Bacterial endophytes of the wildflower *Crocus albiflorus* analyzed by characterization of isolates and by a cultivation-independent approach

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Abstract: The presence and taxonomy of endophytic bacteria of the entire aerial parts of crocus (*Crocus albiflorus*), a wildflower native in the Alps, were investigated. A combination of plating of plant macerates, isolation and sequence identification of isolates, and direct 16S rDNA PCR amplification followed by whole-community fingerprinting (T-RFLP) and by construction of a bacterial clone library was used. The results clearly indicated that a wide range of bacteria from diverse phylogenetic affiliation, mainly γ-*Proteobacteria* and *Firmicutes*, live in association with plants of *C. albiflorus*. The community composition of the culturable component of the microflora was remarkably different from that of the clone library. Only three bacterial divisions were found in the culture collection, which represented 17 phylotypes, whereas six divisions were identified in the clonal analysis comprising 38 phylotypes. The predominant group in the culture collection was the low G + C Gram-positive group, whereas in the clone library, the γ-*Proteobacteria* predominated. Interestingly, the most prominent bacterium within the uncultured bacterial community was a pseudomonad closely related to a cold-tolerant *Pseudomonas marginalis* strain. The results suggest that *Crocus* supports a diverse bacterial microflora resembling the microbial communities that have been described for other plants and containing species that have not been described in association with plants.

Key words: crocus, endophytes, 16S rRNA, 16S rDNA clone library, T-RFLP analysis, community analysis.

Résumé : La taxonomie des bactéries endophytes présentes sur toutes les parties aériennes du crocus (*Crocus albiflorus*), une fleur sauvage alpine, a été étudiée. Nous avons utilisé une combinaison d'approches incluant l'ensemencement d'extraits de plantes macérées, l'isolement et l'identification de séquences des isolats et l'amplification PCR de l'ADNr 16S suivie par l'empreinte de toute la communauté (RFLP-T), ainsi que la construction d'une banque de clones bactériens. Les résultats indiquent clairement qu'un vaste éventail de bactéries d'affiliations phylogéniques diverses, dont principalement γ-*Proteobacteria* et *Firmicutes*, vivent en association avec le crocus. La composition de la communauté des composantes cultivables de la microflore était remarquablement différente de celle de la banque de clones. Trois divisions bactériennes seulement, représentant 17 phylotypes, ont été trouvées en culture, alors que six divisions comprenant 38 phylotypes ont été identifiées lors de l'analyse des clones. Le groupe prédominant des cultures était constitué de bactéries Gram-positives à faible contenu en G + C, alors que dans la banque de clones, les γ-*Proteobacteria* prédominaient. Fait intéressant, la bactérie prédominant à l'intérieur du groupe non cultivé consistait en une souche de *Pseudomonas marginalis* tolérante au froid. Ces résultats suggèrent que le crocus supporte une microflore bactérienne variée, qui ressemble aux communautés microbiennes qui ont déjà été décrites chez d'autres plantes, et qui comporte des espèces qui n'ont pas encore été décrites quant à leur association avec des plantes.

Mots clés : crocus, endophytes, ARNr 16S, banque de clones d'ADNr 16S, analyse en RFLP-T, analyse de communauté.

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Introduction

The term endophyte refers to microorganisms that colonize intercellular and sometimes intracellular spaces of plants without exhibiting pathogenicity. For a long time, endophytic bacteria have been regarded as latent pathogens or as contaminants from incomplete surface sterilization (Thomas and Graham 1952). Meanwhile, both Gram-positive and Gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species, and several different bacterial species have been found to colonize a single plant (for reviews, see Hallmann et al. 1997; Kobayashi and Palumbo 2000). Furthermore, many endophytes show beneficial effects on plant growth and health (reviewed in Sturz

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et al. 2000). Natural endophyte concentrations in different crops range between 10^3 and 10^6 CFU/g fresh mass (Frommel et al. 1991; Hallmann et al. 1997; McInroy and Kloepper 1995).

Despite the broad application of culture-independent techniques for the analysis of microbial communities in a wide range of natural habitats (Morris et al. 2002), studies on the diversity of bacterial endophytes have been mainly approached by characterizing isolates obtained from internal tissues (Adhikari et al. 2001; Zinniel et al. 2002; Elvira-Recuenco and van Vuurde 2000). Owing to insufficient knowledge about the growth requirements of many microorgansims as well as the fact that cells may enter a viable but not culturable status (Tholozan et al. 1999), culture-dependent methods do not accurately reflect the actual bacterial community structure but rather the selectivity of growth media for certain bacteria. However, only a few studies have been published yet that report the application of 16S rDNA based community fingerprint techniques, such as denaturing gradient gel electrophoresis (Araújo et al. 2002; Garbeva et al. 2001; Sessitsch et al. 2002) and terminal restriction length polymorphism analysis (T-RFLP) (Krechel et al. 2002; Reiter et al. 2002; Sessitsch et al. 2002; Idris et al. 2004), to assess the bacterial diversity in plants. The presence and high concentration of organelle small-subunit rRNA in plants appears to be a major drawback for the cultivation-independent community analysis of endophytes. This is particularly problematic for the direct cloning of the 16S rRNA gene pool in plants. The major part of the clone library constructed of a partial 16S rDNA fragment, which was amplified from DNA isolated from potato plants by using universal eubacterial primers, contained mitochondrial sequences and to a smaller extent chloroplast small-subunit rRNA sequences (Sessitsch et al. 2002). Recently, Chelius and Triplett (2001) described a bacterial 16S rDNA primer for the selective amplification of bacterial sequences directly from maize roots that excluded eukaryotic and chloroplast DNA and allowed separation of bacterial and mitochondrial 16S rRNA gene fragments. With the primer pair suggested by Triplett and Chelius (2001), most bacterial species are addressed; however, the high abundance of *Proteobacteria* sequences in the 16S rDNA clone library along with a strong discrepancy between cultured *Actinobacteria* and actinobacterial 16S rDNA clones indicates a certain preference of this primer to proteobacterial 16S rDNA. In contrast, Garbeva et al. (2001) mechanically dislodged bacterial cells from inside the plant tissue to minimize the amount of nonbacterial sample material prior to DNA isolation.

