions has resulted in many endemic lineages. Although recent studies have indicated more complicated pictures of the glacial and postglacial history of the European biota than initially anticipated, the importance of the Mediterranean peninsulas for the evolution of European flora and fauna cannot be overestimated (Médail and Diadema, 2009). Focusing on the Apennine Peninsula, several studies have provided evidence for the existence of multiple, distinct glacial refugia distributed along the peninsula (see Vettori et al., 2004; Canestrelli et al., 2006, 2008; Magri et al., 2006; Ansell

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et al., 2008; Canestrelli and Nascetti, 2008; Grill et al., 2009; Vega et al., 2010), in accordance with the "refugia-within-refugia" concept proposed by Gómez and Lunt (2006). The Apuan Alps, the southern Apennines, the regions of Campania, southern Calabria, Gargano, and Sicily have repeatedly been identified as local refugia and regional hotspots harboring increased genetic and species diversity (Médail and Diadema, 2009). Nevertheless, studies devoted to plant phylogeography and evolution in the Apennine Peninsula are still very scarce, and additional detailed studies are needed. The genus *Alyssum* (Brassicaceae) appears to be a suitable model for such studies.

Alyssum is represented by ca. 195 species worldwide, with 70 of them occurring in Europe (Ball and Dudley, 1993; Warwick et al., 2006). Despite this high species diversity, recent studies on the genus are rather scanty, and species delimitations and relationships are mostly unknown. Only a few *Alyssum* species have been included in phylogenetic studies at the levels of family and tribe (*Alysseae*) (Bailey et al., 2006; Warwick et al., 2008; German et al., 2009; Cecchi et al., 2010). The only study to date with a larger sample of *Alyssum* species is that by Mengoni et al. (2003), which addressed the evolution of Ni-hyperaccumulation and presented the first phylogenetic hypotheses.

Our recent investigation of Central European populations of the *A. montanum-A. repens* complex (Španiel et al., 2011), exploring morphological, genetic, and cytotype variation, contradicted current taxonomic concepts and indicated that substantial taxonomic revision will be needed in the genus. In that study, three ploidy levels were discovered: diploids and tetraploids were common and sympatric, whereas hexaploids were very rare. *Alyssum repens*, represented by diploids and tetraploids found in Romania and Austria, was confirmed to be a distinct species. Within *A. montanum*, the circumscription of two subspecies, traditionally based on habitat preferences (*A. montanum* subsp. *montanum* on rocky sites and subsp. *gmelinii* on sandy sites), was not supported. Most of the Central European populations of *A. montanum* (including that of the type of *A. montanum* subsp. *gmelinii*) formed a genetically homogeneous group, consisting of diploids and tetraploids. Only the populations from the southern edges of the study area appeared to show more pronounced variation and structure. Populations from Switzerland and southwestern Germany (including the type population of *A. montanum* subsp. *montanum*, Marhold et al., 2011) were found to be morphologically and genetically well separated from the rest, indicating that the name *A. montanum* should be applied in a more restricted sense than previously. Additionally, the hexaploids representing *A. montanum* subsp. *pluscanescens*, a Slovenian and Croatian narrow endemic, were confirmed to be a distinct taxon, most likely of an allopolyploid origin.

For the Apennine Peninsula, two closely related taxa, *A. montanum* subsp. *montanum* and *A. diffusum,* were reported from the *A. montanum-A. repens* complex (Pignatti, 1982; Jalas et al., 1996), but their circumscription in this area, their morphology, distribution, and genetic variation have not been explored in detail. *Alyssum diffusum* has been described by Tenore (1812) from the former kingdom of Naples and recorded from Abruzzo (Tenore, 1815), Puglia (Gargano), and Calabria (Pollino) (Tenore, 1830). Later, some authors considered *A. diffusum* as a variety of *A. montanum* (Bertoloni, 1846; Fiori, 1924) or included it as a synonym of *A. montanum* (Caruel, 1893). According to Pignatti (1982), *A. montanum* subsp. *montanum* is distributed throughout most of the Apennine Peninsula, reaching altitudes from 100 to 1500 m a.s.l., whereas *A. diffusum* occupies altitudes between 800 and 2000 m a.s.l. in the central and southern parts of the peninsula. The main morphological characters suggested for their distinction in Italy are the ratio of the fruit and style lengths, leaf size, and plant height (Zangheri, 1976; Pignatti, 1982). Whereas three different ploidy levels are known within *A. montanum* (Warwick and Al-Shehbaz, 2006; Španiel et al., 2011), only diploids and tetraploids have been reported from Italy (Küpfer, 1974; La Valva, 1976; Kieft and van Loon, 1978). Specifically for *A. diffusum*, only the diploid chromosome count has been recorded from Italy (Polatschek, 1983).

In addition, two other species names have been used previously for populations in the border regions of Italy and France: *Alyssum orophilum* was described by Jordan and Fourreau (1868) from the Dauphiné Alps (the vicinity of Briançon), and *A. pedemontanum* was described by Ruprecht (1869) from the alpine pastures on Mt. Cenis between the Cottian and Graian Alps and from above the commune of Tende between the Maritime and Ligurian Alps. Both names, however, have mostly been treated as synonyms of *A. montanum*.

In the present study, we aimed to characterize the morphological, genetic (AFLPs and cpDNA sequences) and cytotype variation in the Apennine and southwestern Alpine populations of the *A. montanum-A. repens* complex to answer the following sets of questions: (1) Which species of the complex occur in the Apennine Peninsula and adjacent regions—is the recognition of two taxa, *A. montanum* and *A. diffusum*, supported, or should a different taxonomic concept be adopted? Are the populations from the southwestern Alpine border region of Italy and France conspecific with *A. montanum*, or do they represent a distinct taxon? (2) Does the phylogeographic structure of these populations support the "refugia-within-refugia" concept? Do the genetic variation patterns indicate the presence of refugial populations that served as a source for postglacial expansion to Central Europe or, rather, favor their long-term isolation and differentiation? (3) Is there any geographic pattern in the distribution of different ploidy levels among the studied populations? What role did the polyploidization play in the evolution and diversification of these populations?

MATERIALS AND METHODS

Plant material—Plant material of the *Alyssum montanum-A. repens* complex was collected in the field during 2006–2010 and consisted of silica-gel-dried leaves (for molecular and flow cytometric analyses), seeds (for chromosome number counting), and herbarium specimens (for morphometric analyses). The localities sampled are listed in Table 1 and Appendix 1 and depicted in Fig. 1. The sampled plant material consisted of 16 populations from Italy and the neighboring areas of southeastern France (including the material from the type localities, or their vicinity, of *A. diffusum*, *A. orophilum*, and *A. pedemontanum*). For reference, five populations of *A. repens* from Austria and Romania (including the type population) and 13 populations of *A. montanum* from a wide Central European area (including the type localities of *A. montanum* subsp. *gmelinii*, *A. montanum* subsp. *montanum*, and *A. montanum* subsp. *pluscanescens*) were included. The selection of Central European populations followed our previous study (Španiel et al., 2011) and included a representative sample of the genetic, morphological, and karyological variability present in that area. These Central European populations are referred to as the core diploids and tetraploids (corresponding to *A. montanum* subsp. *gmelinii*), the Swiss-SW German group (corresponding to *A. montanum* subsp. *montanum*), the Serbian group (*A. montanum* s.l. with uncertain assignment), and *A. montanum* subsp. *pluscanescens*, following the results by Španiel et al. (2011).

Our sampling for ploidy level (flow cytometry, FCM), morphometric, and AFLP analyses was designed with the intention of analyzing the same sets of

individuals and/or populations as much as possible. All plants included in the AFLP studies were analyzed for ploidy levels by FCM, and populations used for morphometric and AFLP studies were almost identical, with only a few exceptions that were mostly due to phenological factors or the scarcity of plants at a locality (see Table 1, Appendix 1; *A. montanum* subsp. *pluscanescens* was omitted from morphometric analyses due to only a few plants growing at two known localities of this taxon). The cpDNA variation was assessed in all of the populations studied herein (Table 1, Appendix 1) and in all of those sampled in Španiel et al. (2011) to encompass the entire cpDNA variation.

For ploidy level determination, all 16 population samples (176 plants) from Italy and SE France and one population sample of *A. repens* (10 plants) from Romania (84RET) were analyzed using FCM. To relate the measured fluorescence intensity to ploidy levels, we counted chromosomes in three populations of different ploidy levels (as assumed from the FCM measurements). The ploidy levels of the rest of the material (the Central European accessions, the other *A. repens* samples) were known from the previous study (Španiel et al., 2011; see Table 1).

For the AFLPs, we analyzed 14 populations (94 plants) from Italy and France, two populations (14 plants) of *A. repens* and 13 Central European populations (88 plants); altogether, 29 populations were analyzed (196 plants). Individuals from two other populations from France (91TDE and 92CEN, 14 plants) and one population of *A. repens* (84RET, six plants) repeatedly failed to produce clear AFLP profiles for one primer pair (of the four primer pairs employed); thus, they were used for scoring the loci generated by only three primer combinations. We also surveyed the polymorphisms in several cpDNA regions and finally selected two intergenic spacers indicating high polymorphisms, *rpoB-trnC* and *rpl32-trnL*$^{(UAG)}$ (Shaw et al., 2005, 2007). Both cpDNA regions were sequenced in a total of 122 individuals (3–4 plants per 16 Italian and French populations; single plants per other populations). The close relatives, *A. turkestanicum, A. minutum*, and *A. alyssoides* (resolved in the well-supported sister position to the *A. montanum-A. repens* complex in a preliminary genus phylogeny, J. Zozomová-Lihová et al., unpublished data) were used as outgroups.

For morphometrics, we analyzed 14 population samples from Italy and France (355 plants, usually 20–30 plants per population), four samples of *A. repens* (100 plants), and 11 Central European samples (262 plants), examining the stem, leaf, and floral (S-L-Fl) characters. Due to phenological reasons, fruit (Fr) characters were measured on a partially different set of specimens composed of eight population samples (170 plants) from Italy, three samples (45 plants) of *A. repens*, and seven Central European samples (129 plants).

Voucher specimens are deposited in the Herbarium of the Institute of Botany, Slovak Academy of Sciences (SAV; Appendix 1).

Chromosome counting and DNA ploidy level estimation—Chromosome numbers were determined in the mitotic metaphases of cells from root tips, following the protocol described in Španiel et al. (2011).

For flow cytometric analyses, plants with known chromosome numbers were first analyzed simultaneously with an internal standard, and the ratios of their G_1 peak positions were recorded. As an internal standard for the diploids and tetraploids, we employed *Lycopersicon esculentum* 'Stupické polní rané' (2C = 1.96 pg; Doležel et al., 1992). To avoid any overlap between the G_2 peaks of *Lycopersicon* and the G_1 peaks of the hexaploid samples, we used *Bellis perennis* as a secondary standard (2C = 3.38 pg; Schönswetter et al., 2007). The DNA content of *Bellis* was calibrated against *Lycopersicon* based on three repeated analyses performed on different days. The DNA ploidy levels of the sampled plants with unknown chromosome numbers were next assessed by their peak position relative to the standard peak. The analyses were performed using a Partec Cyflow ML instrument, equipped with an HBO-100 mercury arc lamp (Partec GmbH, Münster, Germany), at the Institute of Botany, Slovak Academy of Sciences, Bratislava.

Fresh leaves were dried in silica gel immediately after collection in the field and stored at −25°C. The DNA ploidy level was estimated from dehydrated plant tissues (Suda and Trávníček, 2006) on the basis of the fluorescence intensity of the nuclei stained using the AT-selective fluorochrome 4′,6-diamidino-2-phenylindole (DAPI). Tissues from the silica-gel-dried leaves (0.5 cm^2) of the analyzed plants were co-chopped with the fresh leaf tissues of a standard plant in a Petri dish with 1 mL of ice-cold Otto I buffer (0.1 mmol/L citric acid monohydrate and 0.5% Tween 20; Otto, 1990) using a razor blade. The obtained suspension was filtered through a 42-μm nylon mesh and incubated for at least 5 min at room temperature. Next, 1 mL of a solution containing Otto II buffer (0.4 mmol/L $Na_2HPO_4 \cdot 12H_2O$, 2-mercaptoethanol [2 μL/mL] and DAPI [4 μg/mL]) was added to the flow-through fraction and incubated for 1–2 min. The flow cytometric histograms were evaluated using Partec FloMax software (v. 2.7d; Partec GmbH). The reliability of the measurements was assessed by computing the coefficients of variation (cv) for the peaks of both the analyzed samples and the standards. Because silica-gel-dried material was used, all values below the cv threshold value of 5% were accepted.

