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# Characterization of Gypsophila species and commercial hybrids with nuclear whole-genome and cytoplasmic molecular markers

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ORIGINAL ARTICLE

# Characterization of *Gypsophila* species and commercial hybrids with nuclear whole-genome and cytoplasmic molecular markers

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**Abstract**
The genus *Gypsophila* contains about 150 annual and perennial flowering plant species native to the temperate regions of Europe and Asia. Nowadays *Gypsophila* species are present worldwide as garden ornamental plants. Although *Gypsophila* is one of the most economically important ornamental crops, little is known about its genetic variability and the relationships among the different wild species, cultivars, and commercial hybrids. The aim of our work was to analyze genetic distances among 5 wild species and 13 commercial hybrids of *Gypsophila* with similar phenotypes but unknown origin. For this purpose, we have used amplified fragment length polymorphism, target region amplification polymorphism, and inter simple sequence repeat whole-genome markers and chloroplast simple sequence repeat (cpSSR), targeting chloroplast DNA. Nuclear markers were found to distinguish all the analyzed samples while cpSSR markers were found to discriminate the different wild species, but could not sufficiently separate the commercial hybrids. This notwithstanding, the data obtained allowed us to cluster the commercial hybrids into different sub-groups and to determine the relationships with the putative species of origin.

**Keywords:** *AFLP, cpSSR, genetic analysis,* Gypsophila*, ISSR, TRAP*

**Abbreviations:** AFLP, amplified fragment length polymorphism; cpSSR, chloroplast simple sequence repeat; ISSR, inter simple sequence repeat; RAPD, random amplified polymorphic DNA; TRAP, target region amplification polymorphism

## Introduction

The genus *Gypsophila* L. belongs to the family *Caryophyllaceae* and includes about 150 species. It is native to the temperate regions of Asia and Europe but a few species have been also found in North-East Africa, Australia, and North America. Most of the *Gypsophila* species have a very limited geographic distribution in an area encompassing Turkey, Caucasia, northern Iraq, and northern Iran, this last being one of the main diversification center of the genus (Barkoudah 1962). Eighteen species have been reported from China, mostly distributed in North-West, with the number of species gradually decreasing eastwards (Dequan 1994). Nowadays, *Gypsophila* is present worldwide, primarily occurring in temperate mid-latitude (30°–60° North) regions with some northern and southern outposts.

The genus includes annual, biennial, and perennial species, several of which are economically important being used in medicine or as ornamentals, often as cut flower plants. In particular, *G. paniculata* L., known as "Baby's breath", is a perennial herbaceous plant, and it is present worldwide being often grown commercially as an annual crop. It is the most important species used in commercial cut flower production (Zvi et al. 2008) and is also the main source of germplasm of commercial varieties. However, the flowers of commercial *G. paniculata* plants are sterile and do not produce seeds. Therefore, breeding programs are severely restricted as shown by the very low number of cultivars obtained (Shillo 1985). Because of the widespread male sterility, new varieties are obtained artificially from wild species through *in vitro* vegetative propagation and selection of clonal variants. The most common methods used for plant production are micropropagation from shoot-tips explants (Han et al. 1991; Lee & Bae 1999; Rady 2006) or regeneration from callus and cell suspension cultures (Salman 2002), leaf explants (Zuker et al. 1997), stem segments

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(Ahroni et al. 1997), and more recently, induced mutagenesis with gamma irradiation and collection of lateral buds (Barakat & El-Sammak 2011) and by using bioreactors (Wang et al. 2013). Moreover, induction systems for *in vitro* flower production have been developed (Rady 2006; Kanchanapoom et al. 2011). Conventional breeding is mainly based on open pollination of wild plants. In patent releases of new varieties, the name of the parental lines can be also reported only as *Gypsophila* plants without any further specification of the genotype used in the cross (see for instance US PP 21041P2, US PP 242240 P2, Esmeralda Breeding B.V., Aalsmeer, Holland, the Netherlands). New cultivars developed through selection from crosses in controlled breeding programs are asexually propagated by removing vegetative cuttings or through tissue culture techniques. As, sometimes, even in controlled breeding programs the origin of one/ or both parent/s is unknown, estimates of genetic relationships between species and cultivars are essential in order to maintain and enlarge the genetic diversity of the breeding material and to select new, improved cultivars.

Historically, morphological parameters and quality data have been studied to determine genetic diversity and to identify cultivars, especially in ornamental plants (Linde et al. 2007; Carovic-Stanko et al. 2011; Santos et al. 2011). Now a major advantage is given by employing DNA markers. Molecular markers are now employed in plant diversity assessment, in crop improvement, in phylogenetic and systematic studies, in conservation biology, in molecular ecology and developmental biology, as well as in forensic analysis (for a review, see Borzatti Von Loewenstern et al. 2013; Malik et al 2013; Parida et al. 2013; Poczai et al. 2013; Bolger et al. 2014; Techen et al. 2014; Tonnabel et al. 2014; van Zonneveld et al. 2014).

In ornamental plants molecular markers have shown to be an extremely efficient tool both for genetic characterization and variety protection (Arús 2000; Dendauw et al. 2001; Mata et al. 2009; Braglia et al. 2011; Gupta et al. 2013; Mahmood et al. 2013). Despite the fact that the *Gypsophila* genus is one of the 20 economically most important ornamental plants worldwide, few studies of genetic variation have been focused on *Gypsophila* cultivars and their wild ancestors. To our knowledge only random amplified polymorphic analysis has been used to characterize the propagated plants derived from *in vitro* culture of *G. paniculata* (Rady 2006; Barakat & El-Sammak 2011) and the amplified fragment length polymorphism (AFLP) fingerprinting technique has been used to characterize *G. fastigiata* (Lachmayer 2009).