The goal of this study was to investigate the culturable and nonculturable endophytic microflora in *Crocus albiflorus*, a wildflower belonging to the *Iridaceae* with a widespread distribution throughout the Alps. It forms small white chalices varyingly stained or striped purple at the base. We have chosen *C. albiflorus* for several reasons. (*i*) Up to now, most studies have been focused on the characterization of endophytic bacteria populations in agricultural crops (Stoltzfus et al. 1998; Zinniel et al. 2001; Berg et al. 2005; Sessitsch et al. 2004), whereas little is known about endophytic bacterial communities in wild plants. (*ii*) *Crocus albiflorus* flowers as the snow melts in spring from February to May when the plants are still subjected to frost and snow. Therefore, we were interested in seeing whether such hostile environmental conditions influence the occurrence and species diversity of the bacterial endoflora. We performed a polyphasic approach based on isolation in combination with direct 16S rDNA cloning and T-RFLP community fingerprinting of DNA extracted from *Crocus* plants.

Materials and methods

Crocus plants and collection

Crocus albiflorus, an early spring flowering alpine plant, was chosen for this study. The above-ground parts of 50 *Crocus* plants (stems and flowers) were collected in March 2002 at the edge of a mixed natural forest at about 760 m height in upper Austria. The forest was composed primarily of indigenous tree species, including broadleaf and needle leaf trees, mainly spruces. The forest floor was covered with moss, and grass did not form a continuous layer.

Isolation and PCR–RFLP analysis of endophytic bacteria

Prior to analysis, plants were washed with sterile distilled water and surface disinfected in 5% sodium hypochlorite for 3 min. Subsequently, plants were rinsed four times in sterile distilled water, rinsed in 70% ethanol, and finally flamed. *Crocus* surfaces were tested for their sterility by plotting them tightly on tryptic soy agar (TSA). After 2 days of incubation at 30 °C, no growth was observed on the TSA plates.

Ten *Crocus* plants were cut into pieces, macerated by grinding in sterile mortars, and suspended in 10 mL of 10% tryptic soy broth. Portions of 100 and 200 µL of the supernatant were plated on 20 10% TSA plates in total. In addition, 7 mL was incubated overnight in a shaking incubator at 28 °C. Again, 100 and 200 µL were plated on 10% TSA. Plates were incubated for 24 h at 21 °C or 72 h at 6 °C. Colonies of each plate that could be distinguished based on their colony morphology were picked and further analyzed.

For the isolation of genomic DNA of isolates, bacteria were grown overnight in 5 mL of tryptic soy broth in a rotatory shaker at 28 °C. Cells were harvested by centrifugation for 10 min at 3420*g* at 4 °C. After decanting the supernatant medium, cell pellets were amended with 0.8 mL of TN150 buffer (10 mmol Tris–HCl/L (pH 8.0), 150 mmol NaCl/L), and bead beating was performed twice for 1 min at full speed with an interval of 30 s in a mixer mill (type MM2000, 200 V, 50 Hz) (Retsch Gmbh & Co KG, Haam, Germany) in the presence of 300 mg of acid-washed glass beads (Sigma Chemical Co., St. Louis, Missouri, USA). After extracting with phenol and chloroform, DNA was precipitated with 0.1 volume of 3 mol sodium acetate/L solution (pH 5.2) and 0.7 volume of isopropanol for 20 min at –20 °C. DNA was collected by centrifugation for 10 min at 10 000*g*, washed with 70% ethanol, and dried. Finally, the DNA was suspended in 60 µL of Tris–EDTA containing RNase (0.1 mg/mL).

Extraction of bacterial DNA from *Crocus* plants

Prior to analysis, plants were disinfected as described above for the isolation of endophytic bacteria. Prior to isolation of bacterial community DNA, bacterial cells were dislodged from plants by using a similar procedure as suggested by

Garbeva et al. (2001). The disinfected sliced material of about 50 *Crocus* plants was incubated in 50 mL of 0.9% sodium chloride with shaking for 1 h at room temperature. Cells were collected by centrifugation at 7000*g* at 4 °C and resuspended in 4 mL of TN150 buffer (10 mmol Tris–HCl/L (pH 8.0), 150 mmol NaCl/L). Then, 0.3 g of 0.1 mm acid-washed glass beads (Sigma) was added to aliquots of 0.8 mL, and bead beating was performed as described above. After extraction with phenol and chloroform, nucleic acids were precipitated with 0.1 volume of 3 mol sodium acetate/L solution (pH 5.2) and 0.7 volume of isopropanol for 20 min at –20 °C. Nucleic acids were centrifuged for 10 min at 10 000*g*, washed, and dried. Finally, the DNA was resuspended in 60 μL of Tris–EDTA containing RNase (0.1 mg/mL).

