

Consequences of geographical habitats on population structure and genetic diversity in *Campanula spp.*

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Abstract

Characterization of populations by means of DNA techniques provides a tool for precise identification and a quantitative estimate of genetic diversity, crucial in evaluation of genetic fragmentation within and among populations. NBS profiling are PCR-based approaches that sample genetic variation in resistance genes (R-gene), and R gene analogs (RGA). To date, *myb* patterns have not been used for evaluating genetic diversity in other species. NBS primers are homologous to the conserved sequences in the Nucleotide-Binding-Site of the NBS-LRR class of R-genes. A total of 12 populations from five *Campanula* species (*C. barbata* L., *C. latifolia* L., *C. rapunculoides* L., *C. spicata* L. and *C. trachelium* L.), autochthonous of the West Italian Alps, were genotyped via nucleotide-binding site (NBS) and *myb* gene profiling. The selected markers produced a total of 361 bands, showing high levels of polymorphism. Genetic diversity among and within species and population structure was evaluated by different statistical analyses performed using TREECON software, Mantel Nonparametric Test, NTSYS package, AMOVA and STRUCTURE. The correlation between genetic variability and geographical location suggests that the five *Campanula* species have been subjected to long-term evolutionary processes consistent with the natural fragmentation of continuous mountains areas.

Introduction

The genetics of species whose preservation is of great importance for biodiversity maintenance has been of heightened interest to evolutionary and conservation biologists in the 21st century.¹ In particular, in conservation studies, an important topic is the evaluation of genetic fragmentation within and among populations. The spatial distribution of genetic

diversity in populations is function of gene flow, mutation, inbreeding, population size, and social organization, and has been modelled by biological, ecological, and historical factors related to geo-climatic events.^{2,4} Over the last few decades, the study and safeguarding of genetic variation in nature for plastic responses has progressed significantly.^{5,8}

Campanula L. species might be considered genetic resources able to persist under a wide range of ecological conditions.⁹ In fact, their habitat can vary considerably, from seashore to very high altitude, with colonies found in all major environments from forests to grasslands, and even in rocks.

In the West Italian Alps, five species deserve highlighting above others: 1) *C. barbata* L. (2n = 34;¹⁰), a hairy short-lived perennial or biennial plant, growing in dry grassland on the siliceous soils of the alpine regions of central and boreal Europe; 2) *C. latifolia* Brantwood (2n = 34;¹¹), a perennial plant originating from Northern Europe, growing in acidic soils and forests in the Italian Alps and Northern Apennines; 3) *C. rapunculoides* L. (2n = 68; 2n = 102;^{12,13}), a perennial plant, growing on stony and wooded areas of Europe, except for the arctic regions and islands; 4) *C. spicata* L. (2n = 34;¹²), a perennial plant, growing on stones and cliffs and widespread in the Italian Alps and Northern Apennines; and 5) *C. trachelium* L. (2n = 34;¹⁴), a perennial species native to Europe, from England, throughout the Mediterranean basin, to Northern Africa.¹⁵

Molecular studies may prove useful in improving spatial genetic variation knowledge and for delineating evolutionary genetic processes.¹⁶⁻¹⁹ Neutral markers such as amplified fragment length polymorphisms (AFLP²⁰) or simple sequence repeats (SSR^{21,22}) are commonly used to screen collections. In previous studies, the diversity and the structure in the genus *Campanula* was studied using nuclear (ISSR) and chloroplast (cpSSR) markers in *C. pollinensis* Podlech and *C. pseudostenodon* Lac.⁴ suggesting that the genetic differentiation between subpopulations was caused by a restricted gene flow.

Recently, nucleotide-binding site (NBS) and *myb* gene profiling techniques have been assessed in various genetic approaches. NBS profiling is a PCR-based methodology for studying genetic variability that specifically targets NBS-TIR resistance genes and R-gene analogs by using a degenerate primer based on conserved motifs of these genes.²³ This technique has already been used in apple,²⁴ lettuce,²⁵ potato, barley, durum wheat, and cauliflower^{23,26,27} to map genes related to disease resistance (up to 95% of the amplified bands relate to RGAs). MYB proteins constitute a class of DNA binding proteins, which is particularly important for transcriptional regulation in plants.²⁸ This protein family is characterized

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by having a structurally conserved DNA binding domain – the MYB DNA binding domain which contains one (I), two (II), or three (III) imperfect repeats. The two-repeat (R2R3) MYB family is the most commonly identified in plants and consists of 125 members in *Arabidopsis*.²⁹ The known functions of plant MYB proteins include the regulation of secondary metabolism, control of cellular morphogenesis, and regulation of the meristem and the cell cycle.³⁰

In the present research, we evaluated NBS and *myb* profiling in *Campanula* as tools for genetic diversity studies, which we used to investigate the i. genetic variations, and ii. population structures in five species (*C. barbata*, *C. latifolia*, *C. rapunculoides*, *C. spicata* and *C. trachelium*).