The aim of our study was to analyze using DNA molecular markers the genetic relationships among 13 commercial hybrids of *Gypsophila*, widely present in the market, and to compare them with a number of wild species with different geographical origin. The species chosen were *G. paniculata* from Europe, *G. pacifica* and *G. oldhamiana*, both coming from Northern China (Wang 2005; Luo et al. 2008), *G. libanotica* coming from Turkey, Syria and Lebanon (Kotschy 1864), and *G. repens*, a long-lived perennial species distributed in the mountains of southern and central Europe (Blamey & Grey-Wilson 1989). To investigate the origin and the genetic differentiation between species and varieties, we used a set of molecular markers and compared the results obtained with different techniques, namely inter simple sequence repeats (ISSRs; Zietkiewicz et al. 1994), target region amplification polymorphism (TRAP; Hu & Vick 2003), AFLP (Vos et al. 1995) and chloroplast microsatellites (cpSSR; Powell et al. 1995).

## Materials and methods

### *Plant material and DNA extraction*

The plant material used in this work (Table I) was obtained from different sources. Seeds of *Gypsophila* wild species were obtained from different market companies in Italy, sown in pots containing a mixture of peat and perlite (1:1) and incubated under standard conditions at $25 \pm 1$°C in a growth chamber for 1 month. *In vitro* micropropagated plantlets of commercial hybrids were kindly provided by Azienda Agricola Meristema S.r.l. (Cascine di Buti, Pisa, Italy).

For DNA isolation, 200–300 mg of fresh leaf material from a pool of at least 10 individual plantlets from each species or variety were ground to powder with pestle and mortar in liquid nitrogen. Total genomic DNA was extracted by using the Plant Genomic DNA Kit (Macherey Nagel, GmbH & Co., KG, Düren, Germany) and stored at −20°C for further use. The quality of the extracted DNA was checked on 1% Tris-borate-EDTA (TBE)-agarose gels stained with ethidium bromide, and the concentration of DNA was estimated using the Qubit® 2.0 Fluorometer (Invitrogen by Life Technologies, Monza, Italy).

### *PCR amplifications*

ISSR analysis was performed in 25 µl reaction mixture containing 20 ng template DNA, 1 U Dream Taq polymerase (Fermentas, Thermo Scientific, Milan, Italy ), 200 µM of each dNTP, 0.5 µM ISSR primer (Biotechnology Laboratories, University of British-Columbia, Vancouver, Canada; Wolfe Laboratory, The Ohio State University, Columbus, Ohio, USA) and 1× Taq polymerase buffer. The reaction mixtures were denatured at 94°C for 7 min

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Table I. Plant material.

| Commercial varieties | Breeder | Flowers | Stems |
|---|---|---|---|
| Million Stars® | Danziger, IL | White/small semidouble blooms | Erect/flexible/65–70 cm |
| Bambino[a] | Danziger, IL | like Million Stars® | Like Million Stars® |
| New Love® | Danziger, IL | White/medium-large semidouble blooms | Erect/strong/60–65–70 cm |
| Perfecta | Danziger, IL | White/large double blooms | Erect/strong/90 cm |
| Blancanieves® | Astee Flowers-BV, NL | White/small blooms | Erect/strong/105 cm |
| Inbal | Miyoshi & Co., J | White | – |
| Mirabella | Miyoshi & Co., J | White/medium-sized blooms | Erect/strong/65–70–75 cm |
| MeriG® | Meristema Srl, I | White/medium-sized semidouble blooms | Erect/strong/90 cm |
| Perfecta Marzano | Meristema Srl, I | White/large double blooms | Erect/strong/90 cm |
| Perfecta Marzano OP[b] | Meristema Srl, I | White/large double blooms | Erect/strong/90 cm |
| Vittoria® | Meristema Srl, I | White/small extradouble blooms | Erect/strong/90 cm |
| Flocon de Neige Double | B & T Seed Co., F | White | – |
| Wild species[c] | Geographical diffusion | Flowers | Stems |
| *G. paniculata* L. | C. Europe, E. Europe | White/rarely light purplish pink | Erect/thick/90–120 cm |
| *G. libanotica* Boiss. | Turkey, Lebanon, Siria | White/pink | Erect/40 cm |
| *G. pacifica* Kom. | Manchuria, Siberia, C. Asia | Pale rose/purple | Erect/90 cm |
| *G. oldhamiana* Miq. | E. Asia—China, Japan, Korea, Manchuria. | Pink | Erect/100–120 cm |
| *G. repens* L. | Europe Alps | Lilac-pink to white | Creeping/15 cm |

[a] EDV (essentially derived variety) of Million Stars®. [b] OP, open pollinated. [c] http://www.rockgardener.com/harkness/seedlist.cfm?Genus=Gypsophila.

and subjected to 45 cycles of 30 s at 94°C, 45 s at 52°C, 2 min at 72°C, with a final extension step of 10 min at 72°C. Of the 20 ISSR primers tested in this study 11 were used in the final analysis (Table II). ISSR amplification products were then visualized through gel electrophoresis on 3% TBE-NuSieve gel-ethidium bromide and run overnight at 40 V. The presence or absence of different bands was scored visually, and only distinct, well-resolved, and consistently reproducible bands were considered in the analysis and used to construct a presence/absence binary matrix.