PCR and RFLP analysis of the 16S rRNA gene and the 16S–23S intergenic spacer

Amplifications were performed with a thermocycler (PTC-100™; MJ Research, Inc., Massachusetts, USA) using an initial denaturation step of 5 min at 95 °C, followed by 30 cycles (each) of 30 s at 95 °C, 1 min of annealing at 52 °C, and a 2 min extension at 72 °C. For amplifying 16S rRNA genes, PCR mixtures (50 μL) contained 1× reaction buffer (Gibco, BRL, Gaithersburg, Maryland, USA); 200 μmol/L (each) dATP, dCTP, dGTP, and dTTP; 2 mmol $MgCl_2$/L; 2.5 U of *Taq* DNA polymerase (Gibco, BRL); 0.2 μmol/L (each) primer 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989); and 1 μL of extracted DNA. The primer pair pHr (5′-TGC GGCTGGATCACCTCCTT-3′) (Massol-Deya et al. 1995) and P23SR01 (5′-GGCTGCTTCTAAGCCAAC-3′) (Massol-Deya et al. 1995) was used for amplification of the 16S–23S rRNA intergenic spacer region. For T-RFLP analysis, a partial 16S rRNA gene fragment was amplified using the PCR conditions described above. The primers used were 8f (Edwards et al. 1989) labeled at the 5′ end with 6-carboxyfluorescein (6-Fam) (MWG) and 926r (5′-CCGTCAATTCCTTT(AG)AGT TT-3′) (Liu et al. 1997). Three independent PCRs were carried out and used for subsequent T-RFLP analysis.

RFLP analysis of the 16S rRNA gene and the 16S–23S rRNA intergenic spacer region was used to group isolates at the species as well as at the strain level. Aliquots of PCR products containing 200 ng of amplified DNA were digested with 5 units of the endonucleases *Hha*I (Gibco, BRL) and *Alu*I (Gibco, BRL) for 3 h at 37 °C. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% agarose gels.

T-RFLP analysis

Approximately 200 ng of fluorescently labeled PCR amplification products was digested with a combination of the restriction enzymes *Hha*I and *Hae*III (Gibco, BRL). Aliquots of 0.75 μL were mixed with 1 μL of loading dye buffer (diluted five times in deionized formamide; Fluka, Buchs SG, Switzerland) and 0.3 μL of the DNA fragment length standard (Rox 500; PE Applied Biosystems Inc., Foster City, California, USA). Mixtures were denatured for 2 min at 92 °C and immediately chilled on ice prior to electrophoretic separation on 5% polyacrylamide gels. Fluorescently labeled terminal restriction fragments were detected using an ABI PRISM® 3100 genetic analyzer (PE Applied Biosystems Inc.) in the GeneScan mode. Lengths of labeled fragments were determined by comparison with the internal standard using the GeneScan 2.5 software package (PE Applied Biosystems Inc.).

Terminal fragments (T-RFs) between 35 and 500 bp and heights of ≥50 fluorescence units were included in the analysis. Taking into account the uncertainties of size determination with our automated DNA sequencer, T-RFs that differed by <1.5 bp were clustered. Three PCRs were analyzed individually, and representative sample profiles were determined as suggested by Dunbar et al. (1999). Essentially, the sum of peak heights in each replicated profile was calculated, indicating the total DNA quantity, and peak intensity was adjusted to the smallest DNA quantity. The representative sample profile is composed of mean values of individual peak heights.

Cloning and clone analysis

For cloning, a 16S rDNA PCR product was purified using the NucleoTraPCR kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA fragments were ligated into the pGEM-T vector (Promega) utilizing T4 DNA ligase (Promega, Mannheim, Germany) and the ligation product was transformed into NovaBlue Singles competent cells (Novagen, Madison, Wisconsin, USA), as recommended by the manufacturer. One hundred recombinants, appearing as white colonies on indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), were picked, resuspended in 80 μL of Tris–EDTA buffer, and boiled for 10 min. Subsequently, cells were centrifuged for 5 min at 13 700*g*, and supernatants (1 μL) were used in PCRs using the primers 8f and pH, respectively, and the conditions described above to amplify cloned inserts. PCR products (7 μL) were digested with 5 units of the restriction endonucleases *Alu*I and *Hha*I (Gibco, BRL) individually. Restriction fragments were separated by gel electrophoresis on 2.5% agarose gels. One individual clone of each ribotype was used for sequence analysis.

DNA sequencing and computer analysis

For sequence identification, 16S rDNA genes of isolates or cloned inserts were PCR amplified using the primers 8f and pH (Edwards et al. 1989) and the conditions described above. PCR products were purified using the NucleoTraPCR kit (Macherey-Nagel), according to the manufacturer's instructions, and were used as templates in sequencing reactions. Partial DNA sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) using an ABI 373A automated DNA sequencer, the ABI PRISM® Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems Inc.), and the 16S rRNA gene primer 518r (5′-ATTACCGCGGCTGCTGG-3′). Sequences were subjected to BLAST analysis (Altschul et al. 1997) with the National Center for Biotechnology Information database to identify the most similar 16S rDNA sequences. Alignments were performed using the program MultAlin (Corpet 1988) with a set of sequences of representatives of the most related groups identified. The TREECON software package (van de Peer and de Wachter 1994) was used to calculate distance matrices by the Jukes and Cantor (1969) algorithm and to generate phylogenetic trees using nearest-neighbor criteria.

Table 1. Sequence analysis of 16S rDNA of the endophytic bacteria isolated from *Crocus albiflorus*.