Materials and Methods

Plant material

We sampled a total of 12 populations belonging to *C. barbata*, *C. latifolia*, *C. rapunculoides*, *C. spicata* and *C. trachelium* (Table 1) in the West Italian Alps (Figure 1³¹). The sampling

Table 1. Sampled *Campanula* species and populations with their identification code (ID), geographical location, soil, latitude (La.), longitude (Lo.), altitude (Al.) and sample size (n).

ID	Species	Location	Soil (substrate)	La. (N)	Lo. (E)	Al. (m)	n
Rap1	<i>C. rapunculoides</i>	Gesso Valley	grassland	44°15'29.65"	7°23'04.30"	830	6
Rap2	<i>C. rapunculoides</i>	Gesso Valley	grassland	44°18'02.66"	7°27'19.47"	719	6
Rap3	<i>C. rapunculoides</i>	Vermenagna Valley	scrub	44°14'06.03"	7°31'49.34"	980	4
Rap4	<i>C. rapunculoides</i>	Troncea Valley	scrub	45°01'30.61"	7°04'23.04"	1818	6
Lat1	<i>C. latifolia</i>	Monferrato	grassland	45°05'18.11"	8°02'22.21"	447	3
Lat2	<i>C. latifolia</i>	Sesia Valley	rockwall	45°51'14.32"	7°56'11.23"	1203	6
Trach1	<i>C. trachelium</i>	Stura Valley	grassland	44°20'39.48"	7°01'55.99"	1245	6
Trach2	<i>C. trachelium</i>	Monferrato	grassland	45°06'32.29"	8°01'30.11"	410	6
Barb	<i>C. barbata</i>	Lanzo Valley	grassland	45°21'55.22"	7°20'25.26"	1880	6
Spic1	<i>C. spicata</i>	Sesia Valley	rockwall	45°49'54.28"	7°57'24.20"	1150	6
Spic2	<i>C. spicata</i>	Sesia Valley	rockwall	45°49'19.93"	8°09'25.43"	614	6
Spic3	<i>C. spicata</i>	Varaita Valley	scrub	44°33'58.33"	7°21'06.96"	1812	6

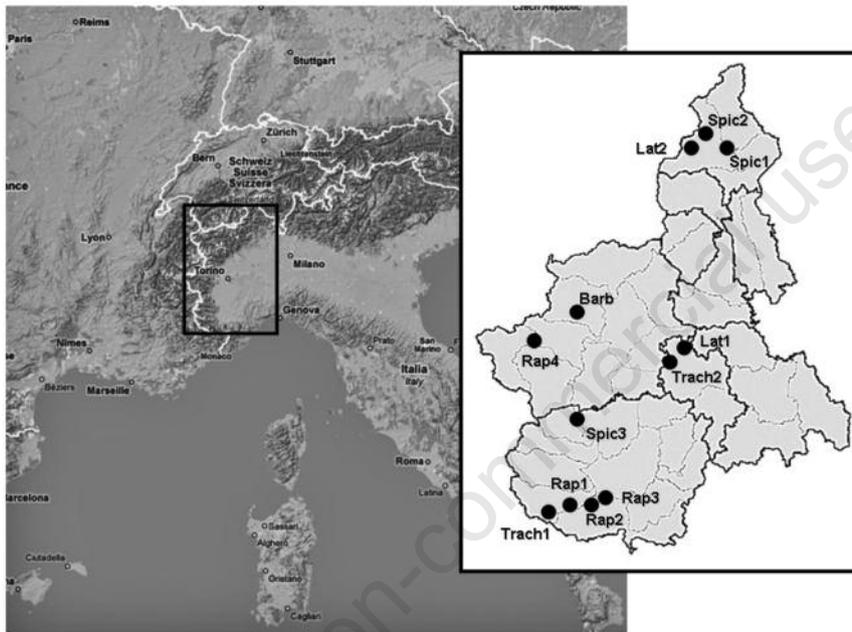


Figure 1. Sampling site distribution of the five *Campanula* species in Piedmont (Northern Italy).

areas ranged in size between 500 m and 6 km; the distance between genotypes within populations ranged between 10 cm and 10 m.