AFLP fingerprinting—Total genomic DNA was extracted from silica-gel-dried leaf samples using the DNeasy 96 Plant kit (Qiagen, Hilden, Germany). The AFLP analysis followed the procedure described by Vos et al. (1995) and the protocol provided by Applied Biosystems (2010), with some modifications that were previously described by Španiel et al. (2011). An initial screening of 53 selective primer pair combinations was performed using 16 individuals from nine populations. The four primer pairs that gave the best AFLP profiles with respect to reproducibility, clarity, and polymorphism were finally selected: *Eco*RI-ATC-(6-FAM)/*Mse*I-CAC, *Eco*RI-AGG-(VIC)/*Mse*I-CAC, *Eco*RI-AGC-(NED)/*Mse*I-CAC, and *Eco*RI-AGC-(PET)/*Mse*I-CAG. The amplification products obtained using the primers labeled with the four different fluorescent dyes were pooled and submitted to the BITCET Consortium, Department of Molecular Biology, Comenius University, Bratislava (ABI 3100 Avant) for fragment analysis. Size calibration was performed using the internal size standard GeneScan −500 LIZ (Applied Biosystems, Foster City, California).

AFLP data analysis—AFLP trace files were read and analyzed using DAx software (Van Mierlo Software Consultancy, Eindhoven, Netherlands). Only well-scorable and unambiguous fragments in the size range of 50–500 bp were recorded and coded as present (1) or absent (0). A binary data matrix was assembled. To estimate the reproducibility of the AFLP data, we calculated the error rate based on the ratio of mismatches (scoring 1 vs. 0) over matches (1 vs. 1) of 28 replicated samples (mostly one individual per population, 14% of the final data set) (Bonin et al., 2004).

The overall genetic structure and relationships among the studied populations were explored by principal coordinate analysis (PCoA; Krzanowski, 1990), cluster analysis based on the neighbor-joining algorithm (NJ; Saitou and Nei, 1987), and the NeighborNet method generating split networks (Huson and Bryant, 2006). The PCoA, using Jaccard's coefficient for calculating pairwise genetic similarities, was performed using FAMD 1.108 beta software (Schlüter and Harris, 2006). Both NJ and NeighborNet were constructed from Sørensen's similarities transformed into a distance matrix ($d = 1 - s$; where d is distance, s is similarity); the NJ tree was computed in FAMD, also assessing group support by bootstrap analyses with 3000 replicates, whereas NeighborNet was generated with the program SplitsTree 4.10 (Huson and Bryant, 2006).

The Bayesian multilocus assignment method based on stochastic optimization was also performed using BAPS 5.2 (Corander et al., 2006) and the module "clustering of individuals", which estimates the highest probability partition (i.e., the optimal number of clusters and assignment of the analyzed individuals). Both the frequencies of the AFLP markers and the number of genetically divergent groups are treated here as random variables. The analysis was carried out with the maximal number of groups (K) set to 100 and repeated 10 times.

The analysis of molecular variance (AMOVA) was used to study the variance partitioning within and among populations and among the population clusters suggested by BAPS and NeighborNet analyses. AMOVA was computed with the program Arlequin 3.11 (Excoffier et al., 2005) using Euclidean pairwise distances and a significance test with 1000 permutations. The genetic diversity within the analyzed populations was assessed by calculating the mean number of AFLP markers scored per individual, the percentage of polymorphic markers (%poly), and Nei's gene diversity (D_{Nei}, expressing the average proportion of pairwise differences between individuals; Nei, 1987) using R-script AFLPdat (Ehrich, 2006).

cpDNA amplification and sequencing—Universal primers were used for both PCR (polymerase chain reaction) and cycle-sequencing: primers rpoB and trnC^{GCA}R for the *rpoB-trnC* intergenic spacer (Shaw et al., 2005) and primers rpL32-F and trnL$^{(UAG)}$ for the *rpl32-trnL*$^{(UAG)}$ spacer (Shaw et al., 2007). The PCR mix contained 0.75 U of *Pfu* polymerase (Fermentas, St. Leon-Rot, Germany), 1× reaction buffer supplied with the enzyme that included $MgSO_4$ at 2 mmol/L, 0.2 mmol/L of each dNTP, 0.2 μmol/L of each primer, and 1 μL of DNA template in a total reaction volume of 25 μL. Amplifications were run in a Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany) using the "*rpl16*" program provided by Shaw et al. (2005). The PCR products were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany). Sequencing was performed on an ABI PRISM 3130*xl* sequencer at the BITCET Consortium, Comenius University, Bratislava.

TABLE 1. List of the studied populations of the *Alyssum montanum-A. repens* complex and their characteristics.

Pop. code	Taxon, Country, region	Locality	Latitude, Longitude	2*n*/Nr	Morph S-L-Fl/Fr	AFLP N_{ind}	AFLP Mean ±SD	AFLP %poly	AFLP D_{Nei}
***A. diffusum* subsp. *diffusum*, central Apennines**				**85**	**137/76**	**46**	**44.2 ± 3.8**	**18.83**	**0.0805**
54SIB	IT, Umbria	Monti Sibillini	42°45.125′–47.937′N 13°11.855′–11.280′E	**6*x*/20**	26/17	5	46.0 ± 2.9	14.35	0.0682
55PAC	IT, Abruzzo	Pacentro	42°3.317′N 14°1.533′E	**2*x*/10**	–/–	6	43.0 ± 2.4	20.18	0.0903
57COL	IT, Abruzzo	San Colombo	42°20.083′N 13°36.533′E	**2*x*/15**	27/26	8	41.4 ± 4.2	19.28	0.0777
58STE	IT, Abruzzo	Santo Stéfano di Sessánio	42°23.145′N 13°39.677′E	**2*x*/10**	23/15	7	41.4 ± 3.2	14.80	0.0645
59CAM	IT, Abruzzo	Valico della Campannelle	42°27.068′N 13°23.183′E	**4*x*/10**	15/18	6	46.7 ± 2.7	20.63	0.0933
60AMA	IT, Abruzzo	Mte Amaro	42°4.820′N 14°4.099′E	**4*x*/10**	27/–	7	46.1 ± 3.6	21.52	0.0841
61MAI	IT, Abruzzo	La Maielletta	42°9.352′N 14°7.375′E	**4*x*/10**	19/–	7	46.1 ± 3.0	21.08	0.0854
***A. diffusum* subsp. *garganicum*, Gargano**				**30**	**44/53**	**14**	**45.5 ± 2.2**	**19.73**	**0.0834**
62ANG	IT, Puglia	Monte Sant´Ángelo	41°44.350′-45.817′N 15°58.960′-59.150′E	**2*x*/20, 2*n* = 16**	22/24	8	45.9 ± 1.8	21.08	0.0873
63MAR	IT, Puglia	San Marco in Lámis	41°43.253′N 15°37.223′E	**2*x*/10**	22/29	6	45.0 ± 2.7	18.39	0.0795
***A. diffusum* subsp. *calabricum*, southern Apennines and Calabria**				**40**	**114/41**	**27**	**53.1 ± 3.7**	**24.44**	**0.1089**
64COC	IT, Calabria	Monte Cocuzzo	39°13.637′N 16°8.215′E	**4*x*/10, 2*n* = 32**	30/23	6	51.5 ± 4.0	23.77	0.1067
65MUL	IT, Calabria	Monte la Mula	39°41.854′-42.014′N 15°58.866′-58.384′E	**4*x*/10**	31/–	7	53.9 ± 2.6	21.97	0.1042
66PRE	IT, Calabria/Basilicata	Serra del Prete	39°54.962′N 16°8.947′E	**4*x*/10**	29/–	7	51.6 ± 4.2	24.66	0.1081
67MOR	IT, Calabria	Morano Cálabro	39°51.852′N 16°6.270′E	**4*x*/10, 2*n* = 32**	24/18	7	55.3 ± 3.3	27.35	0.1166
***A. orophilum* (SE France)**				**21**	**60/–**				
89GLR	FR, Dauphiné Alps	Col du Galibier	45°5.250′N 6°26.150′E	**4*x*/7**	–/–	7	50.7 ± 1.6	17.04	0.0764
91TDE	FR, Alpes-Maritimes	Col de Tende	44°8.367′N 7°33.000′E	**6*x*/7**	30/–	7	—	—	—
92CEN	FR, Cottian/Graian Alps	Lac du Mont Cenis	45°14.567′N 6°56.767′E	**4*x*/7**	30/–	7	—	—	—
***A. montanum* subsp. *gmelinii* (core diploids)**				**46**	**103/38**	**32**	**36.1 ± 3.4**	**12.67**	**0.0491**
*94SAN	DE, Baden-Württemberg	Sandhausen	49°19.917′N 8°39.620′E	2*x*/16	29/16	7	35.7 ± 2.9	12.56	0.0517
*133KEL	RS, Severna Bačka	Kelebija	46°9.154′N 19°38.627′E	2*x*/10	24/22	9	35.7 ± 5.3	14.80	0.0556
*213ZLA	CZ, Český kras	Zlatý kůň	49°54.985′N 14°4.002′E	2*x*/10	25/–	8	37.9 ± 2.0	13.90	0.0553
*220KRY	PL, Województwo świętokrzyskie	Kichary Nowe	50°44.284′N 21°45.198′E	2*x*/10	25/–	8	35.0 ± 1.9	9.42	0.0340
***A. montanum* subsp. *gmelinii* (core tetraploids)**				**40**	**94/54**	**27**	**42.0 ± 2.9**	**16.37**	**0.0689**
*5DOM	SK, Slovenský kras	Domica	48°28.690′N 20°28.128′E	4*x*/10	25/24	6	43.0 ± 3.2	14.80	0.0664
*15CSA	HU, Komárom-Esztergom	Császár	47°31.230′N 18°8.020′E	4*x*/10	17/24	7	40.6 ± 3.4	17.49	0.0709
*211HRU	CZ, Znojemsko-brněnská pahorkatina	Hrubšice	49°5.548′N 16°17.763′E	4*x*/10	25/–	7	43.1 ± 2.7	18.39	0.0777
*225CIE	PL, Województwo Kujawsko-Pomorskie	Ciechocinek	52°51.975′N 18°48.491′E	4*x*/10	27/6	7	41.6 ± 1.9	14.80	0.0606
***A. montanum* subsp. *montanum* (Swiss-SW German group)**				**22**	**42/12**	**13**	**44.0 ± 2.5**	**8.74**	**0.0405**
*95BAS	CH, Baselland	Basel	47°27.175′N 7°35.649′E	2*x*/10	22/12	6	42.2 ± 0.8	4.04	0.0182
*147TRO	DE, Baden-Württemberg	Trochtelfingen	48°20.156′N 9°14.946′E	2*x*/12	20/–	7	45.6 ± 2.3	13.45	0.0628
Serbian group (*A. montanum* s.l.)									
*134SUM	RS, Južni Banat	Šumarak	44°49.106′N 21°8.581′E	2*x*/10	23/25	7	37.4 ± 1.9	11.21	0.0491
A. montanum* subsp. *pluscanescens				**9**	**–/–**	**9**	**48.0 ± 3.9**	**11.44**	**0.0593**
*96ZIC	SI, Predalpsko območje	Žiče	46°18.731′N 15°27.997′E	6*x*/5	–/–	5	50.8 ± 1.9	13.45	0.0664
*207SME	HR, Zagrebačka županija	Smerovišće	45°46.400′N 15°38.542′E	6*x*/4	–/–	4	44.5 ± 2.6	9.42	0.0523

Table 1. Continued.