Clone[a]	T-RF (bp)[b]	Closest NCBI database match	Identity (%)	Tentative phylogenetic group
β-*Proteobacteria*				
Cafi3	**67**	*Zoogloea ramigera* AB126355	98	β-*Proteobacteria*, *Rhodocyclus* group
Cafi4	nd	*Janthinobacterium lividum* Y08846	98	β-*Proteobacteria*, *Oxalobacteraceae*
Cafi15c	**67**	β-Proteobacterium Wuba73 AF336362	97	β-*Proteobacteria*, *Oxalobacteraceae*
Cafi2	nd	β-Proteobacterium Wuba73 AF336362	97	β-*Proteobacteria*, *Oxalobacteraceae*
γ-*Proteobacteria*				
Cafi12c	**39**	*Pseudomonas* sp. E3 AY745742	98	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafi16c	**39**	*Pseudomonas* sp. K94.23 AY456705	97	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafi17c	**39**	*Pseudomonas trivialis* AJ492831	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Low G + C Gram-positives				
Cafi1	nd	*Bacillus* sp. SAFR-048 AY167860	99	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi5	**160**	*Bacillus silvestris* AJ006086	98	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi6	nd	*Bacillus* sp. SWS7 AB126768	96	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi7	**160**	*Bacillus silvestris* AJ006086	99	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi8	**160**	*Bacillus* sp. 433-D9 AY266991	99	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi9	328	Glacial ice bacterium G200-SD1 AF479349	99	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi11c	231	*Bacillus* sp. 9B_1 AY689061X70312	99	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi13c	**309**	*Bacillus subtilis* BAFS AY775778	98	*Firmicutes*, *Bacillus/Staphylococcus* group
Cafi14c	233	*Bacillus* sp. 433-D9 AY266991	99	*Firmicutes*, *Bacillus/Staphylococcus* group

[a]Isolates indicated with a "c" were gained by cultivation at 6 °C.
[b]Corresponding peaks were found in the T-RFLP profile (bold-faced values). For other values no corresponding peak was found. nd, not determined.

Nucleotide sequence numbers

The 16S rDNA sequences determined in this study were submitted to the GenBank database with the accession Nos. AY859742–AY859757 (isolates) and AY881653–AY881690 (16S rDNA clones).

Results

Bacterial isolates

Based on colony morphology, we selected 25 colonies from plates incubated at 21 °C and eight colonies from those incubated at 6 °C. Integrating RFLP data of the 16S rRNA gene and 16S–23S rRNA intergenic spacer region analysis resulted in 13 (21 °C) and seven (6 °C) ribotypes, of which three types were found in both treatments. From each of the 17 different types, one isolate was chosen for partial 16S rRNA sequence analysis. All isolates had at least 97% sequence identity to already described bacteria (Table 1). Three bacterial divisions that comprised five genera were represented in the culture collection. The low G + C Gram-positives were predominant, and all of these strains belonged to *Bacillus* spp. The remaining isolates matched with γ- and β-*Proteobacteria*. The isolates that fell within the γ-subgroup belonged to the *Pseudomonadaceae*, whereas the closest relatives of the β-*Proteobacteria* were an uncultured member of the *Oxalobacteriaceae* (Cafi2 and Cafi15c), a *Zoogloea ramigera* (Cafi3) strain, and a *Janthinobacterium lividum* strain (Cafi4).

16S rDNA clone library

One ribotype of the 16S rRNA gene library representing the majority (15%) of the clones was identified as a chloroplast small-subunit rDNA. In addition, 38 bacterial ribotypes were found, with the ribotypes Cafc4 and Cafc16 being predominant and reflecting about 8% and 9% of the 16S rDNA clones, respectively (Table 2). Cafc4 shared the highest sequence identity with *Pseudomonas* sp. TUT1023 found in a study on the microbial community dynamics during acclimation to household biowaste in a flowerpot-using sequence-batch composting system (Hiraishi et al. 2003). Cafc16 was most closely related to a plant-associated and biocontrol active *Pseudomonas* sp. strain. With a share of about 6% in the total clone library, Cafc7 was the second most abundant ribotype, followed by Cafc31, which represented 5% of the screened clones. The closest relative of Cafc7 was closely related to a *Mycobacterium wolinskyi* recently characterized (Adekambi and Drancourt 2004). Cafc31 was identical in the 500 bp sequence information to an uncultured bacterial clone most probably of pseudomonad origin. The remaining 57% of the clones were equally distributed among the other ribotypes.

The clone library comprised six bacterial divisions and 13 genera, with the overwhelming majority of the clones belonging to the *Proteobacteria* (Table 2). The γ-*Proteobacteria* made up the largest fraction, and all but two of these clones were pseudomonads. The latter clones originated most probably from members of the *Moraxellaceae*. Cafc28 showed high sequence homology to *Acinetobacter* spp., whereas clone Cafc21 shared only 90% sequence identity with *Moraxella* spp. sequences and clustered with *Pseudomonas* spp. (Fig. 1). The α-*Proteobacteria* represented the second most abundant group. The clones belonged to the *Sphingomonadaceae*, *Rhizobiaceae*, and *Hyphomicrobiaceae*. In addition, the clone library contained four sequences of β-*Proteobacteria*. Two clones were most likely strains of *Varivorax paradoxus* (Cafc11) and *Z. ramigera* (Cafc19). Cafc13 clustered be-