AFLP fingerprinting was performed using the AFLP Ligation and Preselective Amplification Module for regular plant genomes (Life Technologies) following the manufacturer's instructions. One hundred nanogram of genomic DNA were digested with 5 U of the restriction endonuclease *Mse* I and 1 U of *Eco* RI (New England Biolabs, Inc., Ipswich, MA, USA), and ligated with T4 DNA-Ligase (Promega Italia, Milan, Italy) to double-stranded adapters, in a final volume of 11 µl. Incubation of the restriction-ligation reactions was performed overnight at 37°C. Pre-amplification and selective amplification reactions were performed according to the AFLP Plant Mapping Protocol. Six pairs of *Eco* RI/*Mse* I primer combinations with three selective nucleotides at the 3′ end were used in our study (Table II).

TRAP assays were performed, as described in Hu et al. (2007), in a final reaction volume of 15 µl containing 1 U Dream Taq polymerase (Fermentas), 200 µM of each dNTP, 10 pmol of the fixed primer, 1 pmol of the arbitrary primer, 2.5 mM $MgCl_2$, 1 × Taq reaction buffer, and 80 ng template DNA. Following Hu et al. (2007), we used as fixed primers the ones designed on *Arabidopsis thaliana* telomeric regions and Ga3, Ga5, Odd26, and Sa12 as arbitrary primers. Arbitrary primers were labeled at 5′ end with FAM or HEX dyes (Eurofins MWG-Operon, Ebersberg, Germany; Table II).

For cpSSR analysis, 12 plastome-derived microsatellite loci were analyzed by using 7 consensus primer pairs (consensus chloroplast microsatellites primers, ccmp) designed by Weising and Gardner (1999) and 5 (consensus chloroplast Simple Sequence Repeats, ccSSR) designed by Chung and Staub (2003). Only 10 of the 12 primer pairs gave amplification products and were chosen for the analysis (Table III). The polymerase chain reaction (PCR), carried out in a final volume of 25 µl, contained 10 ng total DNA, 1 U Dream Taq polymerase (Fermentas), 1 × Taq buffer, 160 µM each dNTP and 5 pmol each primer. The reaction mixtures were denatured at 94°C for 5 min and subjected to 30 cycles of 1 min at 94°C, 1 min at each primer-specific annealing temperature (see Table III), 1 min at 72°C and to a final extension step of 10 min at 72°C. Total 5 µl of each PCR products were run on 1% agarose gel and, on the basis of each signal intensity, 0.1–0.2 µl of amplified DNA were used for capillary electrophoresis. To this aim, one of each primer was 5′ labeled with different dyes, FAM, HEX, and TET.

All AFLP, TRAP, and cpSSR samples were run on an ABI™ 310 DNA Sequencer (Applied Biosystems Italia, Monza, Italy) and detection time, signal peak height and surface for each fragment were estimated using GeneScan® Analysis software package (Applied Biosystems). The sizes of the amplified

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Table II. Levels of polymorphism obtained with ISSR, AFLP, and TRAP markers in the *Gypsophila* examined genotypes.

| Primer | Total bands | Polymorphic bands | Polymorphism (%) | Unic bands |
|---|---|---|---|---|
| **ISSR** | | | | |
| ISSR[a]12 | 28 | 28 | 100 | 3 |
| MAO[a] | 39 | 39 | 100 | 5 |
| UBC[b] 808 | 25 | 25 | 100 | 0 |
| UBC[b] 823 | 20 | 20 | 100 | 2 |
| UBC[b] 827 | 25 | 25 | 100 | 0 |
| UBC[b] 825 | 18 | 18 | 100 | 1 |
| UBC[b] 843 | 9 | 8 | 88.9 | 1 |
| UBC[b] 845 | 17 | 17 | 100 | 0 |
| UBC[b] 848 | 23 | 22 | 95.6 | 1 |
| UBC[b] 850 | 25 | 22 | 88 | 1 |
| UBC[b] 881 | 12 | 8 | 66.7 | 0 |
| Total | 241 | 232 | 96.3 | |
| **AFLP** | | | | |
| *Eco* RI-AAC$^{(NED)}$/*Mse* I-CTA | 107 | 93 | 86.9 | 8 |
| *Eco* RI-ACA$^{(FAM)}$/*Mse* I-CAA | 112 | 40 | 35.7 | 10 |
| *Eco* RI-ACT$^{(FAM)}$/*Mse* I-CAT | 115 | 92 | 80 | 17 |
| *Eco* RI-ACT$^{(FAM)}$/*Mse* I-CAC | 114 | 83 | 72.8 | 19 |
| *Eco* RI-ACC$^{(NED)}$/*Mse* I-CTT | 119 | 106 | 89.1 | 18 |
| *Eco* RI-AAC$^{(NED)}$/*Mse* I-CAG | 110 | 97 | 88.2 | 32 |
| *Eco* RI-AGC$^{(NED)}$/*Mse* I-CAT | 77 | 61 | 79.2 | 10 |
| Total | 754 | 572 | 75.9 | |
| **TRAP** | | | | |
| TeloRD/Ga3[c] | 101 | 101 | 100 | 22 |
| TeloRD/Ga5[c] | 102 | 100 | 98 | 22 |
| TeloRD/Odd2 6[c] | 58 | 56 | 96.55 | 18 |
| TeloRD/Sa12[c] | 70 | 69 | 98.6 | 6 |
| TeloTRG/Ga3[c] | 49 | 42 | 85.7 | 8 |
| TeloTRG/Ga5[c] | 80 | 48 | 60 | 15 |
| TeloTRG/Sa12[c] | 99 | 98 | 99 | 22 |
| Total | 558 | 514 | 92.1 | |

[a] http://www.biosci.ohaio-state.edu/awolfe/ISSR/ISSR.html. [b] UBC primer set 9, Biotechnology Laboratory, The University of British Columbia, Canada. [c] Hu et al. (2007).

fragments detected ranged between 50 and 500 bp. In the case of cpSSR, amplification reactions that produced only one selective fragment for each sample were previously pooled providing that the size of the amplicons were sufficiently wide apart and labeled with different primers so that they were analyzed with only two runs for sample. Sizing of the fragments was performed with the GeneMapper software version 4.0 (Applied Biosystems) that was used to assign each fragment to the corresponding category (i.e., presence or absence of the marker) and to generate a scoring table (1/0) for each sample.