Pop. code	Taxon, Country, region	Locality	Latitude, Longitude	2*n*/Nr	Morph S-L-Fl/Fr	AFLP N_{ind}	AFLP Mean ±SD	AFLP %poly	AFLP D_{Nei}
A. repens				**60**	**100/45**	**20**	**49.1 ± 9.1**	**14.57**	**0.0660**
*71CAR	RO, Prahova	Caraiman	45°24.333′N, 25°28.334′E	4*x*/10	23/–	—	—	—	—
*72POS	RO, Braşov	Postăvarul	45°34.138′N, 25°34.073′E	4*x*/10	26/17	7	57.4 ± 3.9	20.18	0.0897
*73PIA	RO, Neamt	Piatra cu Apă	46°57.838′N, 25°57.725′E	4*x*/20	29/14	—	—	—	—
84RET	RO, Hunedoara	Munţii Retezat	45°16.948′N 22°50.933′E	**4x/10**	–/–	6	—	—	—
*150LAV	AT, Kärnten	Lavamünd	46°38.391′N 14°57.038′E	2*x*/10	22/14	7	40.9 ± 1.6	8.97	0.0423

Notes: Population code, country and region, brief locality (full localities including the voucher information are listed in Appendix 1), and geographic coordinates are given for each site. Population samples marked by asterisks were taken from a previous study (Španiel et al., 2011). Country abbreviations: AT, Austria; CH, Switzerland; CZ, Czech Republic; DE, Germany; FR, France; HR, Croatia; IT, Italy; PL, Poland; RO, Romania; RS, Serbia; SI, Slovenia; SK, Slovakia; HU, Hungary. 2*n*/Nr, DNA ploidy levels determined by flow cytometry with the number of analyzed individuals and the chromosome number (if determined); values in boldface refer to the DNA ploidy levels/chromosome counts determined in the present study; those in normal type are from Španiel et al. (2011). Morph, number of the analyzed plants in morphometric studies, using characters on stems, leaves and flowers (S-L-Fl)/characters on fruits (Fr). AFLP columns: N_{ind}, the number of the individuals analyzed for AFLPs; mean ± SD, average number of markers per individual ±SD; %poly, percentage of polymorphic markers; D_{Nei}, Nei's gene diversity.

cpDNA data analysis—The cpDNA sequences were proofread, trimmed of ambiguous ends, and aligned manually with the program BioEdit (version 7.0.4.1; Hall, 1999). Indels were scored as additional gap characters, coded as binary (following the simple gap coding of Simmons and Ochoterena [2000]) or multistate characters (in the case of a few tandem repeats, with different states defining the number of repeats) appended to the final alignment. The two cpDNA alignments were concatenated, and the method of statistical parsimony was employed to determine the relationships among the haplotypes and to construct the haplotype network (TCS version 1.18; Clement et al., 2000). TCS was run with a 90% connection limit and gaps treated as missing data (but with the gap scoring appended to the nucleotide data set). Phylogenetic reconstructions were also performed using Bayesian inference (Huelsenbeck and Ronquist, 2001) as implemented in the program MrBayes 3.1.2. Adequate models of nucleotide substitutions were identified by the program MODELTEST 3.7 (Posada and Crandall, 1998) in conjunction with the program PAUP*, version 4.0b10 (Swofford, 2001). The Akaike information criterion (AIC) and hierarchical likelihood ratio test (hLRT) were used to determine the models that best fit the data sets. In the case of conflict, a likelihood ratio test was applied (Huelsenbeck and Crandall, 1997). Indel scoring, appended to the nucleotide data set, was defined as a separate partition and treated under the standard discrete model implemented in MrBayes. For the nucleotide partition, we set the GTR+G model (being the closest to the determined K81uf+G model, see Results: cpDNA variation): six substitution rates (nst = 6) and gamma distribution (rates = gamma). The Bayesian analysis was performed with the four chains for five million generations and with Temp = 0.03 (a value achieving efficient swaps between the chains). The generated trees were summarized by computing a majority-rule consensus tree, excluding the trees of the burn-in phase. The percentage of trees recovering an individual node was indicated on the consensus tree by the posterior probability (PP) of the node.

For the assessment of genetic diversities based on cpDNA sequence data, we calculated gene diversity (H), nucleotide diversity (π_n; Nei, 1987) and the mean number of pairwise differences (π; Tajima, 1993) with the program Arlequin 3.11. Population expansion was examined by mismatch distribution of pairwise differences among haplotypes; agreement between the observed and the expected distribution under a model of sudden population expansion was tested using the sum of squared deviations and 500 bootstrap replicates. Statistical significance of population expansion was also estimated by neutrality tests, Tajima's D (Tajima, 1989) and Fu's (1997) F_S statistics (using 1000 simulations for estimating the P value). Both mismatch distribution and the neutrality tests were computed in Arlequin 3.11. All these computations were performed only for the groups of Central European core (*A. montanum* subsp. *gmelinii*) and the Italian accessions, which were represented by a comparably high number of analyzed individuals (50 and 51, respectively).

Morphometric analyses—Methods of multivariate morphometrics (Marhold, 2011) were used to test whether the groups resolved by molecular markers are morphologically differentiated. The morphological characters measured or scored included those reported as diagnostic within the *A. montanum-A. repens* complex in determination keys and floras, as well as characters that appeared to be variable during our observations in the field (for the list of characters, see Table 2). Most characters were measured or scored directly on the herbarium specimens. Floral characters were measured on scanned floral parts. Fresh floral parts were attached by adhesive transparent tape to a paper, dried to fix their original size and shape, and scanned using a ScanMaker 9800XL (Microtek International, Hsinchu, Taiwan); measurements were performed using the software CARNOY (Schols et al., 2002). Trichomes on stem leaves were observed and measured using a stereomicroscope (Olympus SZ61) and the software QuickPHOTO Micro 2.3. Two characters were semiquantitative (trichome coverage on the upper and lower surface of stem leaves), and all of the other characters were quantitative. The following primary matrices were assembled: (1) character values/states measured/scored on stems, leaves, and flowers (denoted as S-L-Fl characters) of individual plants (717 plants × 20 characters), (2) character values measured on fruits (Fr characters) of individual plants (344 plants × 6 characters), and (3) population means of Fr characters (18 populations × 6 characters). Partial data sets based on these matrices were also generated and used for particular analyses.

As the first step, the Shapiro-Wilk statistic for the test of normality of distribution was computed for each character. Then the Pearson and nonparametric Spearman correlation coefficients (Zar, 1999) were computed to reveal correlation structure among the characters and to ensure that no very high correlations (>0.95) were present (potentially distorting some of the further multivariate analyses). The multivariate morphometric methods applied included canonical discriminant analyses (CDA) and classificatory discriminant analyses (Klecka, 1980). In CDA, the discriminant functions were derived to express the extent of morphological differentiation between the predefined groups. Nonparametric k-nearest neighbors classificatory discriminant analyses were performed to estimate the percentage of plants correctly assigned to the predefined groups. A cross-validation procedure was used, in which the classification criterion was based on $n - 1$ individuals and then applied for the individual left out. Discriminant analyses generally require multivariate normal distribution of the characters; nevertheless, they have been shown to be considerably robust against deviations in this respect (Thorpe, 1976; Klecka, 1980). Last, descriptive statistics were computed for the groups of populations/taxa delimited here. The analyses were performed using SAS 9.1.3 software (SAS Institute 2007).

RESULTS

Chromosome counting and DNA ploidy level estimation—Flow cytometric analyses revealed three ploidy levels in the populations collected in Italy and France. They were identified

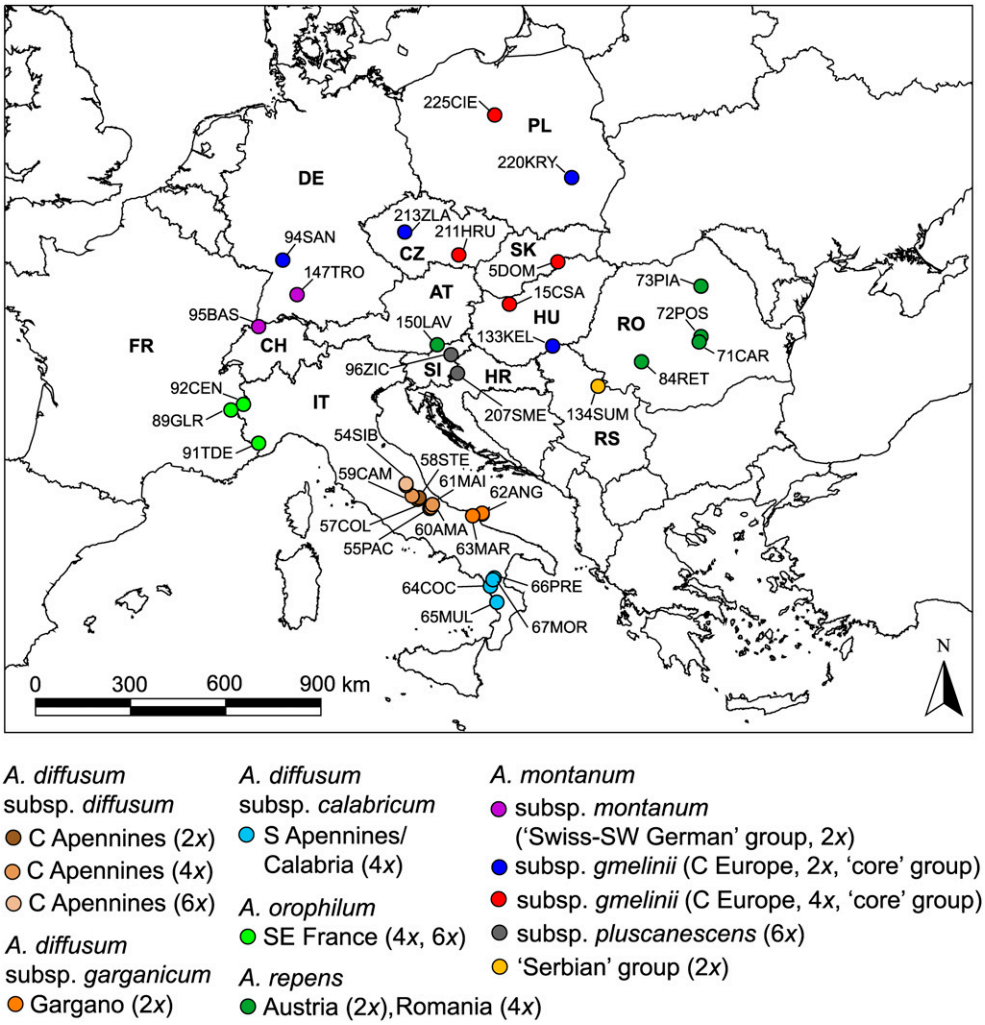

Fig. 1. Distribution map of the *Alyssum* samples analyzed in this study. For details on localities, see Table 1.

as diploid ($2n = 16$, based on the chromosome counts for two individuals from the population 62ANG, see Table 1), tetraploid ($2n = 32$, based on the chromosome counts for two individuals from the populations 64COC and 67MOR), and DNA hexaploids inferred from the relative DNA content (not confirmed by chromosome counting). Among the three populations from SE France, two were found to be tetraploid, and one was DNA hexaploid. Two of these populations (one tetraploid and one DNA hexaploid) were from the localities referred to in the protologue of *A. pedemontanum*; the third one (tetraploid) was from the close neighborhood of the locality of the type of *A. orophilum*. The populations analyzed from Gargano (a region of Puglia) were found to be entirely diploid. In the region of Abruzzo (from where the lecto- and epitype of the name *A. diffusum* is designated, S. Španiel et al., unpublished manuscript), both diploids and tetraploids were determined, and in the adjacent Umbria region, a DNA hexaploid population was revealed. Populations sampled in the southern Italian regions of Calabria and Basilicata were found to be uniformly tetraploid. Each population analyzed exhibited only a single ploidy level (inferred from the data of at least 10 individuals analyzed per population).

AFLPs—Because three of the populations (84RET, 91TDE, 92CEN) repeatedly produced poor AFLP profiles for one of the primer combinations (*Eco*RI-ATC-(6-FAM)/*Mse*I-CAC), two data sets were initially assembled—a data set including AFLP markers generated by three primer pairs only and all of the populations (216 individuals and 174 loci) and a data set of the markers of all of the four primer pairs but excluding the three populations (196 individuals and 223 loci). The former data set was used to ascertain the position of those three populations, but all of the other analyses were performed (and are reported) on the latter data set.

Of the 223 scored AFLP markers (loci), 218 were polymorphic. Replicate samples indicated a high reproducibility of the AFLP data (1.7% error rate). A total of 196 different multilocus AFLP phenotypes were detected; thus, we did not find individuals with identical AFLP profiles. The distance-based analyses of AFLP data may sometimes be considered problematic when analyzing individuals representing different ploidy levels. Nevertheless, this does not seem to be a problem here, as the average number of AFLP markers does not differ markedly between the ploidy levels (Table 1).

Table 2. List of the characters and their acronyms used in morphometric analyses.