DNA extraction and molecular analyses

Total genomic DNA was extracted from freeze-dried leaves using the Dneasy Plant Mini Kit (QIAGEN, Santa Clarita, California, CA, USA) according to the manufacturer's instructions. At the end of the procedure, the columns were eluted twice with 100 μ L of AE buffer, after which they were stored at -20°C. Three different restriction enzymes (*Mse*I, *Rsa*I and *Hae*III) and 10 primers (NBS1, NBS2,

NBS3, NBS5, NBS5a, NBS6, NBS7, NBS8, GLPL6 and MYB2²³) were used in a total of 33 primer-enzyme combinations.

The NBS profiling was applied according to the protocol described by van der Linden *et al.*²³ with some modifications. Restriction digestion and adaptor ligation were performed in a single reaction by incubating 400 ng of DNA overnight at 37°C. Amplification of NBS-specific fragments was achieved through a two-step PCR as described in the original protocol.²³ The first (linear) PCR reaction (5 μ L of restriction-ligation template, 2.5 μ L of PCR buffer, 1 μ L of 5mM dNTP, 0.08 μ L of HotstarTaq polymerase-Qiagen, 2 μ L of 10 pmol/ μ L NBS-specific primer and adaptor primer in a final volume of 25 μ L)

was performed in a PTC-200 thermocycler (MJ Research) using the following thermal profile: 15 min at 95°C to activate HotStarTaq polymerase, 35 cycles with 30 s at 95°C for denaturing, 1 min and 40 s at 55°C (GLPL6, MYB2 and all the NBS primers except NBS2) or 60°C (NBS2) for annealing and 2 min at 72°C for extension and a final extension at 72°C for 20 min at the end of the cycles. The linear PCR was followed by an exponential PCR with NBS/*myb* primer and an adapter primer fluorescent labeled with IRD 700 or IRD 800 by adding to the linear PCR product 10 pmol/ μ L of each primer, 2.5 mM of dNTPs, 1 μ L Supertaq 10 X PCR buffer and 5 U/ μ L supertaq polymerase in an end volume of 10 μ L. The cycling program was performed for 15 min at 95°C and 20 cycles of 30 s at 94°C, 1 min and 40 s at 55°C or 60°C, 2 min at 72°C and a final extension at 72°C for 20 min. For visualization, 10 μ L of Li-Cor formamide with loading dye was added to the PCR products. Samples were denatured for 3 min at 95°C, cooled on ice and loaded on a 6% denaturing polyacrylamide gel using the Li-Cor IR2 Genetic Analyzer (LICOR Biosciences, Lincoln, NE USA).

Data analysis

We coded NBS and *myb* bands as present (1) or absent (0) for each plant, creating a binary data matrix.

Genetic distance were computed using the formula proposed by Nei *et al.*³² $1-2xy/x+y$, where xy is the number of shared bands by the pair and $x+y$ is the total number of bands of the pair. We performed the cluster analysis, applying Neighbor-joining, using arithmetic means³³ with the TREECON software.³⁴ By applying the same software, we estimated the statistical stability of the branches in the tree by bootstrap analysis with 1,000 replicates. Mantel analysis³⁵ was conducted by using the Mantel Non-parametric Test Calculator for Windows, version

Table 2. Number of unique banding patterns in the studied *Campanula* species obtained by means of *myb* and NBS primer-enzyme combinations. In brackets, the percentage of unique banding patterns per species is reported.

N. of Accessions	Total 67	<i>C. rapunculoides</i> 22	<i>C. latifolia</i> 9	<i>C. trachelium</i> 12	<i>C. barbata</i> 6	<i>C. spicata</i> 18
Marker						
NBS2- <i>Hae</i> III	24 (0.35)	10 (0.45)	6 (0.66)	8 (0.66)	1 (0.16)	8 (0.44)
GLPL6- <i>Mse</i> I	22 (0.32)	6 (0.27)	5 (0.55)	4 (0.33)	1 (0.16)	11 (0.61)
GLPL6- <i>Rsa</i> I	13 (0.19)	4 (0.18)	7 (0.77)	2 (0.16)	2 (0.33)	5 (0.27)
NBS profiling	17 (0.25)	8 (0.36)	4 (0.44)	8 (0.66)	1 (0.16)	5 (0.27)
MYB- <i>Mse</i> I	20 (0.29)	8 (0.36)	3 (0.33)	3 (0.25)	1 (0.16)	7 (0.38)

Table 3. Mean pairwise similarity matrix based on Nei and Li matrix between the five *Campanula* species performed on: (a) joined *myb* and NBS data, (b) *myb* data and (c) NBS data. In brackets the mean similarity values within the species.