Table III. Levels of polymorphism detected at cpSSR loci in *Gypsophila* species and commercial hybrids.

| Primer | Source | Ta (°C) | Repeat motif | PCR product (bp) | Number of alleles |
|---|---|---|---|---|---|
| ccmp1$^{FAM}$ | Weising and Gardner (1999) | 64 | $T_{10}$ | 127 | 1 |
| ccmp3 $^{FAM}$ | Weising and Gardner (1999) | 58 | $T_{11}$ | 78 | 1 |
| ccmp5$^{HEX}$ | Weising and Gardner (1999) | 58 | $C_7T_{10}$ | 94 | 1 |
| ccmp6 $^{HEX}$ | Weising and Gardner (1999) | 54 | $T_5(CT)_{17}$ | 108–(–)[a] | 2 |
| ccmp7$^{TET}$ | Weising and Gardner (1999) | 54 | $A_{13}$ | 131–139 | 2 |
| ccmp8$^{HEX}$ | Weising and Gardner (1999) | – | $(T)_6C(T)_{14}$ | (–) | – |
| ccmp9$^{FAM}$ | Weising and Gardner (1999) | – | $(T)_{11}$ | (–) | – |
| ccssr4$^{TET}$ | Chung and Staub (2003) | 64 | $T_8$ | 208–211–215–217 | 4 |
| ccssr9$^{TET}$ | Chung and Staub (2003) | 58 | $A_{13}$ | 196–200 | 2 |
| ccssr21$^{FAM}$ | Chung and Staub (2003) | 62 | $T_{13}$ | 278 | 1 |
| ccssr22$^{HEX}$ | Chung and Staub (2003) | 58 | $T_6$ | 185 | 1 |
| ccssr23$^{FAM}$ | Chung and Staub (2003) | 68 | $A_{14}$ | 366 | 1 |

[a] (–), absence of the band.

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All PCR reactions were carried out on a MJ Research PTC-200 Thermal Cycler (Biorad Italia, Milan, Italy).

*Data analysis*

All data were analyzed using the computer package NTSYSpc version 2.02 (Rohlf 1997). For each of the four molecular assays, pair-wise comparisons of all samples analyzed, based on the presence or absence of unique and shared amplification products, were used to determine the similarity matrices. The Jaccard's (1908) similarity coefficient, and Simple Matching coefficient (Sokal & Michener 1958) were calculated by using the SIMQUAL (similarity for qualitative data) module on each matrix. The similarity coefficients were then used to construct dendrograms, by using the unweighted pair group method with arithmetic averages (UPGMA; Sokal & Michener 1958) employing the SAHN (sequential, agglomerative, hierarchical and nested clustering) routine. To find the robustness of the cluster analyses, for each dendrogram, we produced the matrix of cophenetic values by using the co-phenetic routine. Then, we compared it to the corresponding similarity matrices by computing their product-moment correlation ($r$) and applying the Mantel test statistic ($Z$) (Mantel 1967) to find the significance of the observed correlation. Reliability of each UPGMA clustering was tested also by bootstrap analyses with 1000 replications by using the PAST software package (Hammer et al. 2001). A principal component analysis (PCA) was performed with the same software on each data matrix to further support the clustering. Finally, in order to draw a consensus dendrogram from patterns of all three whole-genome nuclear marker techniques used, we have pooled an equal amount of markers (200 per technique) inferring a consensus tree by using a bootstrapping procedure. In particular, 100 matrices were generated by random sampling 200 marker data per technique, and a consensus tree was obtained from them by applying the majority rule consensus method in the CONSEN module of the NTSYSpc software. We have chosen the confidential value of 200 marker data because the number of 150–200 bands is considered high enough to provide accurate estimates of genetic distance (Pejic et al. 1998; Garcia et al. 2004).

## Results

*Levels of polymorphism: Nuclear and cpSSR markers analysis*

The 17 *Gypsophila* genotypes studied in our work (Table I) were analyzed with different marker systems: three nuclear molecular markers producing a fingerprinting DNA pattern (namely ISSR, AFLP, and TRAP); and cpSSRs markers with plastid inheritance.

A total of 20 ISSR primers were screened, and 11 of them, all giving reproducible and clearly identifiable bands were used in further PCR analyses (Table II). Some amplification profiles, reported as an example, are displayed in Figure 1. The 11 primers yielded total 241 scorable bands with an average of 22 bands per primer. The number of amplified DNA fragments ranged from 9 for UBC 843 to 39 for MAO, the most informative primer. Fragment sizes ranged from 100 to 3000 bp; 232 of 241 bands (96.3%) were polymorphic across 17 genotypes (Table II).

The AFLP–PCR products amplified by six pairs of fluorescent-*Eco* RI/*Mse* I primer combinations from 17 DNA samples yielded total 754 AFLP markers with an average of 126 bands per primer (Table II). Among these fragments, 572 were polymorphic across genotypes indicating a total polymorphic rate of 75.9%. A maximum of 132 scorable fragments was detected with the *Eco* RI-ACA/*Mse* I-CAG primer pair and a minimum of 62 fragments with the *Eco* RI-AGC/*Mse* I-CAG primer pair. The percentages of polymorphic fragments per each primer pairs ranged from 35.7% to 89.1%.