Characters
Vegetative
StemLength – length of longest stem on plant, measured from bottom (including its ascending part) to pedicel base of lowermost silicula/flower (mm)
NrLatBranches – number of lateral branches on main stem (excluding branches in basal, ascending part of stem)
Length8thLeaf – length of 8th stem leaf (counted downward from pedicel base of lowermost silicula/flower) (mm)
Width8thLeaf – width of 8th stem leaf (counted downward from pedicel base of lowermost silicula/flower) (mm)
Length15thLeaf – length of 15th stem leaf (counted downward from pedicel base of lowermost silicula/flower) (mm)
Width15thLeaf – width of 15th stem leaf (counted downward from pedicel base of lowermost silicula/flower) (mm)
Dist8-15thLeaf – distance between 8th and 15th stem leaf (counted downward from pedicel base of lowermost silicula/flower) (mm)
LengthTrichRay – length of longest ray of stellate trichomes on lower surface of middle stem leaf (mean value of three measurements) (mm)
NrRaysTrichLower – number of rays of stellate trichomes on lower surface of middle stem leaf (mean value of three counts)
TrichDensityLower – number of trichomes on the area of 0.5 mm^2 on the lower surface of middle stem leaf
TrichCoverageLower – coverage of trichomes on lower surface of middle stem leaf (0: 0–33% coverage, 1: 33–66% coverage, 2: 66–95% coverage, 3: 95–100% coverage)
NrRaysTrichUpper – number of rays of stellate trichomes on upper surface of middle stem leaf (mean value of three counts)
TrichDensityUpper – number of trichomes on area of 0.5 mm^2 on upper surface of middle stem leaf
TrichCoverageUpper – coverage of trichomes on upper surface of middle stem leaf (0: 0–33% coverage, 1: 33–66% coverage, 2: 66–95% coverage, 3: 95–100% coverage)
Floral
PetalLength – maximum petal length in one of largest flowers (mm)
PetalSinus – deepness of sinus on emarginate petal tip (mm)
PetalWidth – width of longest petal in one of largest flowers (mm)
SepalLength – maximum sepal length in one of largest flowers (mm)
FilamentLength – length of longest filament in one of largest flowers (mm)
StyleLength – length of style in one of largest flowers (mm)
Fruit
FruitStyleLength – length of style persisting on silicula (mm)
RacemeLength – length of raceme (measured from base of lowermost fruit pedicel, at stage when fruits are present along 2/3 of its length) (mm)
PedicelLength – length of longest pedicel in the lower part of raceme (mm)
SiliculaLength – length of largest mature silicula (mm)
SiliculaWidth – width of largest mature silicula (mm)
PedicelDistance – distance between bases of two lowermost silicule pedicels (mm)

Both the NeighborNet diagram (Fig. 2) and the NJ tree (figure not shown) showed a very similar, largely star-shaped structure with short internal branch lengths. Nevertheless, five main clusters can be clearly delimited on the NeighborNet, corresponding to (1) all of the Italian populations (bootstrap support on the NJ tree, BS < 50%); (2) the diploid *A. montanum* subsp. *montanum* (the Swiss-SW German group; BS = 87%), including the type population of the name (95BAS, Switzerland) and a population from SW Germany (147TRO); (3) *A. montanum* subsp. *gmelinii* (the Central European core group; BS = 59%) comprising diploids and tetraploids and including the type locality of the name; (4) the Serbian group represented by the diploid population 134SUM (BS = 100%); and (5) a group (BS < 50%) formed by *A. repens*, a population from Col du Galibier (89GLR, SE France) and two hexaploid populations of *A. montanum* subsp. *pluscanescens* from the Croatian-Slovenian border (96ZIC, 207SME; the former being the type population of the name). Further substructure can be recognized within clusters (1) and (5) that is correlated with geography and/or ploidy level (Fig. 2). Within the Italian cluster (1), populations from the Gargano region (diploids) were clearly separated from the others (BS = 98%), and further clusters corresponded to the populations from the southern Apennines and Calabria (all tetraploid; BS = 69%), and the central Apennine populations (Abruzzo, Umbria; BS = 50%), which consisted of one hexaploid (BS = 99%), three diploid (BS = 71%), and three tetraploid populations (placed in two separate subclusters, BS < 50%). The cluster (5) contained four clearly differentiated subclusters: (5a) *A. repens* (BS = 62%) comprising the Austrian diploid (150LAV) and the Romanian tetraploid population (72POS) (plus the population 84RET when considering the analyses based on the three primer combinations; *A. repens* receiving here BS = 65%, figure not shown); (5b) a SE French tetraploid population (89GLR, BS = 100%), clustering with the hexaploid 91TDE and tetraploid 92CEN populations when considering the analyses based on the three primer combinations (all of the three populations having BS = 73%, figure not shown); and (5c) and (5d) *A. montanum* subsp. *pluscanescens* represented by two populations, each placed in a separate subcluster (BS = 99% and 100%). In both the NeighborNet graph and NJ tree, individuals from the same (or geographically close) populations clustered together.

PCoA ordination (Appendix S1, see Supplemental Data with the online version of this article) illustrated a rather complex genetic pattern within the data set, confirming the results of the NeighborNet and NJ tree. The Italian populations and the Central European core populations (*A. montanum* subsp. *gmelinii*) were resolved into two distinct groupings separated along the first axis (extracting 16.13% of the overall variation), whereas the other populations were placed in several smaller clusters differentiated from each other, mostly with uncertain affinities among them. The PCoA based on the data of the three primer combinations only (figure not shown) resulted in the same overall structure and placed the population, 84RET, close to the other populations of *A. repens*, and intermixed the French populations, 91TDE and 92CEN, with 89GLR.

To gain further insights into the genetic structuring of the large, rather loose cluster of the Italian populations, we performed a separate PCoA on the reduced data set (87 Italian individuals and 158 AFLP loci). The populations from the central

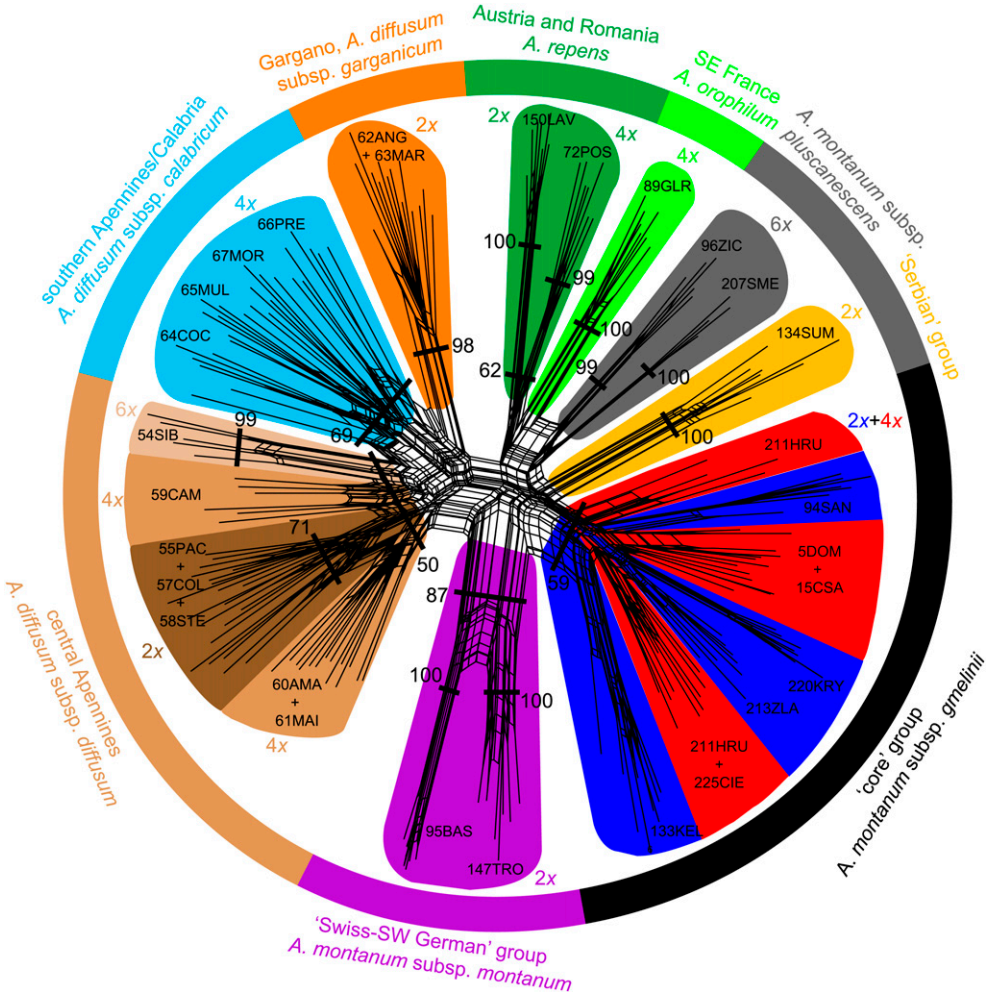

Fig. 2. NeighborNet diagram derived from 223 AFLP markers of 196 individuals of the *Alyssum montanum-A. repens* complex using Sørensen's similarities. The numbers along branches are bootstrap values above 50% taken from the neighbor-joining analysis. Eight genetic clusters inferred from the BAPS analyses are indicated by a multicolored circle drawn around the diagram. The population acronyms follow those in Table 1.

Apennines (Abruzzo and Umbria), Gargano, and the southern Apennines/Calabria were resolved into three separate groups, occupying clearly distant positions in the ordination space. The three cytotypes of the central Apennine populations were also separated quite well from each other (online Appendix S2).

The Bayesian (BAPS) optimal partition estimates resulted in congruent assignments of the analyzed individuals to eight clusters that were consistent with the above analyses (see Fig. 2). The same clusters as displayed by NeighborNet and PCoA are emphasized here (i.e., three geographical clusters of Italian populations, one Central European core cluster [i.e., *A. montanum* subsp. *gmelinii*], *A. repens*, the Swiss-SW German group [i.e., *A. montanum* subsp. *montanum*], and the SE French population), with an exception that the Serbian diploid population, 134SUM, clustered with the hexaploid *A. montanum* subsp. *pluscanescens*.

The nonhierarchical AMOVA revealed that a large portion of the variation occurred within the populations (40.11% vs. 59.89% among populations, $F_{ST} = 0.60$, df = 28, $P < 0.001$). Population groups, as defined by BAPS (eight clusters), explained 32.71% ($F_{CT} = 0.33$, df = 7, $P < 0.001$) of the variation, whereas those suggested by the NeighborNet (five main clusters) explained 23.73% ($F_{CT} = 0.24$, df = 4, $P < 0.001$) of the total variation.

The estimates of the genetic diversity for the studied populations and groups are given in Table 1. The mean numbers of AFLP markers scored per individual were lowest in the diploid populations of *A. montanum* subsp. *gmelinii* (min. 35.0 ± 1.9, in 220KRY) and highest in the tetraploid *A. repens* (max. 57.4 ± 3.9, in 72POS) and in the populations from the southern Apennines/Calabria. Genetic diversity values, expressed as %poly and D_{Nei}, showed that the populations from the southern Apennines/Calabria (max. values of %poly = 27.35% and $D_{Nei} = 0.12$ was in 67MOR) were the most diverse. All of the Italian populations, those from SE France and the tetraploid population of *A. repens* were generally more diverse than all of the other Central European populations. Specific (private) markers were found in very few populations and in low numbers. Considering the BAPS and NeighborNet clusters, each of them was supported by several private AFLP markers, supporting their divergence; the highest numbers were harbored by the central Apennine populations (16), followed by the populations from the southern Apennines/Calabria (10), *A. montanum* subsp. *montanum* (9), *A. montanum* subsp. *gmelinii* (9), and, last, those

from Gargano (7), *A. repens* (6), the Serbian group (5), *A. montanum* subsp. *pluscanescens* (4), and the population from SE France (3).

cpDNA variation—All sequences have been deposited in GenBank with accession numbers JF703911–JF704037 for *rpoB-trnC*, and JF703784–JF703910 for *rpl32-trnL*$^{(UAG)}$ (see Appendix 1). The *rpoB-trnC* alignment was 932 bp long with 10 indels; the *rpl32-trnL*$^{(UAG)}$ alignment was 947 bp long with 13 indels introduced. The concatenated cpDNA alignment comprised 1879 bp (of which 76 sites were variable) and 23 scored indels (online Appendix S3). Altogether, 48 ingroup haplotypes were recognized. The model K81uf+G was determined for both cpDNA data sets as best fitting the data. The Bayesian majority-rule consensus tree (online Appendix S4) displayed a polytomy with five well-supported clades (PP = 0.92–1.00) at the backbone, comprising (1) *A. montanum* subsp. *pluscanescens* (PP = 1.00), (2) central Apennine populations (Abruzzo and Umbria; PP = 0.92), with individual cytotypes resolved into three separate subclades (with an exception of a diploid accession, 58STE5, placed among the tetraploids; PP = 0.85–1.00), (3) Italian populations from the Gargano region (PP = 1.00), (4) Central European core populations (i.e., *A. montanum* subsp. *gmelinii*; PP = 0.93), and (5) a clade (PP = 1.00) containing a few smaller subclades in polytomy—populations from SE France (PP = 1.00), those from the southern Apennines/Calabria together with the Serbian group and two diploid central Apennine individuals (PP = 0.97), *A. montanum* subsp. *montanum* (the Swiss-SW German group) with two N Polish accessions of the core tetraploids and a single SE French accession (PP = 0.96). Accessions of *A. repens* were split between two main clades of the tree: the clade of the Central European core populations (clade 4) and clade 5.