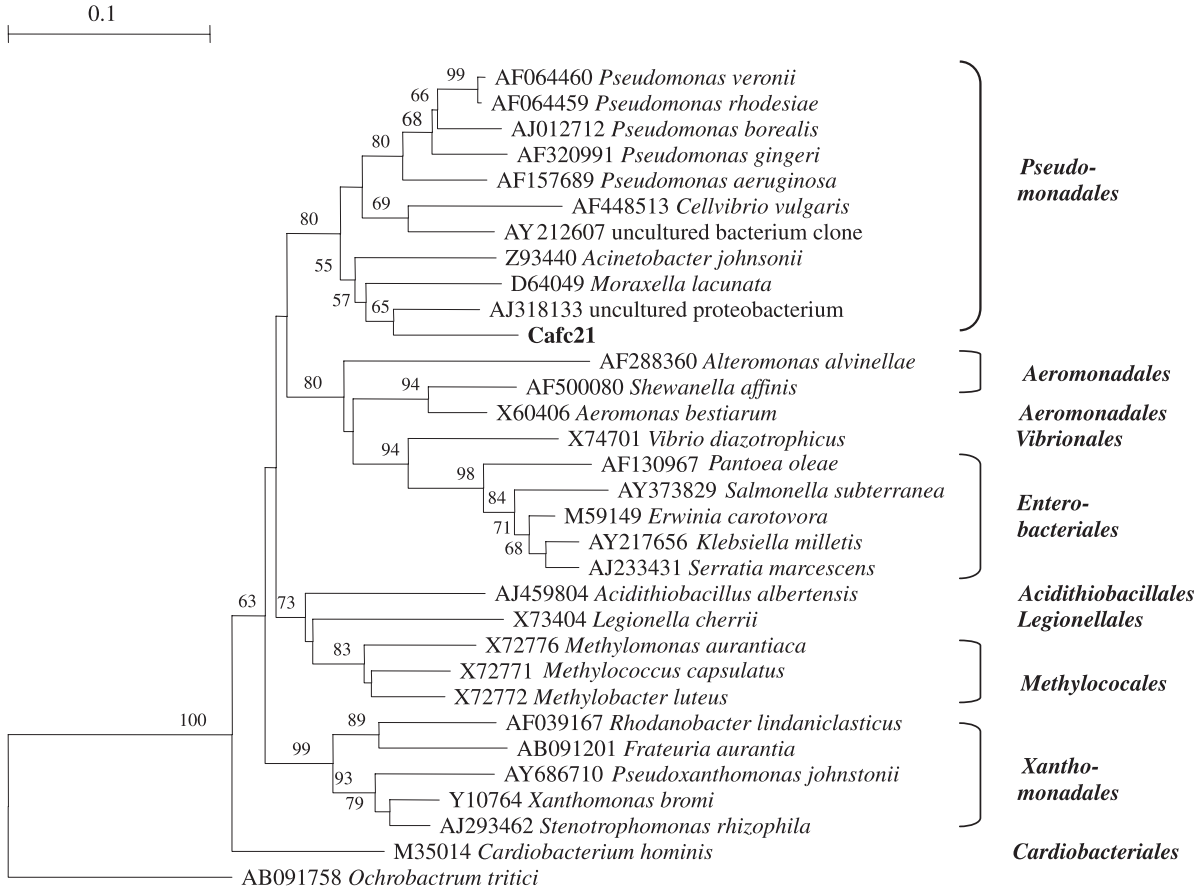
Table 2. Sequence analysis of partial 16S rDNA (approx. 480–500 bp) clone library of DNA isolated from *Crocus albiflorus*.

Clone	T-RF (bp)[a]	Closest NCBI database match	Identity (%)	Tentative phylogenetic group
α-Proteobacteria				
Cafc2	**85**	*Sphingomonas* sp. AY336550	98	α-*Proteobacteria*, *Sphingomonadaceae*
Cafc23	71	*Sphingomonas* sp. pfB27 AY336556	99	α-*Proteobacteria*, *Sphingomonadaceae*
Cafc24	225	Uncultured α-proteobacterium AF445680	98	α-*Proteobacteria*, *Hyphomicrobiaceae*
Cafc26	**297**	*Sphingomonas* sp. M3C203B-B AF395031	98	α-*Proteobacteria*, *Sphingomonadaceae*
Cafc29	195	*Bradyrhizobium* sp. PAC48 AY624135	99	α-*Proteobacteria*, *Rhizobiaceae*
Cafc37	229	Uncultured bacterium AJ744893	97	α-*Proteobacteria*, *Rhizobiaceae*
β-Proteobacteria				
Cafc11	nd	*Variovorax* sp. AY571831	99	β-*Proteobacteria*, *Comamonaodaceae*
Cafc13	**205**	Uncultured *Variovorax* sp. clone AY599727	95	β-*Proteobacteria*, *Comamonaodaceae*
Cafc14	**207**	Uncultured bacterium clone 171ds20 AY212622	98	β-*Proteobacteria*, *Comamonaodaceae*
Cafc19	**67**	*Zoogloea ramigera* X74914	99	β-*Proteobacteria*, *Rhodocyclus* group
γ-Proteobacteria				
Cafc1	**39**	Uncultured bacterium clone AY216460	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc3	**39**	*Pseudomonas* sp. E-3 AB041885	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc4	**39**	*Pseudomonas* sp. TUT1023 AB098591	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc5	**39**	*Pseudomonas veronii* AB056120	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc6	**39**	*Pseudomonas corrugata* AF348508	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc8	**39**	*Pseudomonas* sp. AC-167 AJ519791	98	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc9	**39**	*Pseudomonas* sp. pfB35 AY336564	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc10	**39**	*Pseudomonas veronii* AB056120	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc12	**39**	*Pseudomonas* sp. PsH AF105386	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc15	**39**	*Pseudomonas* sp. NZ096 AY014817	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc16	**39**	*Pseudomonas* sp. Ki353 AY366185	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc18	**39**	*Pseudomonas putida* AS90 AY622320	100	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc20	**39**	Uncultured *Pseudomonas* sp. clone cRI31d AY364068	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc21	247	Uncultured proteobacterium AJ318133	90	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc25	**39**	Unidentified γ-proteobacterium AB015251	97	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc27	**39**	Uncultured bacterium clone S1-2-CL7 AY725255	100	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc28	**200**	*Acinetobacter* sp. Wuba16 AF336348 ZR16SRRNB	99	γ-*Proteobacteria*, *Moraxellaceae*
Cafc30	**39**	Uncultured *Pseudomonas* sp. clone YJQ-20 AY569295	98	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc31	**39**	Uncultured bacterium clone KM94 AY216460	100	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc32	**39**	*Pseudomonas graminis* Y11150	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc33	**207**	Unidentified γ-proteobacterium JTB247 AB015251	98	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc34	**39**	*Pseudomonas* sp. Ki353 AY366185	99	γ-*Proteobacteria*, *Pseudomonadaceae*
Cafc36	**39**	*Pseudomonas* sp. LCY17 AY510004	99	γ-*Proteobacteria*, *Pseudomonadaceae*
High G + C Gram-positives				
Cafc7	**67**	*Mycobacterium wolinskyi* AY457083	97	*Firmicutes*, *Actinomycetales*
Cafc22	**67**	Uncultured *Rhodococcus* sp. AJ631300	99	*Firmicutes*, *Actinomycetales*
Low G + C Gram-positives				
Cafc17	**311**	*Bacillus mycoides* 10206 AF155957	99	*Firmicutes*, *Bacillus*/*Staphylococcus* group
Cafc35	**311**	*Bacillaceae* bacterium C22 AY504448	99	*Firmicutes*, *Bacillus*/*Staphylococcus* group
Cyanobacteria				
Cafc38	242	Uncultured bacterium clone N12.44WL AF432708	93	Cyanobacteria, *Chroococcales*