a

Species	<i>C. rapunculoides</i>	<i>C. latifolia</i>	<i>C. trachelium</i>	<i>C. barbata</i>	<i>C. spicata</i>
<i>C. rapunculoides</i>	1 (0.568)				
<i>C. latifolia</i>	0.314	1 (0.521)			
<i>C. trachelium</i>	0.352	0.283	1 (0.510)		
<i>C. barbata</i>	0.288	0.380	0.243	1 (0.676)	
<i>C. spicata</i>	0.381	0.336	0.327	0.305	1 (0.526)

b

Species	<i>C. rapunculoides</i>	<i>C. latifolia</i>	<i>C. trachelium</i>	<i>C. barbata</i>	<i>C. spicata</i>
<i>C. rapunculoides</i>	1 (0.606)				
<i>C. latifolia</i>	0.343	1 (0.614)			
<i>C. trachelium</i>	0.382	0.295	1 (0.548)		
<i>C. barbata</i>	0.326	0.458	0.305	1 (0.761)	
<i>C. spicata</i>	0.361	0.293	0.321	0.309	1 (0.540)

c

Species	<i>C. rapunculoides</i>	<i>C. latifolia</i>	<i>C. trachelium</i>	<i>C. barbata</i>	<i>C. spicata</i>
<i>C. rapunculoides</i>	1 (0.564)				
<i>C. latifolia</i>	0.324	1 (0.424)			
<i>C. trachelium</i>	0.380	0.304	1 (0.512)		
<i>C. barbata</i>	0.309	0.339	0.291	1 (0.688)	
<i>C. spicata</i>	0.399	0.349	0.368	0.325	1 (0.509)

2.00³⁶ (<http://www.terc.csiro.au/mantel.htm>) in order to compare the matrices of the *myb* and NBS data. The significance of the statistic was evaluated by permutations (1000x) and expressed as a probability.³⁷

We performed Principal Coordinate Analysis (PCoA) based on the estimated similarities. We plotted the first two axes according to the extracted Eigen vectors, using the software package NTSYS-pc version 2.1 (Applied Bio-statistics Inc., NY, USA³⁸).

We calculated the total genetic diversity in variance components among and within populations through the Analysis of Molecular Variance (AMOVA³⁹) with 1,000 permutations using AMOVA 1.5 package software.

The program STRUCTURE 2.2.3 based on a Markov chain Monte Carlo (MCMC) algo-

rithm was used further to detect genetic stratification using the admixture model.⁴⁰ Patterns of genetic structure in the 12 populations comprised from five *Campanula* species were analyzed in the entire data set with four separate runs at each K from 6 to 14. After preliminary analyses to determine the adequate burn-in and number of MCMC Repts generations, we decided to use a burn-in period of 50,000 generations, a run length of 200,000 generations and 10 iterations at each K. We used a modal value of ΔK to assess the most likely K.⁴¹ Specifically, we classified an individual into a cluster when the assignment probability was more than 0.75. In other words, when an individual showed a color for more than three-quarters of its area, it was allocated to the correspon-

ding population. When it did not, the genotypes displayed admixture, indicating gene flow between populations or even hybridization between species.

Results

Level of polymorphism and discriminating capacity

We tested three samples from each population against thirty-three primer-enzyme combinations. The generated fingerprints were evaluated for the overall clearness of the banding patterns. We selected four primer-enzyme combinations for further screening on 67 *Campanula* samples: GLPL6-*Mse*I, GLPL6-*Rsa*I,