The statistical parsimony network resulted in a single network connecting all 48 haplotypes (Fig. 3). Several groups of related haplotypes, corresponding to the clades and subclades of the Bayesian tree, can be recognized. They are connected through several unsampled or extinct haplotypes. A central loop indicates uncertain relationships among the haplotype groups, which is in accordance with the polytomy in the Bayesian tree. Two haplotypes found in the populations from Gargano appear as most distinct; as many as 16 mutations separate them from the closest Central European *A. montanum* subsp. *gmelinii* haplotypes. The central Apennine populations, with 11 haplotypes, form another distinct group, with only an exceptional haplotype sharing among the three cytotypes (a diploid accession, 58STE5, placed among the tetraploid ones). The cytotype-specific haplotypes are all derived from a common but not sampled (or extinct) haplotype. In two diploid accessions (58STE2 and 57COL3), however, a completely distinct haplotype was detected, connected to one southern Apennine/Calabrian haplotype by three mutations. Clade 5 of the Bayesian tree is recognized here as a series of rather distantly related haplotype groups, connected to each other by several unsampled (extinct) interior haplotypes. Populations from the southern Apennines/Calabria harbor five related haplotypes, which are close to the haplotypes retrieved from the Serbian group (the most common haplotype is even shared with one accession from the Serbian group). In the populations from SE France, two closely related and one more differentiated haplotype were revealed. *Alyssum montanum* subsp. *montanum* (two closely related haplotypes), *A. montanum* subsp. *pluscanescens* (a single haplotype), and two individuals of *A. repens* (72POS1 and 71CAR4, two closely related haplotypes) harbor their own, distinct haplotypes as well. Last, the haplotypes found in the Central European *A. montanum* subsp. *gmelinii* display a star-like topology, with two widespread and common haplotypes in the interior positions, and a series of 14 rare, derived haplotypes usually restricted to single individuals, which are connected to the widespread haplotypes (mostly) by a single (or up to 2–3) mutational step(s). In addition, another two analyzed individuals of *A. repens* (150LAV2 and 73PIA5) harbored two haplotypes that were directly connected to these widespread core haplotypes.

Estimations of genetic diversity, the results of mismatch distribution and neutrality tests are shown in Table 3. Although the number of cpDNA haplotypes observed in the Italian samples (20 haplotypes) was similar to central European *A. montanum* subsp. *gmelinii* (18), the nucleotide diversity and the mean number of pairwise differences were markedly higher in the Italian accessions. The observed mismatch distribution showed a unimodal distribution for *A. montanum* subsp. *gmelinii* (SSD = 0.0459, $P = 0.110$, not rejecting the sudden expansion model), while it was clearly multimodal for the Italian accessions (SSD = 0.013, $P = 0.074$) indicating distributional stasis and lack of population expansion. Both Tajima's D and Fu's F_S statistics yielded negative values for *A. montanum* subsp. *gmelinii* (although only D value was significant, Table 3), consistent with the scenario of rapid population expansion, in contrast to the Italian populations with positive D and F_S values.

Morphometric analyses—The distribution of most of the measured characters departed from the normal distribution, and therefore, the nonparametric correlation coefficient (Spearman) (apart from the Pearson parametric one) and nonparametric classificatory discriminant analyses were used. The correlation coefficients did not exceed 0.95 for any character pair, and, thus, all of the measured characters were retained for further analyses. The highest correlations (0.92 for both Spearman and Pearson coefficients) were found between the characters NrRaysTrichLower and NrRaysTrichUpper (see Table 2 for character explanations).

A series of discriminant analyses (CDA) based on S-L-Fl and Fr characters presented below aimed at exploring the morphological differentiation among the geographical/taxonomic groups supported by molecular data (Figs. 2, 3). First, we analyzed all of the individuals based on the S-L-Fl characters in a single CDA. Then, to visualize their distinction in more detail, we divided them into two groupings that were analyzed separately. Last, we focused on the differentiation between the three Italian groups (i.e., the central Apennines, Gargano, and southern Apennines/Calabria groups). Fruit characters were analyzed both at the level of population means and individual plants.

In the CDA of the S-L-Fl characters performed on all of the individuals and with the eight genetically supported groups (online Appendices S5 and S6, column CDA 1), most of the genetic groups occupied compact areas in the morphological space but showed considerable overlaps. Two large groupings (denoted as A and B) with less overlap could be identified. Grouping A consisted of the populations from the southern Apennines/Calabria, SE France, and *A. repens*. Grouping B consisted of the rest of the analyzed populations. The overlap between these groupings was caused mostly by the central Apennine hexaploid population (54SIB). The classificatory discriminant analysis (DA) based on the same eight groups and characters (with $k = 11$) showed a

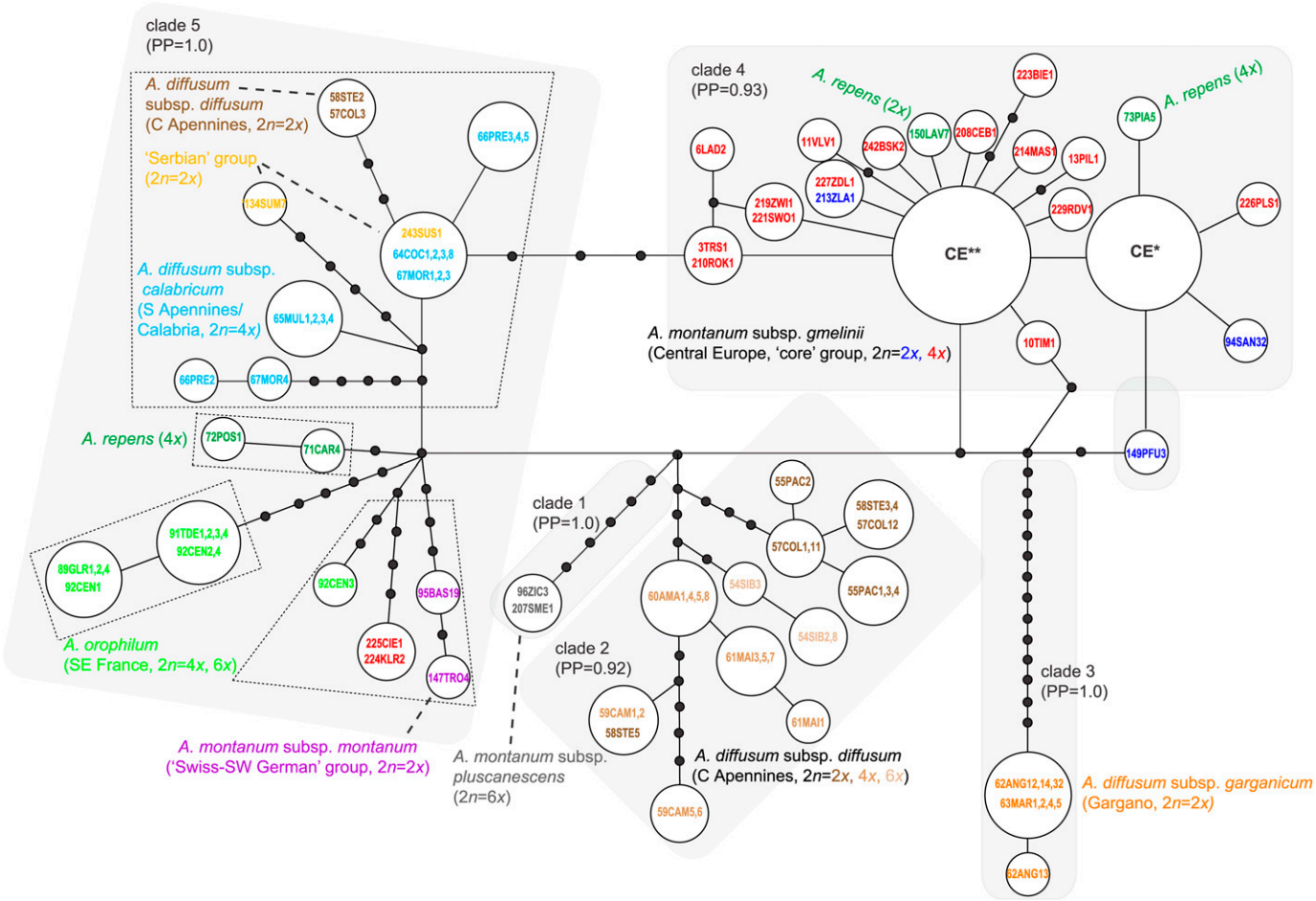

Fig. 3. Maximum parsimony network of cpDNA (*rpoB-trnC* and *rpl32-trnL*$^{(UAG)}$ intergenic spacers) haplotypes of the *Alyssum montanum-A. repens* complex (excluding outgroup). The circle sizes are proportional to frequencies, the lines represent mutational steps, and the black dots are not sampled haplotypes. Gray fields indicate the main clades resolved by the Bayesian analysis (denoted as clades 1–5, including Bayesian posterior probabilities, PP); dashed frames stand for smaller subclades. CE* and CE** haplotypes include numerous Central European core accessions (*A. montanum* subsp. *gmelinii*), CE*: 220KRY1, 233MDL1, 237MKL1, 238LSV1, 234KRM1, 7KPS9, 148SEE, 133KEL1, 21TUR10, 20NAG8, 2PLP1, 5DOM6; CE**: 17ORO2, 85GEM2, 209MOH7, 16GYO1, 211HRU9, 15CSA2, 236PVL1, 232UHR1, 8DRE10, 4LUK1, 9SBR3, 228ZHM1, 18SZE1, 1DVK4, 235ERL2, 215SED1, 12BUD10, and 52KRA3. The population acronyms follow those in Table 1; populations not analyzed here for AFLPs and morphology have acronyms according to Španiel et al. (2011).

relatively high classification success rate (80–100%) for most of the groups, except for the central Apennine populations (66.42%, which were mostly misclassified into the southern Apennine/Calabrian populations and *A. montanum* subsp. *gmelinii*).

The CDA of grouping A illustrated a rather clear morphological differentiation among the populations from SE France, those from the southern Apennines/Calabria, and *A. repens* (Fig. 4A; online Appendix S6, column CDA 2). In the classificatory DA, the percentage of individuals correctly assigned to these three groups was above 92% for each group.

The CDA performed on the grouping B provided insight into the differentiation of the populations from Gargano, the central Apennines, those of *A. montanum* subsp. *montanum* (the Swiss-SW German group), subsp. *gmelinii* (core populations), and the Serbian population, 134SUM (Fig. 4B; Appendix S6, column CDA 3). In the classificatory DA, a relatively high

TABLE 3. Genetic diversity, mismatch distribution, and neutrality tests derived from cpDNA sequence data (concatenated *rpoB-trnC* and *rpl32-trnL*$^{(UAG)}$ intergenic spacers) of the Italian populations (*Alyssum diffusum*) and the Central European core accessions (*A. montanum* subsp. *gmelinii*).

Accession	N_{ind}	N_{hapl}	H (SD)	π_n (SD)	π (SD)	SSD (P-value)	Tajima's D (P-value)	Fu's F_S (P-value)
A. diffusum	51	20	0.9443 (0.0139)	0.0172 (0.00846)	31.8478 (14.1262)	0.0130 (0.074)	0.007 (0.589)	8.111 (0.973)
A. montanum subsp. *gmelinii*	50	18	0.8180 (0.0426)	0.0037 (0.00196)	6.8555 (3.2815)	0.0459 (0.110)	−2.2022 (0.000)	−1.462 (0.336)

Notes: N_{ind}: no. of individuals; N_{hapl}: no. of haplotypes; H: gene diversity; π_n: nucleotide diversity; π: mean no. of pairwise differences; SD: standard deviation; SSD: sum of squared deviations between observed and expected mismatch distribution under a model of sudden population expansion.

classification success rate was obtained at above 80% for each of these five groups.