Seven primer combinations were used for TRAP–PCR analysis, which yielded total 558 products; 514 fragments were found to be polymorphic, showing a high percentage of polymorphism (92.1%) among the 17 examined genotypes. The number of amplified DNA fragments ranged from 42 to 101, depending on the primer and the DNA sample, with a mean value of approximately 80 bands per primer (Table II).

As for cpSSR, 12 universal chloroplast microsatellite primer pairs were tested on all DNA samples. Sizes of the amplified products obtained for each genotype with each primer are shown in Table III. All primer combinations, with the exception of ccmp8 and ccmp9, underwent amplification. Monomorphic alleles were detected with the ccmp1, ccmp3, ccmp5, ccmp6, ccssr21, ccssr22, ccssr23 primer pairs, while no amplification was detected with ccmp6 primer pairs in *G. oldhamiana*. Two polymorphic alleles were observed at the ccmp7 and ccssr9 loci and four polymorphic alleles at the ccssr4 locus. Overall, four different chlorotypes were found, two of which only in wild species. Moreover, most commercial hybrids showed the same haplotype shared with *G. repens*. A comparative analysis of the level of polymorphism obtained with the different techniques showed that the highest number of amplicons was evidenced by AFLPs (754) followed by TRAP (558) and ISSR (241). On the other hand, the highest percentage of polymorphism was revealed by ISSR markers

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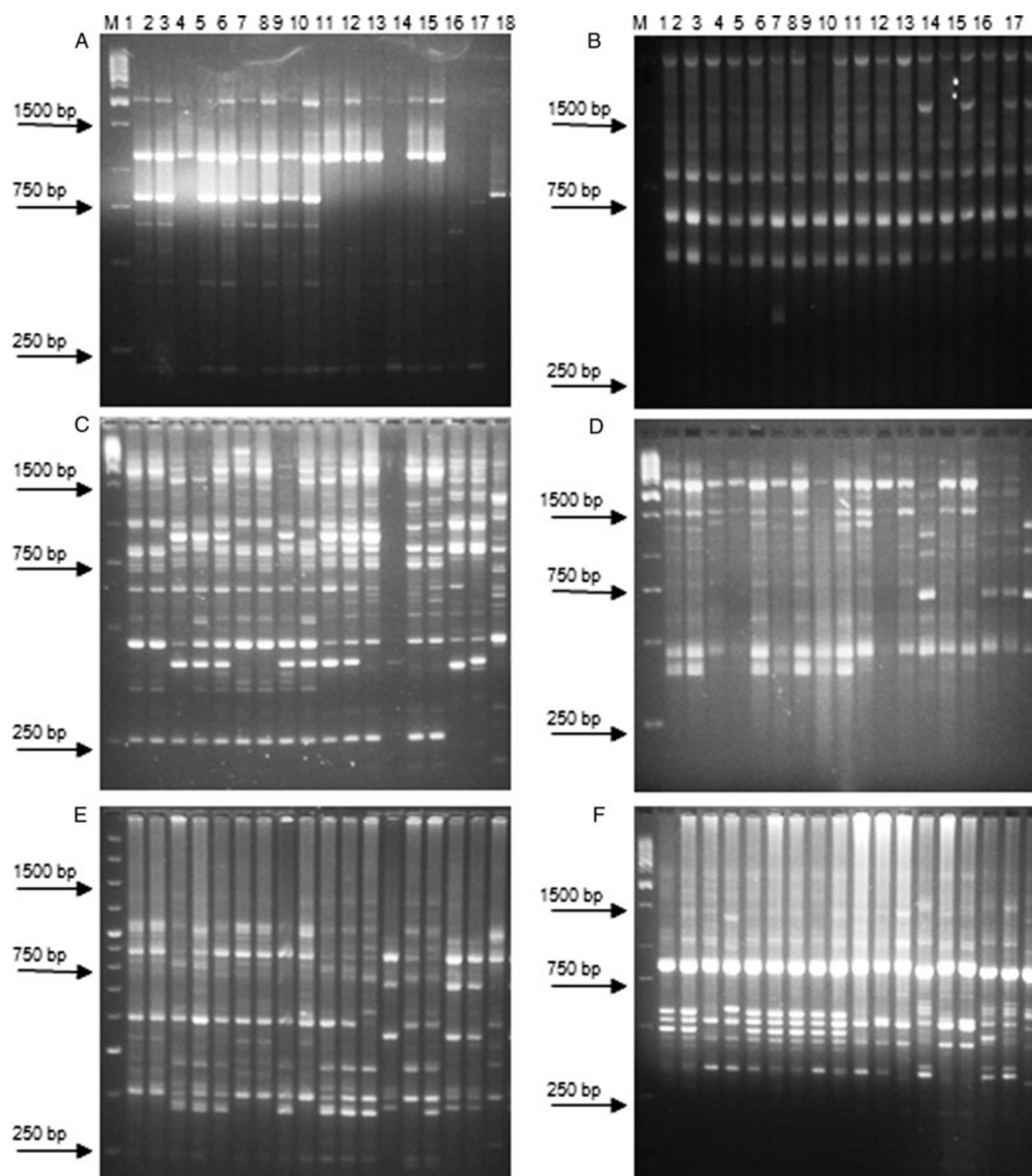


Figure 1. Examples of ISSR fingerprinting generated by UBC 843 primer (A), UBC 881 primer (B), UBC 850 primer (C), UBC 854 primer (D), MAO primer (E), and UBC 848 primer (F) in the *Gypsophila* analyzed genotypes.