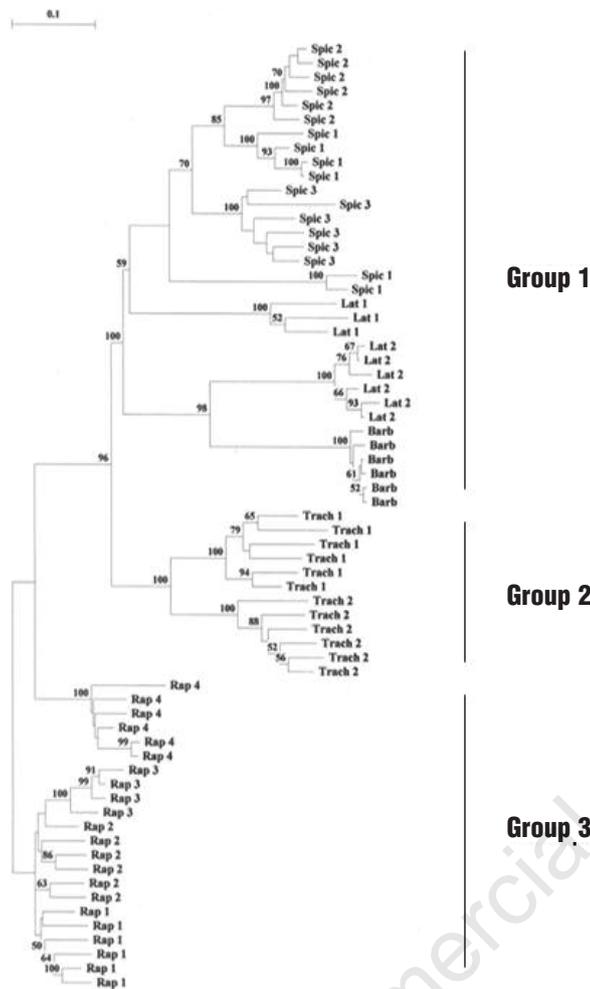


Figure 2. Consensus tree between *Campanula* populations and species for the presence/absence of the joined *myb* and NBS markers. Numbers at the internodes indicate bootstrap values from 1,000 re-sampling cycles.

NBS2-*Hae*III and MYB2-*Mse*I. We identified a total of 361 unambiguously scorable bands with the number of bands generated by each primer-enzyme combination ranging from 47 (NBS2-*Hae*III) to 131 (MYB2-*Mse*I), with an average of 90 polymorphic loci per combination.

In order to provide information on the differentiating capacity of each marker, we calculated the number of unique banding patterns per primer-enzyme combination (Table 2). In the 67 campanulas, the NBS and *myb* profiling primers produced a similar number of banding patterns, 17 and 20, respectively. Only in *C. trachelium* the number of banding patterns for NBS was more than double the value found for *myb* profiling. Among NBS markers, *C. rapunculoides* and *C. trachelium* were better discriminated by the combination of NBS2-*Hae*III while *C. latifolia* and *C. barbata* were best by GLPL6-*Rsa*I and *C. spicata* was best by GLPL6-*Mse*I.

Genetic diversity

We used several approaches (AMOVA, Principal Co-ordinate Analysis, assignment test), comparing for consanguinity, to estimate the genetic diversity among and within species.

Between species, the mean similarity values (*H*_t) obtained by the joined NBS and *myb* data sets ranged from 0.243 (*C. barbata* and *C. trachelium*) to 0.381 (*C. spicata* and *C. rapunculoides*; Table 3a). When *myb* profiling was used, the values ranged between 0.382 (*C. rapunculoides* and *C. trachelium*) and 0.293 (*C. spicata* and *C. latifolia*; Table 3b), and when NBS profiling was employed the values ranged between 0.399 (*C. spicata* and *C. rapunculoides*) and 0.291 (*C. trachelium* and *C. barbata*; Table 3c). Looking at the populations, the highest similarity values were obtained for all the molecular markers in *C. rapunculoides*

(NBS + *myb* = 0.578, *myb* = 0.618 and NBS = 0.596) while the lowest were found in *C. latifolia* (NBS + *myb* = 0.334, *myb*=0.294 and NBS=0.279).

To compare the two molecular data sets (*myb* and NBS profiling), the Mantel test was performed. By means of a permutation procedure, the correlation between the two Nei and Li similarity matrices was tested against multiple randomizations of one of them. Results showed that the data were statistically correlated ($g = 27.204$, critical value = 1.645 for $P = 0.05$, $r = 0.804$). Consequently, to improve our understanding of these new molecular markers, NBS and *myb* profiling were evaluated both joined and as single data sets.

Cluster analysis with bootstrapping was applied to analyze the genetic relationships among the 67 genotypes by means of NBS and *myb* profiling combined data sets (Figure 2). The NJ tree derived from the joined data sets divided the samples into three distinct major groups. All populations of *C. spicata*, *C. latifolia*, and *C. barbata* were clearly grouped into the same branch (Group 1) and separated according to a high bootstrap value (100%) from the second cluster comprised of the *C. trachelium* populations Trach1 and Trach2 (Group 2). Group 3, comprised of the *C. rapunculoides* genotypes, formed a cluster distinct from the other groups with very high bootstrap support (96%). The tree also revealed a clear separation among populations within species, in accordance with their geographical provenance, except for *C. spicata* in where Spic1 is scattered into two subgroups. Small differences in the clustering results, based on the *myb* and NBS data analyzed separately, were noted (trees not shown). Cluster analysis performed on the single *myb* gene profiling (data not shown) confirmed a *C. trachelium* cluster (Group 2); however, within the *C. rapunculoides* species genetic variability as high as in the NJ tree based on the combined markers (Group 3) were not revealed. The *myb* data set also showed a strong separation (100%) of Barb and Lat2 from Group 1. In the dendrogram obtained from the NBS data (data not shown), Rap1, Rap2 and Rap3 populations were intermingled, yet clustered apart from Rap4, as shown in the combined tree. Whereas, in the same dendrogram, Spic3 and Trach1 were grouped, based on high bootstrap values (96% and 99%, respectively), into the main cluster containing all samples derived from *C. spicata*, *C. barbata*, *C. latifolia* and *C. trachelium*.