The CDA of the three Italian groups (Fig. 5; Appendix S6, column CDA 4) differentiated the individuals from the southern Apennines/Calabria along the first axis (correlated with the characters measured on trichomes), with some overlap with the central Apennine populations caused by the hexaploid population 54SIB. The populations from the central Apennines and those from Gargano partly overlapped, but showed a distinct shift along the second axis due to the quantitative characters measured on leaves and stems. In the classificatory DA, high percentages of correctly assigned plants were obtained for the regions of Gargano and the southern Apennines/Calabria (both above 97%), whereas a lower value (79%) was assessed for the individuals from the central Apennines. Fruit characters were found to contribute less to the differentiation than the S-L-Fl characters; analyses based on individual plants showed very little resolution here (figures not shown), but a certain structure was evident when considering the population means. The CDA based on the population means of the entire material in the fruit (seven predefined groups, excluding the plants from SE France that we had in the flowering stage only) showed that most genetic groups/species occupied a separate ordination space (online Appendix S7). In addition, all three of the Italian groups were separated from the remaining populations.

Descriptive statistics of the measured morphological characters for all studied taxa/groups is summarized in online Appendix S8.

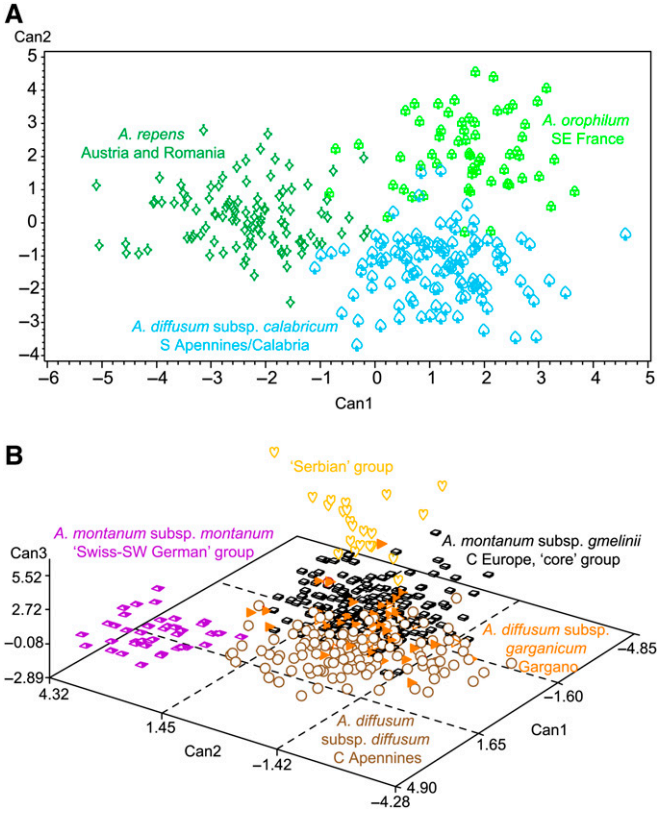

Fig. 4. Canonical discriminant analyses (CDA) performed on the reduced data sets of the *Alyssum montanum-A. repens* complex, based on individual plants and 20 characters measured on stems, flowers and leaves. (A) CDA of *A. diffusum* subsp. *calabricum* from the southern Apennines and Calabria (blue spades), *A. orophilum* (SE France; green clubs) and *A. repens* (green diamonds). (B) CDA of *A. montanum* subsp. *montanum* (violet pyramids), *A. montanum* subsp. *gmelinii* (black cubes), *A. diffusum* subsp. *garganicum* from Gargano (orange flags), *A. diffusum* subsp. *diffusum* from the central Apennines (brown circles), and the Serbian group (yellow hearts).

DISCUSSION

Position of the populations from SE France and Italy within the* Alyssum montanum-A. repens *complex—Our previous study based on AFLP and morphometric analyses (Španiel et al., 2011) highlighted the relationships in the *A. montanum-A. repens* complex within the wider Central European area and revealed incongruence with the traditional taxonomical treatments. In the present study, we focused on the populations from the Apennines and the SW Alpine region, which have typically been reported as *A. montanum* and *A. diffusum* in the literature (see the introduction). Using genetic data and supported by morphometric analyses, we show here that the Italian populations and those from SE France form two clearly delimited groupings within the studied complex, being distinct from any of the Central European reference groups/taxa. Based on the literature survey and plant material from type localities, we conclude that the Italian populations should be attributed to *A. diffusum*, whereas for the populations from SE France, the name *A. orophilum* is applicable (for details, see the Taxonomic conclusions section later).

The morphological patterns in the whole *A. montanum-A. repens* complex are rather complicated, and the species limits cannot be easily defined. No reliable qualitative discriminating characters exist, and, due to large phenotypic variation and overlaps in the quantitative traits, distinction between the taxa is often not straightforward. However, here we show that when a multivariate approach is employed, genetically defined groups can be morphologically distinguished. We have demonstrated here that *A. diffusum* and *A. orophilum* can be differentiated from the other taxa of the *A. montanum-A. repens* complex by a

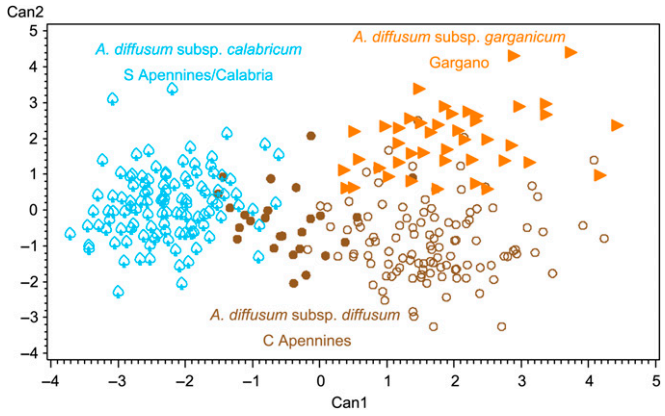

Fig. 5. Canonical discriminant analysis of three genetic and geographical groups of *Alyssum diffusum* (Italian populations, three different subspecies), based on individual plants and 20 characters measured on stems, flowers, and leaves: central Apennines (*A. diffusum* subsp. *diffusum*, brown circles; full circles indicate the 54SIB population), Gargano (*A. diffusum* subsp. *garganicum*, orange flags), southern Apennines and Calabria (*A. diffusum* subsp. *calabricum*, blue spades).

combination of characters, including the size and shape of flower parts, the leaf width and length, the number of trichome rays, and the density of trichomes on the leaf surface (online Appendices S6 and S8).

cpDNA variation patterns in the* A. montanum-A. repens *complex: (In)congruence with the AFLP data—Both the Bayesian phylogenetic tree and the parsimony network based on cpDNA data depicted a series of well-supported haplotype groups/clades, however, with uncertain relationships among them. Apparently, the observed cpDNA variation is part of the much larger ancestral variation, as indicated by numerous inferred missing (most likely extinct) haplotypes placed in interior positions (Hudson, 1990). Low resolution at the backbone of the cpDNA phylogeny agrees with the AFLP-based neighbor-joining tree and NeighbourNet, which showed low bootstrap values and short lengths of internal branches/splits; thus, although the individual taxa/population groups were supported as distinct lineages, the relationships among them remained largely unresolved. The lack of resolution at basal nodes of either cpDNA or nuclear DNA-derived phylogenetic reconstructions has quite commonly been observed in other species complexes as well, and it has been attributed to diverse evolutionary processes, such as rapid radiation, ancient hybridization and backcrossing, extensive ancestral polymorphisms, and stochastic lineage sorting or extinctions (e.g., Marhold et al., 2004; Flagel et al., 2008; Gurushidze et al., 2010).

The star-like structure of the cpDNA variation in *A. montanum* subsp. *gmelinii* (the Central European core group), supported by the results of mismatch distribution and neutrality tests, indicate recent expansion and radiation in this subspecies (according to coalescent theory; see Hudson, 1990). Past bottleneck and a more recent (probably postglacial) colonization of the present area from a single or a few source populations can be assumed. A similar scenario of recent expansion and diversification has also been inferred for *Arabidopsis thaliana* (Beck et al., 2008), *Capsella bursa-pastoris* (Ceplitis et al., 2005), and *Dianthus broteri* (Balao et al., 2010). Only two populations of *A. montanum* subsp. *gmelinii* from northern Poland (224KLR and 225CIE) do not fit this pattern. They harbor strongly divergent haplotypes, and because the AFLP variation attributed these populations unequivocally to the other populations of subsp. *gmelinii*, this discrepancy may indicate ancient hybridization (chloroplast capture) and, possibly, a different refugium and migration route to Central Europe, compared with the rest of the subspecies.

The haplotypes of *A. repens* were placed in two distinct clades; two of them formed their own clade, whereas the other two were resolved among those of *A. montanum* subsp. *gmelinii*. The presence of such divergent haplotypes in a single species, which (considering the AFLP markers) is otherwise genetically coherent, can be explained either by incomplete lineage sorting of ancestors polymorphic for cpDNA haplotypes or by past hybridization with another taxon and the retention of an introgressed haplotype (chloroplast capture; Maddison, 1997; for examples, see Frajman and Oxelman, 2007; Flagel et al., 2008; Gurushidze et al., 2010). The latter explanation appears more likely here, as the haplotypes close to *A. montanum* subsp. *gmelinii* are in the derived positions, which is incompatible with the scenario of ancestral polymorphisms. Analyzing additional populations of *A. repens* and other relatives from the Balkans could provide a clue to the evolutionary history of this species.

The cpDNA haplotypes as well as the AFLP data suggest a close relationship between *A. repens* and *A. orophilum*, which is also supported morphologically. This finding could imply their common origin or at least close evolutionary relationships among populations from the peri-Alpine and perhaps the Eastern Carpathian region. High genetic variation was revealed in the Italian populations (*A. diffusum*), which was geographically structured, reflecting three regions (the central Apennines, Gargano, southern Apennines/Calabria). Still, while the three AFLP clusters were apparently closely related, the cpDNA haplotypes of this species formed three clearly distant clades, separated by a high number of mutations, which might indicate a long history of isolation. Gene flow among the populations from these three disjunct regions has presumably been highly restricted, although with exceptions. Two individuals from the central Apennines harbored a haplotype derived from the dominant southern Apennine/Calabrian haplotype, which probably illustrates an occasional gene flow and persistence of an introgressed haplotype.

In conclusion, the cpDNA data presented here indicate a rather complex evolutionary history of the *A. montanum-A. repens* complex, involving both extinctions and recent diversification. Most taxa are characterized by monophyletic or at least closely related haplotypes, but exceptions can be found, which we putatively attribute to introgression and chloroplast capture. Further studies should also focus on the areas not sampled here (i.e., the Balkans, France, and Iberian Peninsula), which may reveal additional diversity of the complex.

Genetic, cytotype, and morphological variation patterns in the Italian populations—The central Apennine populations of *A. diffusum* from the regions of Umbria and Abruzzo form a highly variable group, regarding both the genetic variation and ploidy levels. Three different cytotypes and as many as 12 cpDNA haplotypes were determined in the seven populations studied. Previous studies on *A. diffusum* (as defined here) have reported diploids and tetraploids from Italy (La Valva, 1976; Kieft and van Loon, 1978; Polatschek, 1983), and, in addition to these counts, we also found a hexaploid population in this area. The three cytotypes found in the central Apennines appear to be genetically differentiated; populations of the same ploidy levels tend to cluster together in AFLP analyses, and importantly, only a limited haplotype sharing was observed among the cytotypes. All the haplotypes of the central Apennine populations seem to be derived from a single ancestral (not sampled, most likely extinct) haplotype, with each cytotype bearing its own set of monophyletic haplotypes. This pattern reflects not only restricted gene flow among different ploidy levels, but, most likely, also a single origin of the tetraploid and hexaploid cytotypes and independent haplotype diversification within each cytotype.