(96.3%) as compared to TRAP (92.1%) and AFLP (75.9%; Table II). Chloroplast microsatellites showed, as expected, the lowest level of genetic diversity (Table III).

## Genetic similarity analysis

Similarity matrices were constructed between pairs of *Gypsophila* genotypes based on shared amplification products for each marker type. The Jaccard's coefficient (Jaccard 1908) was used for nuclear markers, whereas in the case of cpSSRs, the Simple Matching coefficient was chosen (Sokal & Michener 1958). Similarity indexes (SIs) ranged from 0.14 to 0.95 suggesting a great variability among the analyzed accessions (data not shown). The largest distances (SI = 0.23; 0.14) were observed between the commercial hybrid Bambino and the wild species *G. repens* with AFLP and TRAP markers, and between Bambino and the wild species *G. oldhamiana* and *G. pacifica* with ISSR (SI = 0.31; 0.32). The smallest distance values were between the commercial hybrids Bambino and Million Star (SI = 0.75; 0.78) as shown with AFLPs and TRAPs and between Bambino and Blancanieves (SI = 0.95) with ISSR. Two major clades were evidenced in the UPGMA trees based on ISSR (Figure 2(A)), AFLP (Figure 2(B)), and TRAP (Figure 2(C)) similarity matrices, one always grouping the wild species *G. pacifica* and *G. oldhamiana*, the other one containing most commercial hybrids together with *G. libanotica* and *G. paniculata*, the elite

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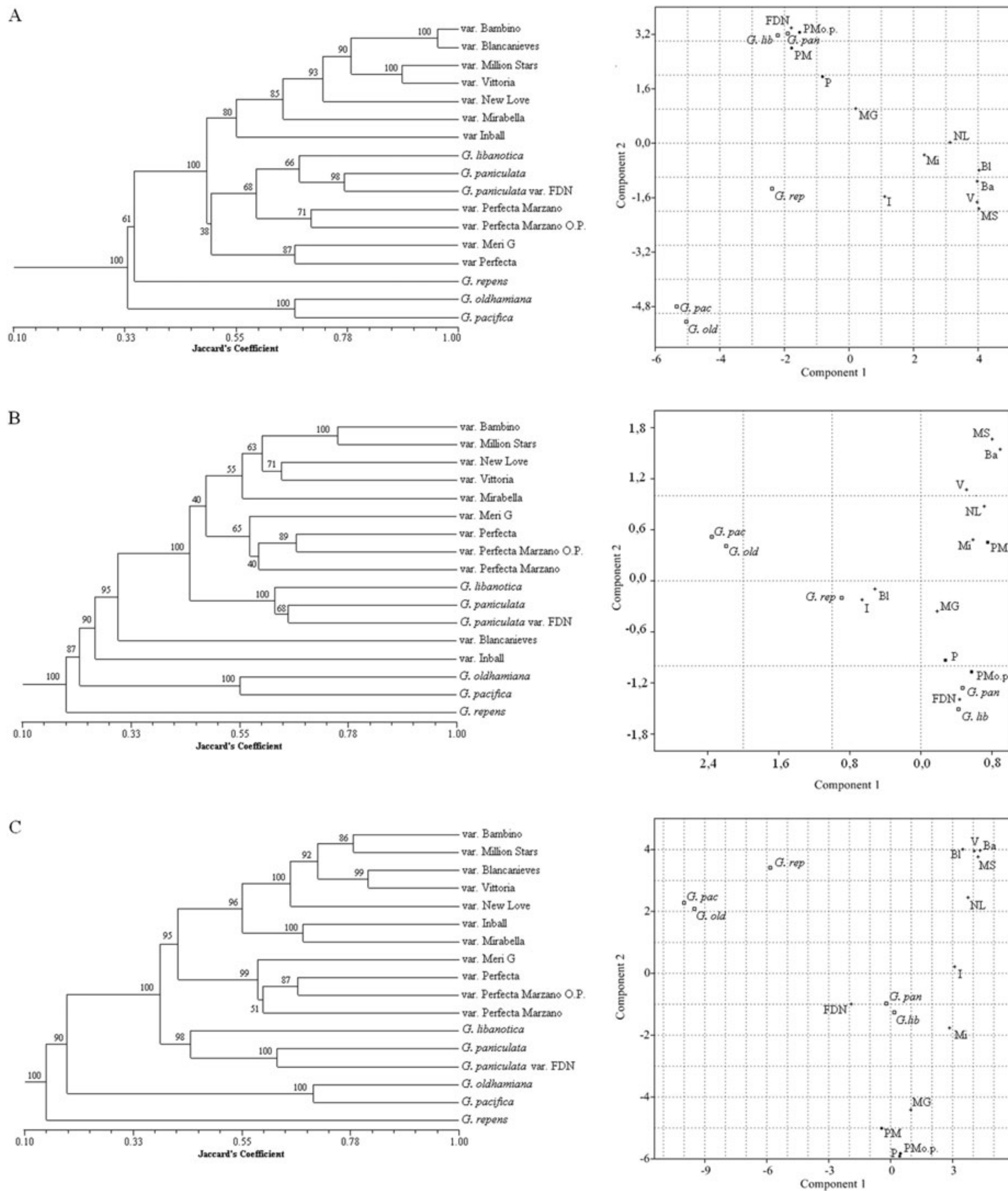


Figure 2. Dendrograms resulting from UPGMA analysis based on Jaccard's similarity coefficient (left) and PCA plots (right) in *Gypsophila* genotypes obtained by using ISSR (A), AFLP (B), and TRAP (C). The correlation *r* between the cophenetic and the similarity matrices were $r \geq 0.97$ for AFLP, $r \geq 0.97$ for TRAP, and $r \geq 0.96$, respectively, for ISSR data.

breeding species according to the extant literature. *G. repens* generally behaved as an out-group as shown by TRAP and AFLP dendrograms. Clustering analysis was supported by high bootstrapping values and was validated by PCA analysis (Figure 2(A)–(C)). The significance of the resulting dendrograms was verified calculating the cophenetic correlation *r* (Mantel 1967) whose values were highly significant ($r \geq 0.96$) for each marker system.