To attribute the genetic variation distribution, a hierarchical AMOVA (Table 4) was performed on three sets: (a) *myb*, and (b) NBS patterns alone, and (c) combined *myb*-NBS profiling. The joined data set results showed highly significant differences ($P = 0.001$, determined from a 1,000 replication bootstrap)

Table 4. Analysis of molecular variance among and within the five species and 12 populations of *Campanula* based on *myb* and NBS combined profiles (a) and NBS (b) and *myb* (c) alone. Levels of significance were based on 1000 iteration steps (SS, sums of squares; MS, means squares; %, proportion of genetic variability; P-value, level of significance).

a

Level of variation	d.f.	SS	MS	%	P
Among species	4	1591.0	397.7	45.19	0.001
Among populations within species	7	711.4	209.3	32.54	0.001
Within populations	55	620.7	21.5	22.27	0.001

b

Level of variation	d.f.	SS	MS	%	P
Among species	4	917.7	229.4	26.55	0.001
Among populations within species	7	619.0	88.4	32.11	0.001
Within populations	55	925.1	16.8	41.34	0.001

c

Level of variation	d.f.	SS	MS	%	P
Among species	4	877.6	219.4	52.66	0.001
Among populations within species	7	319.7	45.6	29.28	0.001
Within populations	55	254.0	4.6	16.06	0.001

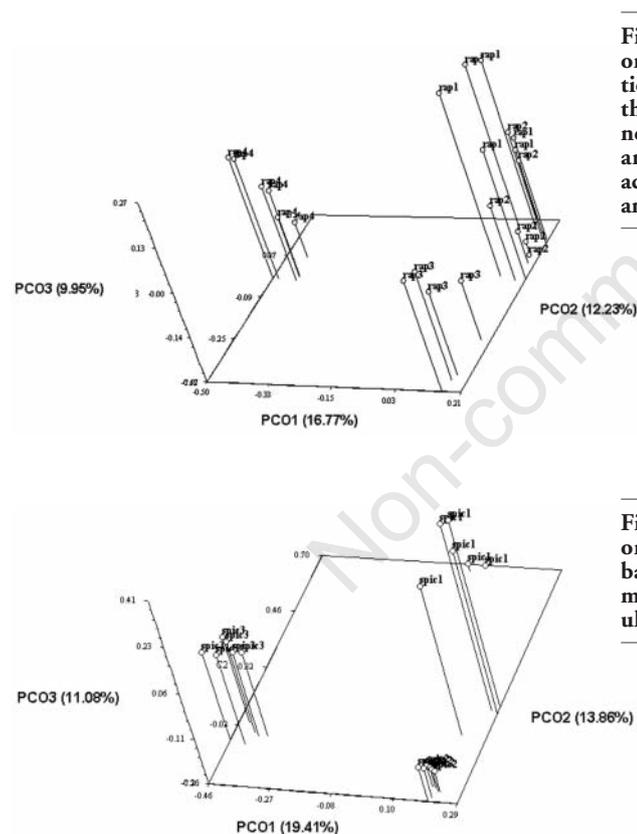


Figure 3. Principal Coordinate Analysis ordination of first, second, and third principal components of genetic similarity among *C. rapunculoides* accessions based on *myb* and NBS profiling data.

the populations, we performed a PCoA on *myb* and NBS profiling in *C. rapunculoides* and *C. spicata*, the two species represented by more than two populations (Figures 3 and 4). PCoA yielded groupings similar to the ones obtained with cluster analysis. The first three principal co-ordinates accounted for 39.00% of the variation in *C. rapunculoides* and 44.35% in *C. spicata*, and differentiated the populations according to their geographical distribution. AMOVA analysis showed (Table 5) that the genetic diversity was equally attributable to differences among and within populations (49.90% and 50.10% in *C. rapunculoides*; 50.53% and 49.47% in *C. spicata*).

Structure comparison

To investigate patterns of genetic structure, we completed a Bayesian cluster analysis using the program STRUCTURE 2.2.3. By mean of the modal value of ΔK , in agreement with Evanno,⁴¹ the number of groups was detected at $K = 9$.