[a]Corresponding peaks were found in the T-RFLP profile (bold-faced values). For other values no corresponding peak was found. nd, not determined.

tween *Varivorax* sp. and *Curvibacter* sp. A phylogenetic tree demonstrating the taxonomic affiliation of Cafc13 is shown in Fig. 2. The fourth clone showed the highest sequence similarity with a yet to be identified bacterium, with the closest described relative being an *Aquaspirillum delicatum* (Cafc14). The remaining clones comprised members of the Gram-positive bacteria. Two of them (Cafc17 and Cafc35) were identified as *Bacillus* spp., whereas Cafc7 and Cafc22 were most closely related to *Mycobacterium* spp. and *Rhodococcus* spp., respectively. A single clone (Cafc38) represented the *Cyanobacteria*, sharing 93% sequence identity with an uncultured bacterium cloned from lodgepole

Fig. 1. Phylogenetic tree showing the affiliation of the 16S rDNA clone Cafc21 obtained from a *Crocus*-associated community with reference sequences of the γ-*Proteobacteria* based on a BLAST homology search. *Ochrobactrum triticii* (AB091758), an α-*Proteobacteria*, was used as an outgroup, and the number of bases used for the alignment was 325.



pine rhizosphere soil DNA (Chow et al. 2002). In addition to the bacterial sequences, one of the sequenced clones contained the chloroplast 16S rRNA gene but no mitochondrial sequence was found.

T-RFLP analysis

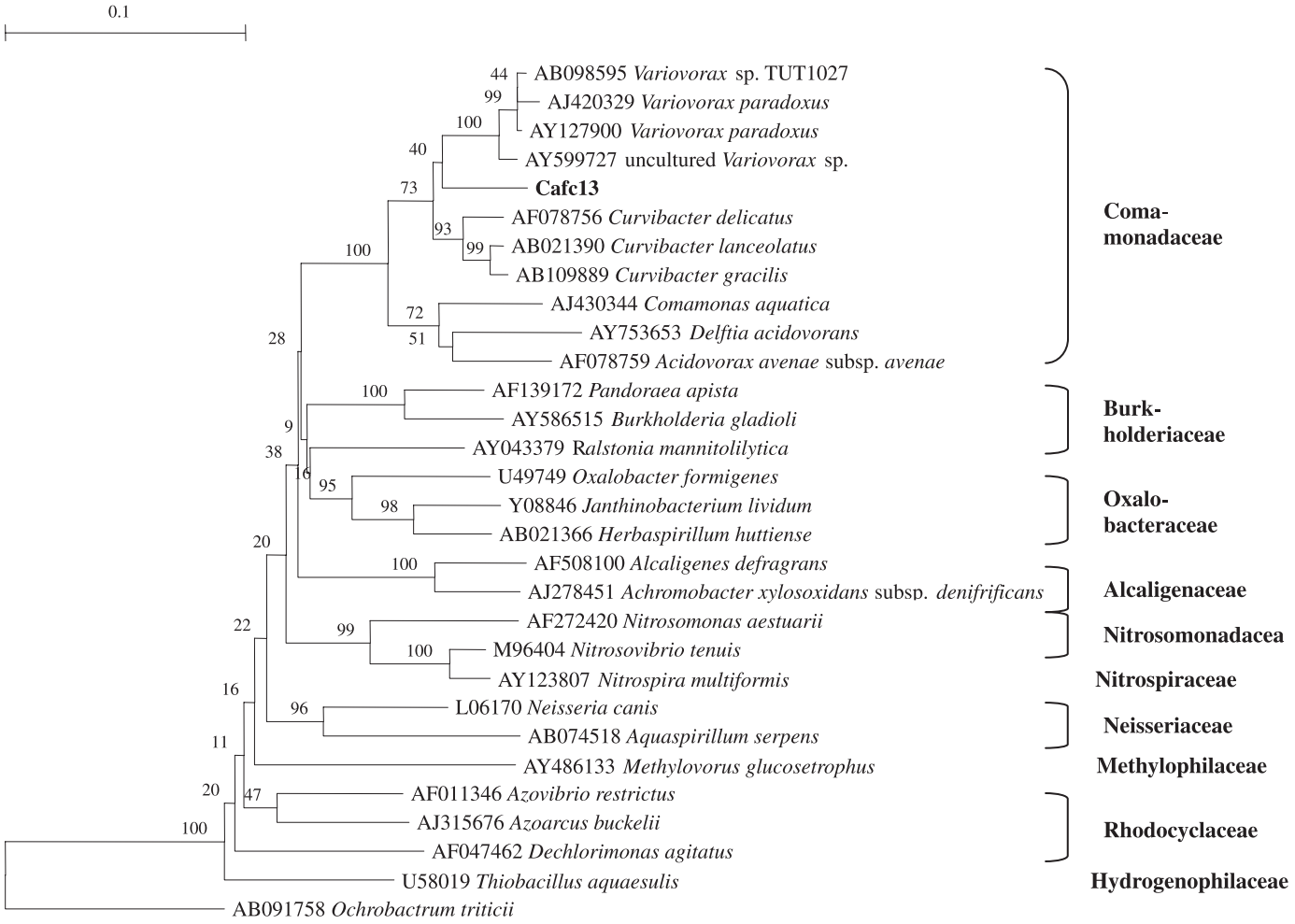
In the T-RFLP profiles one T-RF was obtained that originated from chloroplast small-subunit rRNA sequences and made up about 15% of the total fluorescent intensity (data not shown). In addition, 15 fragments of bacterial origin were identified (Fig. 3). Using the estimation that T-RFs found in a T-RFLP profile and in a DNA sequence that differed by <2 bp are identical, nine T-RFs (39, 65, 87, 162, 199, 205, 209, 297, and 310 bp) could be clearly assigned to certain clones or isolates. Six fragments (77, 216, 218, 266, 303, and 377 bp) remained unidentified, and another eight T-RFs (71, 195, 225, 229, 231, 233, 242, and 247 bp) predicted from the sequence data were not found in the T-RFLP pattern. The T-RF with 39 bp, representing the pseudomonads, was predominant with a 35% share in the total profile. The second most abundant fragments were those with 87 and 162 bp and accounted for 15.6% and 14.6%, respectively. The 87 bp peak correlated with a *Spingomonas* sp. sequence (Cafc2), whereas the 162 bp fragment matched with *Bacillus* sp. 16S rDNA fragments (Cafi5, Cafi7, and Cafi8). The fragments with 205 bp (4.4%), 209 bp (4.2%), and 310 bp (3.5%) could be assigned to *Comamonaodaceae* and *Bacillaceae*. Only two of the minor represented T-RFs (199 bp (0.6%) and 297 bp (0.7%)) could be identified and corresponded to *Acinetobacter* sp. (Cafc28) and *Sphingomonas* sp. (Cafc26), respectively.