A consensus tree obtained by the pooled data from each nuclear marker is shown in Figure 3. The combined analysis generated a dendrogram that clearly distinguishes the 17 genotypes.

Finally, the clustering pattern for cpSSR profiles obtained using the Simple Matching coefficient method is reported in Figure 4. Despite the very low level of genetic diversity, cluster analysis showed also in this case that commercial hybrids are likely to

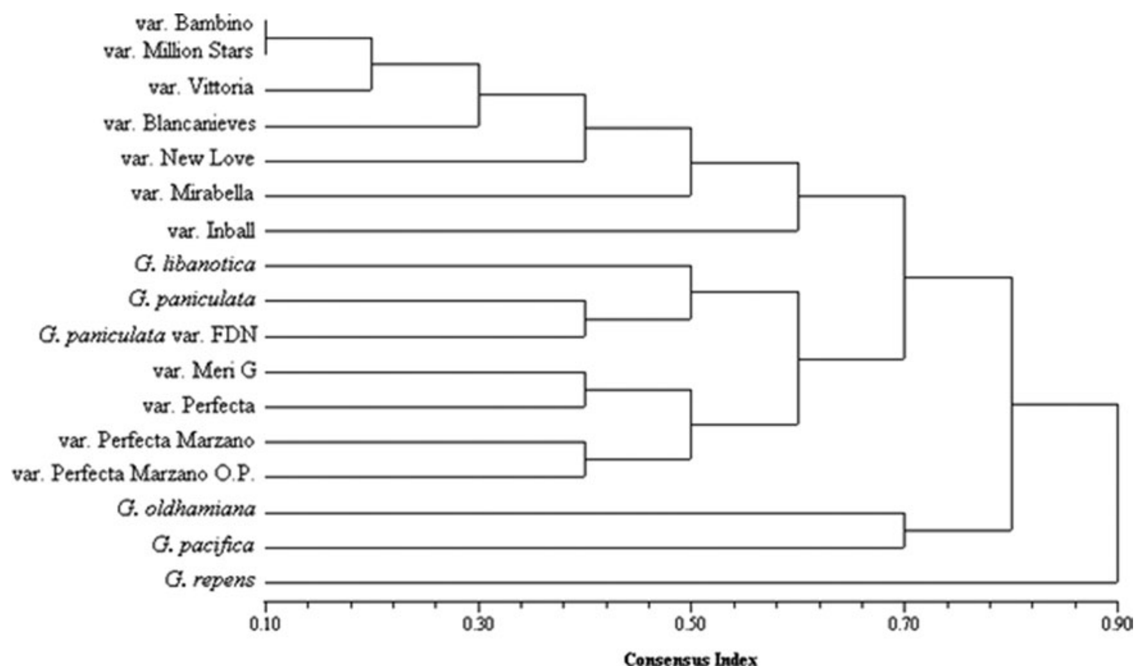


Figure 3. Consensus tree in *Gypsophila* commercial hybrids and wild species calculated using combined molecular markers data.

group in two main separate clusters, one comprising the Perfecta group and the species *G. paniculata* and *G. libanotica*, while the other commercial varieties form a different cluster with *G. repens*. In accordance with nuclear markers, *G. oldhamiana* and *G. pacifica* were closely associated.

## Discussion

In this article, we reported a preliminary assessment of genetic diversity among species and hybrids of the genus *Gypsophila* using molecular markers. The aim of our work was to get further insight into the genetic diversity and relatedness of a number of *Gypsophila* commercial varieties known to show very little variation (Zuker et al. 1997) thus putatively obtaining reliable data on the now unknown origin of most commercial hybrids. The characterization of the genetic structure of *Gypsophila* may in our opinion be very useful for the construction of breeding strategies yielding the selection and the introduction in the market of new attractive varieties. Four different types of molecular markers highly informative and reproducible, ISSR, TRAP, AFLP,