Figure 5 displays the assignment probabilities of individuals in the nine inferred genetic clusters. The four populations of *C. rapunculoides* formed one structure group, Trach1 and Trach2 formed a second group, and the population of *C. barbata* formed a third. The populations of *C. latifolia* (Lat1 and Lat2) were assigned to two different structure groups. Within *C. spicata*, Spic2 and Spic3 populations, together with the 66% of Spic1, formed another group. In the Spic1 population, variable levels of admixture from an unknown population were observed, showing the possible presence of hybridization.

among the five species (45.19%), among populations within species (32.54%) and within populations (22.27%). Looking at the single molecular marker technique, by means of *myb* profiling 26.55% of the total variation was attributed to differences among species, 32.11% among populations within species and the major part of variation (41.34%) was attrib-

uted to variability within populations. By means of the three NBS primer-enzyme combinations, the 52.66% of variation was attributed to differences among species, 29.28 % among populations within species and 16.06 % within populations.

To give a more comprehensive representation of the intra-specific relationships among

Figure 4. Principal Coordinate Analysis plot based on *myb* and NBS markers among three populations of *C. spicata*.

Table 5. Analysis of molecular variance performed on the populations of *C. rapunculoides* and *C. spicata*.

Level of variation	d.f.	SS	MS	%	P
<i>C. rapunculoides</i>					
Among populations	3	212.1	70.7	49.90	0.001
Within populations	18	197.8	10.9	50.10	0.001
<i>C. spicata</i>					
Among populations	2	212.5	106.2	50.53	0.001
Within populations	15	223.6	14.9	49.47	0.001

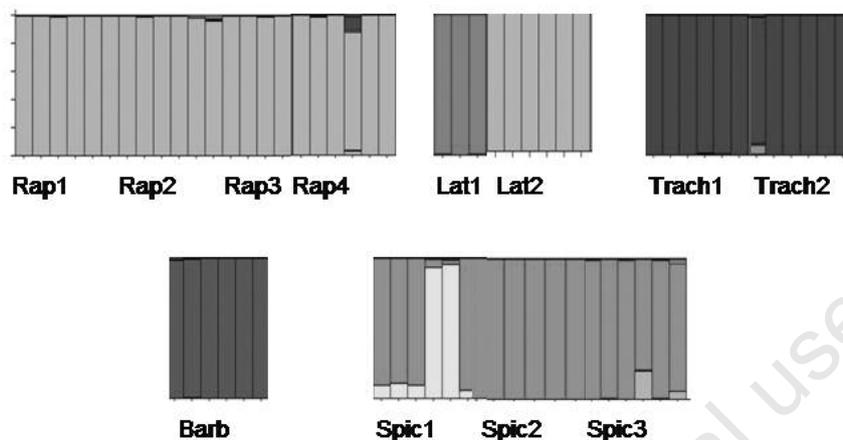


Figure 5. Estimated genetic structure for $K = 9$ obtained with STRUCTURE program from *myb* and NBS data sets. Each individual is represented by a vertical bar, which is partitioned into different colored segments that represent the individual's estimated membership fractions in nine clusters.

Discussion

The globalization of the floriculture industry is contributing to the genetic erosion process because the rapid adoption of few modern flower cultivars worldwide is rapidly replacing many traditional cultivars causing their disappearance.⁴² Most ornamental plant breeding programs are focusing primarily on flower esthetic qualities and plant architecture, resulting in an inadvertent narrowing of the genetic base of many modern cultivars. With the purpose of creating an efficient gene bank to safely store a vast number of plant genera and species, the USDA Herbaceous Ornamental Crop Germplasm Committee composed a list of priority genera of herbaceous ornamentals. The genus *Campanula* was included in the 15 most important genera for the development of the germplasm acquisition center.⁴³

In contrast to the expectation that endemic plant species are genetically depauperate, it has been demonstrated that many endemic

species maintain high levels of genetic variability compared to their widespread congeners.⁴⁴ In view of the preservation and exploitation in floriculture of new *Campanula* species with interesting ornamental values,³¹ the evaluation of the genetic diversity within the genus appeared necessary. In the present study, NBS and *myb* profiling were used for the first time for measuring genetic variation in five *Campanula* species (*C. barbata*, *C. latifolia*, *C. rapunculoides*, *C. spicata* and *C. trachelium*), widespread in all the West Italian Alps.⁴⁵ This research represents the first genetic investigation on these species and populations. The results can provide important information from both an ecological and a horticultural point of view.