Discussion

Although surface sterilization of *C. albiflorus* plants proved to be effective, it was unclear whether DNA of cells that were treated with hypochloride reside on the plant surface and thus might be detected by molecular methods. As aseptical peeling of the *Crocus* plants was impractical, the bacterial community analyzed in this study by cultivation-independent methods was defined as plant-associated microflora potentially including epiphytic bacteria. The DNA extraction method used in this study proved to be valid to minimize disturbance caused by plant organelle small-subunit rDNA and allowed the construction of a bacterial 16S RNA gene library, although chloroplasts could not be completely excluded.

Our research goals were to survey *C. abliflorus*, an alpine wildflower, for the presence of mainly endophytic bacteria and to determine their taxonomic positions. *Crocus* plants flowered in the early alpine spring when plants were still subjected to snow and frost. This could be expected to be a hostile environment for bacteria, and one might expect specific climatic adaptation of the plant-associated microflora.

Fig. 2. Phylogenetic tree showing the affiliation of the 16S rDNA clone Cafc13 obtained from a *Crocus*-associated community with reference sequences of the β-*Proteobacteria* based on a BLAST homology search. *Ochrobactrum triticii* (AB091758), an α-*Proteobacteria*, was used as an outgroup, and the number of bases used for the alignment was 472.



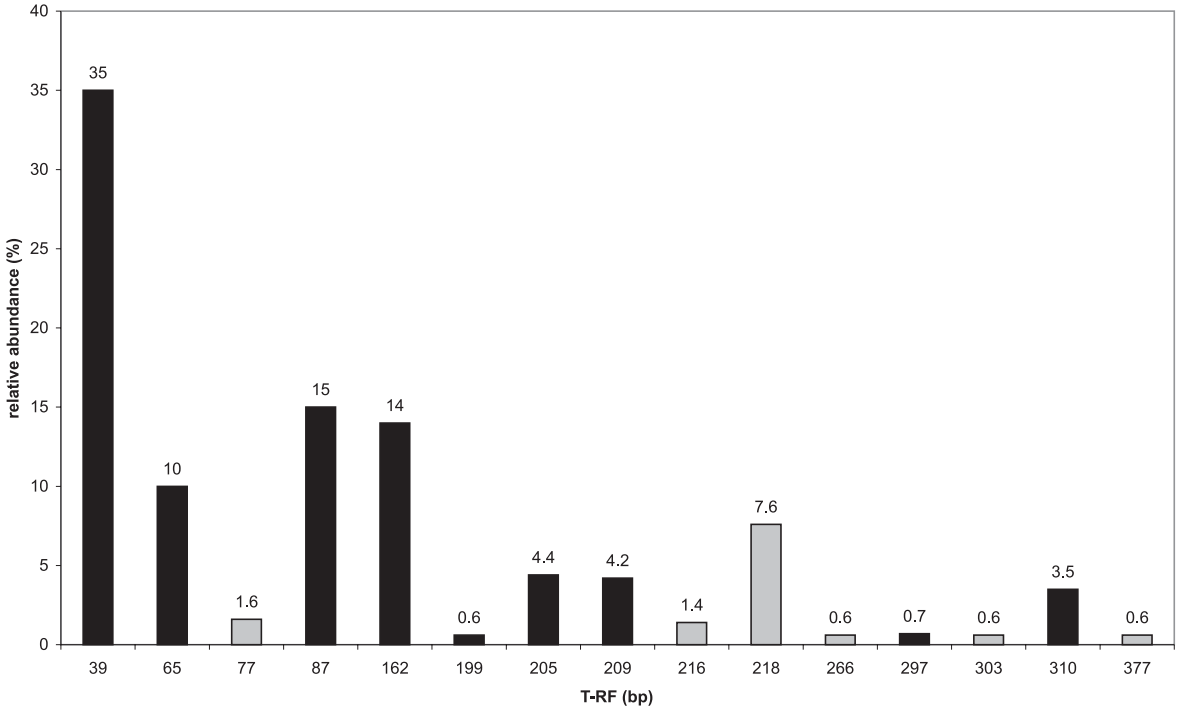
Therefore, it was not surprising that the uncultured bacterial community was co-dominated by a pseudomonad (Cafc16) closely related to a cold-tolerant *Pseudomonas marginalis* strain (Godfrey and Marshall 2002). Furthermore, several isolates, including a *P. marginalis* strain, were able to grow at 6 °C. Nevertheless, the bacterial microflora colonizing *C. albiflorus* generally resembled those that have been described for agronomic crops. In contrast, a previous study on endophytes of the Ni-hyperaccumulating plant revealed an extremely high richness of endophytes, including divisions that have not been identified before as endophytes (Idris et al. 2004). However, this particular microflora may be due to the high concentration of Ni within plants.