The populations of *A. diffusum* from the southern Apennines and the northern Calabrian Arc were exclusively tetraploid, exhibiting high intrapopulational variation as revealed by AFLP markers. Their distinct position from the other Italian populations, suggesting their own evolutionary history, is well documented by both AFLP and cpDNA data. A distinct position of the Calabrian and the southern Apennine biota has also been illustrated in several other cases, where either phylogeographic or phylogenetic patterns have suggested a pronounced divergence of the populations from this region (*Bombina*, Canestrelli et al., 2006; *Sciurus*, Grill et al., 2009; *Sorex*, Vega et al., 2010; *Himantoglossum hircinum*, Pfeifer et al., 2009; *Plantago brutia*, Palermo et al., 2010). The Calabrian Arc, consisting of several mountain ranges separated by lowlands, which formed a

chain of islands from the Pliocene to the Middle Pleistocene, undoubtedly favored long-term survival and isolation (Blasi et al., 2007). At the same time, the proximity of the northernmost Calabrian massif, Catena Costiera, to the southern Apennines in the northern Calabria/southern Basilicata region might have allowed gene exchange and dispersal between these adjacent ranges. Such affinities have, indeed, been revealed for the Italian pygmy shrews (*Sorex minutus*; Vega et al., 2010) and are documented here for *A. diffusum*. One of the intriguing findings of our study, nevertheless, is a very close relationship between the southern Apennine/Calabrian populations and the Serbian populations (134SUM and 243SUS) shown by cpDNA data. Our preliminary analyses indicate that such close affinity holds true also for some other Balkan representatives from the *A. montanum-A. repens* complex (S. Španiel et al., unpublished data). We hypothesize that the southern Apennine/Calabrian populations are either direct descendants of the Balkan ancestors or that gene exchange between the populations from these two Peninsulas has occurred in the past. Indeed, close relationships between the southern Italian and Balkan species have already been reported for many other plant taxa (Morgante et al., 1998; Passalacqua, 1998; Gömöry et al., 1999; Fineschi et al., 2002; Vettori et al., 2004; Musacchio et al., 2006; Simeone et al., 2009; Bellusci et al. 2010; Kučera et al., 2010). This trans-Adriatic affinity has mainly been attributed to the lowering of the sea level during the Pleistocene (Blasi et al., 2007) and also to the extensive desiccation of the Mediterranean Sea (Messinian Salinity Crisis) in the Messinian Age of the Miocene (Passalacqua and Bernardo, 1998; Blasi et al., 2007).

The populations from the Gargano region in Puglia were diploid and, interestingly, bore strongly divergent cpDNA haplotypes, although AFLP data did not support such a pronounced differentiation. These populations also differ from the rest of *A. diffusum* ecologically; in contrast to the other Italian populations, which are typically high-altitude mountain plants growing from 1000 to 2000 m a.s.l., those from Gargano are found in mid-altitude hilly habitats at ca. 600–700 m a.s.l. Thus, ecological adaptation might have reinforced their distinct evolution. Indeed, a unique position of the Gargano region within the flora of Italy has been shown in other genera as well (e.g., *Quercus*, Fineschi et al., 2002; *Anacamptis*, Cozzolino et al., 2003). Gargano belongs to the Mediterranean region (Pedrotti, 1996), not to the Apennine one, even if it shares some orophytic plants with the Apennine flora (Marchiori et al., 2000).

From the cytotype perspective, we can conclude that the polyploid populations of *A. diffusum* from different Italian regions are genetically clearly differentiated and arose independently. Morphologically, the southern Apennine/Calabrian populations can be distinguished quite well, whereas the differentiation between the populations from the central Apennines and those from Gargano, in contrast to the genetic findings, is weaker. In addition, the hexaploid central Apennine population (54SIB) clearly deviates morphologically from the other central Apennine populations (diploids and tetraploids) and appears to be closer to the southern Apennine/Calabrian specimens. Such incongruence between the genetic and morphological data might suggest an allopolyploid origin for this hexaploid. Such discrepancies between morphological and genetic patterns have been observed in confirmed polyploid hybrids (see e.g., Lihová et al., 2007).

The Apennine Peninsula: A center of diversity and evolution—The Apennine Peninsula, one of the three Mediterranean peninsulas, has been recognized as an important hotspot of genetic and species diversity in Europe (Thompson, 2005). Two of the mainland regions of Italy, Calabria and Abruzzo, host the highest numbers of endemic vascular plants, comprising 205 and 177 endemic taxa (species and subspecies), respectively (Abbate et al., 2007). The high species diversity in the central Apennines (Abruzzo) is also due to the occurrence of many Central European and Alpine species that reach the central Apennines but not the southern Apennines (Conti, 1998; Lucchese and De Simone, 2000). A bio- and phylogeographical synthesis by Médail and Diadema (2009) identified, on a finer geographical scale, several Mediterranean regions that have provided favorable conditions for a long-term persistence of species, allowing the accumulation of genetic and species diversity, as well as active speciation by means of isolations and secondary contacts. The Apennine hotspot regions highlighted by Médail and Diadema (2009), located in the Apuan Alps, Gargano, Campania, southern Apennines and Calabria (see Fig. 1 in Médail and Diadema, 2009), can be illustrated by several recent studies on the endemics of these regions, such as *Allium garganicum*, *Cardamine apennina*, *C. battagliae*, *C. silana*, *Ornithogalum umbratile*, *Plantago brutia*, *Polygala apiculata*, *Rhaponticoides calabrica*, and *Sesleria calabrica* (Cesca and Peruzzi, 2002; Tornadore et al., 2003; Lihová et al., 2004; Perný et al., 2005; Peruzzi et al., 2005; Di Pietro, 2007; Brullo et al., 2009; Puntillo and Peruzzi, 2009; Palermo et al., 2010). The presence of three genetically differentiated lineages within the *Alyssum diffusum* complex in the present study, inhabiting Gargano, the central Apennines (Abruzzo and Umbria), and the southern Apennines/northern Calabrian Arc, coincides very well with the patterns in other plant genera and reiterates the evolutionary importance of these regions. We assume that these three intraspecific lineages represent the descendants of separate refugial populations, in congruence with the "refugia-within-refugia" hypothesis, favored by several groups of organisms, including plants (Magri et al., 2006; Ansell et al., 2008), mollusks (Fiorentino et al., 2010), amphibians (Canestrelli et al., 2006, 2007, 2008; Canestrelli and Nascetti, 2008), reptiles (Böhme et al., 2007), and mammals (Ruedi et al., 2008; Vega et al., 2010).

A certain environmental stability in refugia and a climatic and topographic heterogeneity, providing a high diversity of ecological niches and the opportunity for species to migrate along the altitudinal gradient, have been crucial for plant survival and explain the present-day diversity. Such vegetation dynamics have been hypothesized for different parts of the Apennine Peninsula, especially for higher altitudes (Médail and Diadema, 2009). Whereas the genetic differences among the populations of *A. diffusum* from the three regions of Italy (Gargano, the central Apennines, and the southern Apennines/Calabria) favor their long-term persistence and isolation, the occurrence of three different ploidy levels in the central Italian mountains also implies more dynamic evolutionary processes there. The local populations in the central Apennines may have experienced one or more cycles of isolations and subsequent secondary contacts on an altitudinal, rather than latitudinal, gradient, leading to hybridization and polyploidization.

A rather low overall genetic variation and weak structure in *A. montanum* subsp. *gmelinii* (the Central European core populations) contrasts with the much higher and more complex diversity in *A. diffusum*, as it can be seen from the diversity measures inferred from both AFLP and cpDNA data (Tables 1 and 3). This pattern coincides with a general trend toward a

lower genetic diversity in the northern parts of Europe and genetic richness in the south, the so called southern richness vs. northern purity paradigm (Hewitt, 1999), which is associated with glacial extinctions, postglacial expansion and genetic bottleneck during recolonizations (Hewitt, 1996, 2004a, b; Schmitt, 2007). Nevertheless, the genetically rich Italian populations of *A. diffusum* analyzed here most likely did not contribute to the postglacial colonization of Central Europe. We assume this based on a strong differentiation of the Italian accessions inferred from AFLP markers (Fig. 2, online Appendix S1), and a completely different set of cpDNA haplotypes resolved in these populations, when compared with the Central European ones (Fig. 3). Similar patterns, showing that the Mediterranean refuge areas existed during glaciations but were not the source areas for the colonization of Central and northern Europe, have been reported in both plants and animals (Bilton et al., 1998; Vettori et al., 2004; Magri et al., 2006; Ansell et al., 2008; Pfeifer et al., 2009). The actual genetic source and/or glacial refugium of the Central European populations of *A. montanum* is as yet unknown, and the role of other areas (mainly the Balkans) should be inspected in future studies.

Taxonomic conclusions—The genetic and morphological variation patterns of the populations sampled from Italy and neighboring southeastern France strongly disagree with previous taxonomic treatments. While two taxa, *A. montanum* and *A. diffusum*, have traditionally been reported from central and southern Italy (Zangheri, 1976; Pignatti, 1982; Kerguélen, 1993), we demonstrated here that the studied Italian populations are clearly distinct from *A. montanum*, whether considering the latter in a strict (subsp. *montanum* from the region of Switzerland and southwestern Germany) or a broad sense (including the widespread Central European populations, subsp. *gmelinii*). Thus, all of the Apennine populations in this study should be assigned to *A. diffusum*, a species described from the former kingdom of Naples (covering central and southern Italy: Tenore, 1812). It is disputable, however, whether *A. diffusum* should be treated as a single, genetically and morphologically highly variable species or split into three taxa following its geographically correlated structure. Considering that each of the three Italian regions harbors differentiated, presumably ancestral, populations and thus encompasses a unique genetic diversity, we recognize here the populations from the central Apennines, Gargano and the southern Apennines/Calabria as three infraspecific entities within *A. diffusum*. The formal recognition of infraspecific taxa may also be beneficial for the conservation of the genetic resources of this species.

A question may arise concerning the species and subspecies concept adopted here. Due to the complexity of the variation patterns observed in the *A. montanum-A. repens* group, it is very difficult to impose unequivocal and consistent criteria for these ranks. Genetic differentiation and affinities often do not parallel the morphological ones and vice versa. Nevertheless, taking into account all available evidence, i.e., the morphological, molecular, cytotype, and geographical data, as well as the practical applicability of the adopted taxonomic treatment, we believe that the taxonomic decision proposed here is most appropriate and attempts to reflect also the evolutionary history of the species. Such an approach is most closely related to the pragmatic species concept advocated by Ehrendorfer (1984).

In the past, *Alyssum diffusum* has also been reported from southern France (departments Ariège and Aude, in the eastern Pyrenees and their foothills), the Iberian Peninsula (Rouy and Foucaud, 1895; Guinochet and de Vilmorin, 1982; Saule, 1991; Kerguélen, 1993; Jalas et al., 1996), and Greece (prefecture of Phocis, Mount Giona; Contandriopoulos, 1970). Nevertheless, in the *Flora Iberica* (Küpfer and Nieto Feliner, 1993, p. 169), the name *A. diffusum* is treated as a synonym of *A. montanum*, and only *A. diffusum* subsp. *corymbosum* from the Sierra Nevada is considered to be a separate taxon (treated as *A. nevadense*). Similarly, in the recent taxonomic treatment of the genus *Alyssum* for the territory of Greece (Hartvig, 2002), *A. diffusum* is not mentioned, not even as a synonym. The taxonomic identity of the plants from the areas outsides the Apennines and their relationships to the Italian *A. diffusum* will require further study.

The distinctness of the populations from the southwestern Alps in France does not support their treatment within *A. montanum* either. Although two names, *Alyssum orophilum* and *A. pedemontanum*, pertinent to the populations from the Italian-French border region have existed, they were mostly treated as synonyms of *A. montanum* (Kerguélen, 1993). Of these two names, *A. orophilum*, by Jordan and Fourreau (1868), has an unequivocal priority over *A. pedemontanum*, by Ruprecht (1869). Whereas the name *A. pedemontanum* has been accepted by at least some authors either at the level of species (Rouy and Foucaud, 1895) or subspecies (*A. montanum* subsp. *pedemontanum*, Küpfer, 1974), the name *A. orophilum* was most probably only used by the original authors. Before final taxonomic decision can be done, more populations attributable to *A. orophilum* should be sampled in future to characterize this taxon in more detail.

TAXONOMY

Here we present the differential diagnoses of the infraspecific units of *A. diffusum*, with the fulfillment of all necessary requirements for valid publication of the names of two new subspecies. Detailed morphological descriptions of the taxa, discussion of the place of publication of the name *A. diffusum*, discussion, and formal designation of its lectotype are presented in S. Španiel et al. (unpublished manuscript).

Alyssum diffusum **Ten., Flora Napol. 1: XXXVII, 1812**
Ind. loc.: [Kingdom of Naples]
Lectotype (designated in Španiel et al., unpublished manuscript): Scrimacavallo / Majella ([Tenore] s.a. **NAP!**)

Alyssum diffusum **Ten. subsp. *diffusum***

Alyssum diffusum **subsp. *garganicum* Španiel, Marhold, Passalacqua & Zozomová-Lihová, subsp. nov.**
Holotype—Italy, Puglia, Gargano, Promontorio del Gargano, quarries near San Marco in Lámis toward San Nicandro Garganico, 41°43′15.2″N, 15°37′13.4″E, 630 m a.s.l., 3.VI.2007, *S. Španiel, M. Perný & V. Kolarčik 63MAR/27* (**SAV**).
Diagnosis—Typo speciei *Alyssum diffusum* Ten. affinis, sed caulibus majoribus, foliis longioribus et latioribus differt.