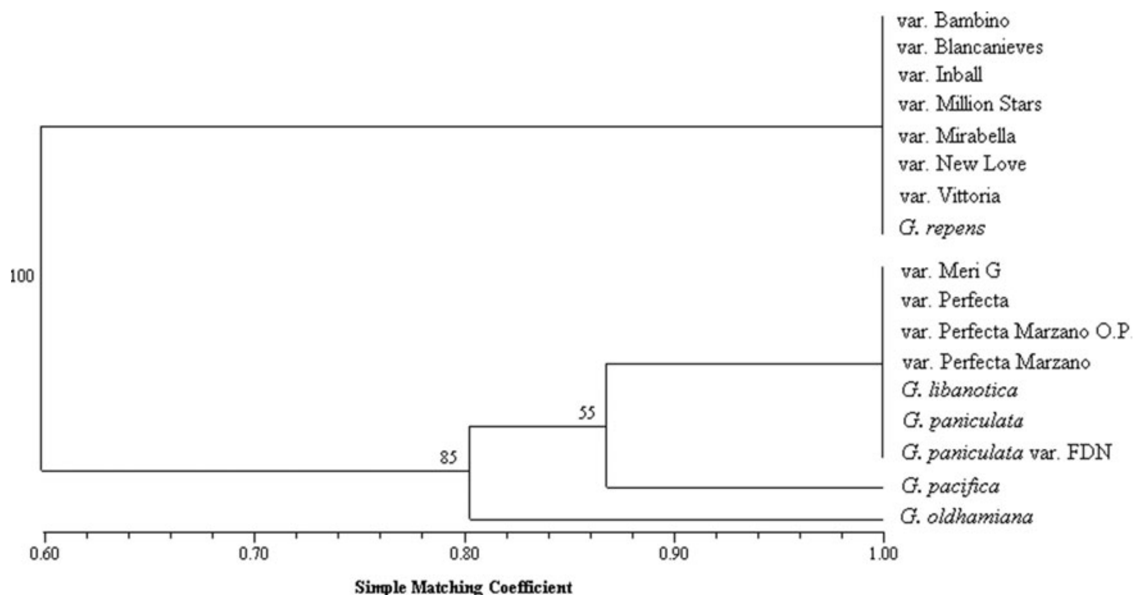


Figure 4. Dendrogram showing UPGMA clustering based on Simple Matching similarity coefficient calculated using cpSSR analysis.

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and chloroplast microsatellites were used to obtain amplification profiles of 13 different cultivars and 5 species belonging to the genus *Gypsophila*. Up to now, in our knowledge, these markers have never been used for the genetic characterization of species and hybrids of *Gypsophila*. The three nuclear marker systems were used to fingerprint the accessions studied, while cpSSRs, targeted the more conservative chloroplast genome relevant for the study of maternal inheritance and produced, as expected, fewer but informative amplicons. Nuclear markers allowed the characterization of all the analyzed samples while cpSSR markers were found to discriminate the different wild species, but could not sufficiently distinguish the commercial hybrids. Overall, the data obtained allowed us to cluster the commercial hybrids into different sub-groups and to determine the relationships with the putative species of origin. As expected, the discrimination power of different markers varied according to the marker used. In particular, results obtained with AFLP (Vos et al. 1995) showed the highest number of PCR products as these markers allowed covering a large proportion of the genome, but the level of polymorphism was lower than that obtained with TRAPs and ISSRs. The level of polymorphism obtained with TRAPs (Hu & Vick 2003) was higher, in spite of the lower number of amplicons produced. On the other hand, ISSRs (Zietkiewicz et al. 1994) using oligonucleotides based on SSR motifs found ubiquitously in plant genomes, were able to detect in *Gypsophila* even fewer loci characterized, however, by the highest level of variation.

To complement the information obtained from nuclear genetic markers, we also used chloroplast microsatellites, also known as cpSSRs (Powell et al. 1995). With the 12 primers used only 4 chlorotypes were found, thus not allowing the classification of the commercial hybrids, but managed to distinguish one from another the different wild species. Despite the low level of variability the data obtained with cpSSRs markers supported the construction of a reliable hypothesis on the maternal origins of commercial hybrids. For instance, the maternal origin of the Perfecta group from the *G. paniculata* and *G. libanotica* seemed to be suggested. Moreover, the other commercial varieties showed a different chlorotype shared with the gynodioecious plant *G. repens* (López-Villavicencio et al. 2003, 2005), thus supporting the hypothesis of a common maternal ancestor with that wild species (Figure 4). Finally, cpSSR markers grouped a series of genotypes of known parental origin such as Million Stars and Bambino. The same group also include New Love and Mirabella, the latter showing some traits common both to New Love and Million Stars. In accordance with nuclear markers, *G. pacifica* and *G. oldhamiana* clustered together, separately from the other genotypes, coherently with their common geographical origin and distribution (North China) (Wang 2005; Luo et al. 2008) and with the fact that both the species show pink flowers, an uncommon flower color among *Gypsophila* genotypes.

Altogether, cluster analysis performed with nuclear molecular markers confirmed the results obtained with cpDNA markers, also providing a better resolution among cultivars due to their higher levels of polymorphism. Dendrograms inferred from each fingerprinting profile, always showed one main cluster that grouped commercial hybrids with *G. paniculata* and *G. libanotica*. It is worth noting that in the literature, *G. paniculata* is considered the putative species of origin of all commercial cultivars, while little is known of *G. libanotica*, described for the first time by Pierre Edmond Boissier in the mid 1800 (Kotschy 1864). Our data suggested a very closely related phylogenetic origin of these two species and a very high similarity of commercial hybrids to both of them. Moreover, the Perfecta genotypes were always divided into sub-groups closely clustered, whereas Million Stars and related genotypes were placed in near sub-groups. All this notwithstanding, some differences were observed among the different trees. Imball and Blancanieves, for instance, fell outside the main cluster of commercial hybrids in the AFLP tree. *G. repens* with nuclear markers behaved generally as an out-group coherently with its morphological characteristics (Table I). Finally, the tree constructed combining all data showed the most likely relationships among commercial hybrids and wild species (Figure 3).

The improvement of *Gypsophila* varieties has so far been modest due to the lack of knowledge on their genetic structure and on the cause of the unfortunately widespread flower sterility. Our results may help therefore to speed up the breeding process, together with other information discussed elsewhere, concerning the ploidy level, chromosome number analysis, or the study of flower biology and morphology (Bogani et al. 2012; Vettori et al. 2013). Overall, these findings may hopefully lay the foundations for selection planning and lead to the development of cultivars endowed with new traits improved for quality and phenotypic characteristics liable to be attractive in the market.

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