Most 16S rRNA genes determined in this study showed high sequence identity to already reported isolates or clones. Numerous *Crocus*-associated bacteria were related to plant-associated and soil bacteria. However, some sampled species have not been described as inhabitants of the plant environment so far.

The results clearly indicated that a wide range of bacteria from six bacterial divisions, mainly γ-*Proteobacteria* and *Firmicutes*, live in association with plants of *C. albiflorus*. The γ-*Proteobacteria* were also most frequently detected in culture collections of maize (McInroy and Kloepper 1995) and potato (Reiter et al. 2002) as well as clone libraries from roots of *Lolium perenne* and *Trifolium repens* (Marilley and Aragno 1999). Sixteen genera comprising 51 strains were represented, with the pseudomonads being predominant. *Pseudomonas* spp. have been frequently sampled in studies analyzing the endoplant microflora (Elvira-Recuenco and van Vuurde 2000; Garbeva et al. 2001; Sessitsch et al. 2002). The low G + C Gram-positives were the second most abundant group, made up largely of *Bacillus* strains. We collected a similar proportion of low G + C Gram-positive bacteria from greenhouse-grown potato plants (Reiter et al. 2002). *Bacillus* spp. were among the most frequently sampled endophytes of potato tubers (Sturz 1995), leaf tissue of citrus rootstocks (Araújo et al. 2001), and citrus branches (Araújo et al. 2002).

The culture collection comprised only three of the six bacterial divisions represented in the clone library. The culturable component of the bacterial community associated with maize roots was remarkably different and less diverse than that of the 16S rDNA clone library (Chelius and Triplett

Fig. 3. Quantitative T-RFLP analysis of mixed DNA isolated from aboveground parts of *Crocus albiflorus*. Terminal restriction fragments (T-RFs) derived from chloroplast 16S rDNA (294 bp) were not included in the analysis. T-RFs that were also found in the sequence analysis data of isolates or 16S rDNA clones (black bars). T-RFs that were not identified in the clone library (grey bars).



2001). In that study, only four bacterial divisions were found in the culture collection, which represented 27 phylotypes, whereas six divisions were identified in the clonal analysis, comprising 74 phylotypes (Chelius and Triplett 2001). The major fraction of the *Crocus*-associated microflora was not culturable on 10% TSA. It is known that in nature, bacterial cells may enter a viable but not culturable state. Such a loss of culturability has been reported, for example, for the biocontrol strain *Pseudomonas fluorescens* CHA0 (Troxler et al. 1997). This could explain why the majority of pseudomonads identified by the cultivation-independent approach could not be isolated, although the genus *Pseudomonas* is in general supposed to be easy to cultivate. We made similar observations when we analyzed the endophytic *Pseudomonas* spp. population of pathogen-infected potato plants. Only one out of 18 pseudomonades identified with genus-specific PCR was also cultured on TSA (Reiter et al. 2003). Interestingly, Berg et al. (2005) isolated hardly any pseudomonades from the endosphere of field-grown potato plants, although they detected *Pseudomonas*-specific peaks in the 16S rDNA based T-RFLP analysis and isolated a variety of different *Pseudomonas* strains from the rhizosphere and endorhiza (Berg et al. 2005). All together, this indicates that a cultivation approach led to an underestimation of the endophytic *Pseudomonas* spp. diversity.

Most cultured endophytes that were not found in the clone library derived from spore-forming members of the *Bacillus*/*Staphylococcus* group. Such differences in the presence of Gram-positive bacteria in culture collections and cultivation-independent analysis have already been shown for the associating microflora of maize roots (Chelius and Triplett 2001) and potato stems (Reiter et al. 2002). Therefore, we suggest that during cultivation in a nutrient-rich medium, such as TSA, particularly fast-growing strains may be enriched that were not highly abundant in vivo. Alternatively, as surface sterilization did prevent isolation of epiphytic bacteria but detection of DNA from those cells cannot be excluded, the disparity in the community composition of the culturable and not culturable component might be due to a disturbance in the molecular analysis caused by bacterial DNA residing on the plant surface.

It should be mentioned that we investigated *Crocus* plants from a single site and thus the community pattern reflects a snapshot of the bacterial endoplant community of *C. albiflorus*. It is now well accepted that biotic as well as abiotic factors significantly affect the community structure of bacterial endophytes. However, our results clearly prove that *Crocus* supports a diverse bacterial microflora resembling the microbial communities that have been described for crop plants. Thus, this study gives clear evidence that internal colonization by nonpathogenic bacteria is a universal feature of plants. Furthermore, this study demonstrated once more the usefulness of an approach combining traditional isolation and molecular techniques to assess bacterial diversity in association with plants.

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