Alyssum diffusum **subsp. *calabricum* Španiel, Marhold, Passalacqua & Zozomová-Lihová, subsp. nov.**
Holotype—Italy, Calabria, Monte Cocuzzo, SW of Cosenza (near the road toward Fiumefreddo Brúzio), 39°13′38.2″N, 16°08′12.9″E, 1367 m, 4.VI.2007, *S. Španiel, N. G. Passalacqua, M. Perný & V. Kolarčik 64COC/8* (**SAV**).

Diagnosis—Typo speciei *Alyssum diffusum* Ten. affinis, sed foliis sparse pubescentibus, pilis stellatis cum ramis minor numerosis differt.

Identification key to the subspecies—

1a Stellate trichomes on lower surface of middle stem leaf with 15–27* rays, those on upper surface with 9–20* rays. Lower surface of middle stem leaf mostly densely hairy, with 7–20 trichomes per 0.5 mm^2 area 2

1b Stellate trichomes on lower surface of middle stem leaf with 7–12* rays, those on upper surface with 6–9* rays. Lower surface of middle stem leaf sparsely hairy, with 2–8 trichomes per 0.5 mm^2 area ..... subsp. *calabricum*

2a Middle stem leaf (usually 8th leaf, counted downward) (4.5–)4.9–10.6(–12.4) mm long. Style persisting on silicula (1.7–)1.8–2.5(–2.5) mm long, silicula (2.9–)3.0–4.5(–4.7) × (2.8–)3.0–4.0(–4.4) mm. Stem densely leafy, distance between base of 8th and 15th leaf is (8–)10–34(–40) mm ..... subsp. *diffusum*

2b Middle stem leaf (usually 8th leaf, counted downward) (9.0–)10.3–18.6(–21.5) mm long. Style persisting on silicula (2.0–)2.4–3.3(–3.4) mm long, silicula (4.1–)4.2–5.4(–5.5) × (3.6–)3.7–4.9(–5.0) mm. Stem less densely leafy, distance between base of 8th and 15th leaf is (19–)32–59(–65) mm ..... subsp. *garganicum*

* Mean of three random counts per leaf surface.

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APPENDIX 1. Collection sites and GenBank accession numbers for the sequences of *rpl32-trnL*$^{(UAG)}$ and *rpoB-trnC* intergenic spacers of the *Alyssum* populations analyzed in this study. Herbarium vouchers are deposited in the herbarium of the Institute of Botany, SAS, Bratislava, Slovakia (SAV). Names of collectors: *SŠ*, S. Španiel; *JZL*, J. Zozomová-Lihová; *KM*, K. Marhold; *MP*, M. Perný; *VK*, V. Kolarčik; *FM*, F. Maggi; *NGP*, N.G. Passalacqua). Population samples marked by asterisks were also analyzed in a previous study (Španiel et al. 2011).

***Taxon* (Geographic region): Population acronym;** Country, Administrative region, Locality, Altitude (a.s.l.), Collection date, Collectors, GenBank accessions: *rpl32-trnL*$^{(UAG)}$, *rpoB-trnC*.

***Alyssum diffusum* subsp. *diffusum* (central Apennines): 54SIB**; Italy, Umbria, Monti Sibillini, two microlocalities—E of Norcia, Forca Canapine, 1519 m & E of Norcia near the road to Castelluccio through the plateau Piano Grande, 1283 m, 28-May-07, *SŠ, FM, MP & VK*, JF703851-JF703853, JF703978-JF703980. **55PAC**; Italy, Abruzzo, Maiella, between Passo San Leonardo and the village Pacentro, 1075 m, 29-May-07, *SŠ, MP & VK*, JF703858- JF703861, JF703985-JF703988. **57COL**; Italy, Abruzzo, Gran Sasso e Monti della Laga, above San Colombo toward Santo Stéfano di Sessánio, 1246 m, 30-May-07, *SŠ, MP & VK*, JF703862-JF703865, JF703989-JF703992. **58STE**; Italy, Abruzzo, Gran Sasso e Monti della Laga, near road from Santo Stéfano di Sessánio to Mte. Cecco d´Antonio, 1638 m, 30-May-07, *SŠ, MP & VK*, JF703791, JF703848-JF703850, JF703918, JF703975-JF703977. **59CAM**; Italy, Abruzzo, Gran Sasso e Monti della Laga, 5 km of Valico della Campannelle toward Fonte Cerreto, 1481 m, 30-May-07, *SŠ, MP & VK*, JF703843-JF703846, JF703970-JF703973. **60AMA**; Italy, Abruzzo, Maiella, Mte. Amaro, western slopes (toward Passo S. Leonardo), 1888 m, 31-May-07, *SŠ, MP & VK*, JF703854-JF703857, JF703981-JF703984. **61MAI**; Italy, Abruzzo, Maiella, La Maielletta, 2010 m, 31-May-07, *SŠ, MP & VK*, JF703839-JF703842, JF703966-JF703969. ***Alyssum diffusum* subsp. *garganicum* (Gargano): 62ANG**; Italy, Puglia, Gargano, Promontorio del Gargano, south of Foresta Umbra, two microlocalities - 4 km after the crossroad Monte Sant´Ángelo—Carpino—Casa Forestale toward Casa Forestale, 680 m & 7 km after the crossroad Monte Sant´Ángelo—Carpino—Casa Forestale toward Casa Forestale, 603 m, 02-Jun-07, *SŠ, MP & VK*, JF703827-JF703830, JF703954-JF703957. **63MAR**; Italy, Puglia, Gargano, Promontorio del Gargano, quarries near San Marco in Lámis toward San Nicandro Garganico, 630 m, 03-Jun-07, *SŠ, MP & VK*, JF703831-JF703834, JF703958-JF703961. ***Alyssum diffusum* subsp. *calabricum* (southern Apennines and Calabria): 64COC**; Italy, Calabria, Monte Cocuzzo, SW of Cosenza near the road toward Fiumefreddo Brúzio, 1367 m, 04-Jun-07, *SŠ, NGP, MP & VK*, JF703795-JF703798, JF703922-JF703925. **65MUL**; Italy, Calabria, Pollino, Monte la Mula, W of San Donato di Ninea, two microlocalities: 1877 m & 1602 m, 05-Jun-07, *SŠ & NGP*, JF703835-JF703838, JF703962-JF703965. **66PRE**; Italy, Calabria/Basilicata, Pollino, Serra del Prete, 2034 m, 07-Jun-07, *SŠ, MP & VK*, JF703805-JF703808, JF703932-JF703935. **67MOR**; Italy, Calabria, Pollino, Morano Cálabro and Campotenese, 971 m, 07-Jun-07, *SŠ, MP & VK*, 17-Jul-07, *NGP,* JF703799-JF703802, JF703926-JF703929. ***A. orophilum*: 89GLR**; France, Dauphiné Alps, Rhône-Alpes, Savoie, below Col du Galibier, 1980 m, 14-Jun-07, *MP*, JF703901-JF703903, JF704028-JF704030. **91TDE**; France, Provence-Alpes-Côte d'Azur, Alpes-Maritimes, Tende, below Col de Tende, 1851 m, 17 Jun 2007, *MP*, 06-Jun-10, *SŠ, JZL & KM*; *A. pedemontanum* Rupr., locus classicus, JF703904-JF703907, JF704031-JF704034. **92CEN**; France, Cottian/Graian Alps, Rhône-Alpes, Savoie, Massif du Mont Cenis, above Lac du Mont Cenis, above road, 2086 m 18-Jun-07, *MP*, 07-Jun-10, *SŠ, JZL & KM*; *A. pedemontanum* Rupr., locus classicus, JF703792, JF703908-JF703910, JF703919, JF704035-JF704037. ***A. montanum* subsp. *gmelinii* (core diploids): *94SAN**; Germany, Baden-Würtemberg, S of Heidelberg, Sandhausen, 110 m, 12-Apr-08, *KM, JZL & J. Paule*; *A. gmelinii* Jord. & Fourr., locus classicus, JF703889, JF704016. ***133KEL**; Serbia, Severna Bačka, Kelebija near the town of Subotica, 129 m, 13-May-08, *SŠ & J. Šibík*, JF703899, JF703787. ***213ZLA**; Czech Republic, Český kras, stone quarry Čertovy schody near nature reserve Zlatý kůň, Koněprusy, 262 m, 20-Apr-09, *SŠ & JZL*, JF703883, JF704010. ***220KRY**; Poland, Województwo Świętokrzyskie, Kichary Nowe, 1 km E of the village in the valley of the river Opatowka, 190 m, 03-May-09, *SŠ & P. Mereďa*, JF703811, JF703938. **('core' tetraploids): *5DOM**; Slovakia, Slovenský kras, Dlhá Ves, slope of the National Nature Reserve Domické škrapy, above the cave Domica, 360 m, 22-May-06, *SŠ*, JF703878, JF704005. ***15CSA**; Hungary, Komárom-Esztergom, Császár, 189 m, 23-Apr-07, *SŠ & N. Riezing*, JF703816, JF703943. ***211HRU**; Czech Republic, Znojemsko-brněnská pahorkatina, Hrubšice, 260 m, 16-Apr-09, *SŠ & JZL*, JF703815, JF703942. ***225CIE**; Poland, Województwo Kujawsko-Pomorskie, Ciechocinek, beside the road toward Raciążek, 40 m, 07-May-09, *SŠ & P. Mereďa*, JF703789, JF703916. ***A. montanum* subsp. *montanum* (Swiss-SW German group): *95BAS**; Switzerland, Baselland, S of Basel, Aesch, below the castle ruin Pfeffingen, 390 m, 13-Apr-08, *T. Brodtbeck, KM & JZL*; *A. montanum* L., locus classicus, JF703804, JF703931. ***147TRO**; Germany, Baden-Würtemberg, Trochtelfingen, 715 m, 17-May-10, *M. Thiv, SŠ, JZL & KM*, JF703803, JF703930. **Serbian group: *134SUM**; Serbia, Južni Banat, Deliblatska peščara, Šumarak between Gaj and Dubovac, 105 m, 13-May-08, *SŠ & J. Šibík*, JF703794, JF703921. ***A. montanum* subsp. *pluscanescens*: *96ZIC**; Slovenia, Predalpsko območje, Žiče near Slovenske Konjice, stone quarry in the village, 310 m [cultivated from seeds obtained from Ljubljana Botanic Garden]; *A. montanum* subsp. *pluscanescens* (Jos. Baumgartner) Trpin, locus classicus, JF703896, JF704023. ***207SME**; Croatia, Zagrebačka županija, Samoborsko gorje, Smerovišće, ca. 250 m, 23-Jun-08, *I. Boršić, S. Bogdanović & T. Nikolić*; *A. samoborense* Kušan, nom. inval., locus classicus, JF703897, JF704024. ***A. repens*: *71CAR**; Romania, Prahova, Munţii Bucegi, Parcul Naţional Bucegi, Babele, near the trekking path to Caraiman (red mark) and in the vicinity of Cabana Caraiman (blue mark), 2191 m, 05-Jul-07, *SŠ, Ľ. Majeský & V. Kolarčik*, JF703823, JF703950. ***72POS**; Romania, Braşov, Poiana Braşov, Postăvarul, 1799 m, 06-Jul-07, *SŠ, Ľ. Majeský & V. Kolarčik*; *A. repens* Baumg., locus classicus, JF703847, JF703974. ***73PIA**; Romania, Neamt, Munţii Ceahlău, Parcul Naţional Ceahlău, Izvoru Muntelui, near trekking path (blue mark) from Cabana Izvorul Muntelui to Piatra cu Apă, 1498 m, 07-Jul-07, *SŠ, Ľ. Majeský & V. Kolarčik*, JF703870, JF703997. **84RET**; Romania, Hunedoara, Munţii Retezat, Parcul Naţional Retezat, Piatra Iorgovanului, 2014 m, 21-Jul-07, *SŠ*. ***150LAV**; Austria, Kärnten, Lavamünd, in the forest near the calvary and the church Dreifaltigkeitskirche, 500 m, 26-May-08, *SŠ, KM & JZL*, JF703898, JF704025.