To date, *myb* patterns have not been used for evaluating genetic diversity in other species while the NBS technique has been previously applied in durum wheat by Mantovani *et al.*,²⁶ in which it could discriminate closely related genotypes within a species. Overall, *myb* profiling with one enzyme – primer combination as well as NBS profiling with three different com-

binations effectively distinguished *Campanula* genotypes. The three primer – enzyme combinations showed differences in their discriminating capacity in relation to the species.

When *myb* and NBS patterns were combined, *Campanula* species were well differentiated. The average similarity (Nei and Li coefficient) across all paired comparisons between plants revealed high genetic distance both between and within species. Supported by AMOVA, the clustering (based on combined markers) efficiently grouped the populations of the same species according to their geographical locations. Results were similar for *Hypericum nummularium* L. populations in the Alps, which were not true in the Pyrenees,⁴⁶ or in *C. pseudostenocodon* in central-southern Apennines.¹⁹ Also, in *Eryngium alpestris* Schultes populations, no significant correlation was found at a geographical distance of 250 km,⁴⁷ while in a study on *Rumex nivalis* Hegetschw, a correlation was found only in a large region of Switzerland.⁴⁸

Results from cluster analysis of single data sets were less related to geographical distribution. In fact, we found that the *myb* marker was more adept at differentiating species while NBS markers were best for discriminating populations. In fact, NBS-LRR genes are responsible for the hypersensitive defence response⁴⁹ demonstrated in *Lolium perenne* L. in which several candida R-genes are co-located in chromosomal regions. This organization could lead to gene rearrangement processes in accordance with new species specificity related to new pathogens.

PCoA was applied on *C. rapunculoides* and *C. spicata* samples to highlight the variation within species. In all two species, we found that genetic connectivity among populations decreases with increasing spatial distance: a result of natural fragmentation. Indeed, as revealed in *Campanula thyrsoides* L., *Epilobium fleischeri* Hochst., and *Geum reptans* L. by Kuss *et al.*,⁵⁰ the plots showed that populations grouped without overlap agreed with their geographical location as supported by AMOVA. The PCoA plot of *C. rapunculoides* genotypes clearly demarcated the differences between the four populations. In particular, the first axis separated the populations into a Maritime Alps group (Rap1, Rap2, Rap3), mainly characterized by grassland a maximum altitude of 980 m, and a Cozie Alps group (Rap4), located in a scrub area at an altitude of 1,818 m. The two regions were approximately 100 km apart. Axis 2 differentiated populations within the Maritime Alps group and also highlighted genetic dissimilarities among populations located in nearby areas. Similarly, the plot of *C. spicata* genotypes showed that the genetic variability was closely related to the habitat and geographical distances. Axis 1 differentiated populations of different valleys

while axes 2 and 3 individualized populations collected in the same valley at different locations. Such high variability suggests that genetic connectivity between populations has a strong stochastic component at all spatial scales and that the populations are not in gene flow/drift equilibrium.⁵¹

By means of STRUCTURE software, a K value of 9 was obtained. Concerning *C. rapunculoides*, *C. trachelium* and *C. barbata* populations, all individuals of each species were assigned to the correct taxonomical group. In *C. latifolia*, Lat1 and Lat2 were allocated to two different groups (Figure 5) based on a low Nei and Li similarity value (0.334) while in *C. spicata*, the 34% of Spic1 genotypes were assigned to a completely different group. Taking into consideration that *C. latifolia* and *C. spicata* plants were sampled in a narrow area, the observed structure could be the result of fragmentation. This result confirms that the altitude associated with natural boundaries is an obstacle to gene flow, indicating that the genetic structure of plant populations reflects the interactions of various long-term evolutionary processes, such as shifts in distribution, habitat fragmentation, population isolation, mutation, genetic drift, mating system, gene flow, and selection.^{52,54}

NBS and *myb* profiling showed themselves to be new and efficient techniques for studying molecular patterns of the *Campanula* genus, previously poorly investigated. The use of neutral markers could provide the means for a more thorough understanding of the processes that have conditioned the observed genetic structure. Because of the small size of available populations, conclusions about the *Campanula* species and population genetic diversity could be a useful basis from which to develop more a comprehensive work in the future.

Conclusion

In conclusion, our results highlighted that the genetic variability at *myb* and R-gene rich regions can be useful to assess the overall genetic diversity of plants on par with microsatellites and AFLPs²⁶.

The high levels of genetic variability detected within and among *Campanula* species of the North West Alps indicate that natural fragmentation has led to a significant decline in relatedness between population pairs with increasing geographical distance, indicating the lack of a genetic bottleneck.^{55,56} The conservation of variability could be crucial for the continuous evolution of the species and their exploitation.

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