

ORIGINAL ARTICLE

The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*

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Our current knowledge of plant-microbe interactions indicate that populations inhabiting a host plant are not restricted to a single microbial species but comprise several genera and species. No one knows if communities inside plants interact, and it has been speculated that beneficial effects are the result of their combined activities. During an ecological study of nitrogen-fixing bacterial communities from *Lupinus angustifolius* collected in Spain, significant numbers of orange-pigmented actinomycete colonies were isolated from surface-sterilized root nodules. The isolates were analysed by BOX-PCR fingerprinting revealing an unexpectedly high genetic variation. Selected strains were chosen for 16S rRNA gene sequencing and phylogenetic analyses confirmed that all strains isolated belonged to the genus *Micromonospora* and that some of them may represent new species. To determine the possibility that the isolates fixed atmospheric nitrogen, chosen strains were grown in nitrogen-free media, obtaining in some cases, significant growth when compared with the controls. These strains were further screened for the presence of the *nifH* gene encoding dinitrogenase reductase, a key enzyme in nitrogen fixation. The partial *nifH*-like gene sequences obtained showed a 99% similarity with the sequence of the *nifH* gene from *Frankia alni* ACN14a, an actinobacterium that induces nodulation and fixes nitrogen in symbiosis with *Alnus*. In addition, *in situ* hybridization was performed to determine if these microorganisms inhabit the inside of the nodules. This study strongly suggests that *Micromonospora* populations are natural inhabitants of nitrogen-fixing root nodules.

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Introduction

Plants are constantly involved in interactions with a wide range of soil bacteria which colonize the rhizosphere, the phyllosphere and inside of plant tissues (Zakhia *et al.*, 2006). Rhizobia are soil microorganisms that can live symbiotically in the roots of leguminous plants and have a key role in nitrogen fixation. These α - and β -proteobacteria are capable of inducing nodules on the roots of their plant hosts and reduce dinitrogen. Endophytic nitrogen-fixing bacteria seem to constitute only a small proportion of total endophytic bacteria (Rosenblueth and Martínez-Romero, 2006). Our

current knowledge of plant–microbe interactions indicate that populations inhabiting a host plant are not restricted to a single microbial species but comprise several genera and species.

In recent studies, Coombs and Franco (2003) reported on the isolation of endophytic actinobacteria from healthy wheat tissues and showed their potential use as biological control agents (Coombs *et al.*, 2004). Furthermore, Tokala *et al.* (2002) showed that a *Streptomyces* strain, WYEC108, was able to colonize the roots of *Pisum sativum* increasing root nodulation frequency. These authors are of the opinion that these plant–microbe interactions may be common in nature.

The genus *Micromonospora* belongs to the phylum *Actinobacteria*. Typically, colonies on agar media are initially light orange, becoming orange, red, brown or purple with the production of spores. *Micromonospora* is the type genus of the family *Micromonosporaceae* and contains microorganisms with potential biotechnological applications (Ismet

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et al., 2004; Thawai et al., 2004). Micromonosporae have been isolated from diverse habitats including soil, water and marine sediments (Kawamoto, 1989; Zhao et al., 2004; Maldonado et al., 2005; de Menezes et al., 2008). Recently, strains of this genus have also been recovered from diverse plant tissues, especially nitrogen-fixing nodules (Valdés et al., 2005; Trujillo et al., 2006, 2007; Garcia et al., 2010). In the last 4 years our research group has isolated many *Micromonospora* strains from nitrogen-fixing nodules of different legume species. To date, we maintain a collection of more than 500 *Micromonospora* isolates obtained from legume plants collected in Spain, Ecuador and Nicaragua. Thus, we have isolated strains from plants of *Arachis hypogaea*, *Cicer arietinum*, *Glycine max*, *Lens culinarium*, *Lupinus angustifolius*, *Lupinus gredensis*, *Medicago sativa*, *Ononis* sp., *Ornithopus* sp., *P. sativum*, *Trifolium* sp., *Vicia* sp. and the tropical legume *Mucuna* sp. Indeed, we have found *Micromonospora* strains in the root nodules of every legume sampled so far (Trujillo et al., 2007; Cerda, 2008; Garcia et al., 2010).

Frankia is a nitrogen-fixing actinobacterium that can grow as a microsymbiont in the root nodules of several woody dicotyledonous plants. Similarly to rhizobia, *Frankia* strains induce root nodules on their host plants and supply them with atmospheric nitrogen. The report of two isolates from *Casuarina* nodules identified as members of the families *Thermomonosporaceae* and *Micromonosporaceae* that contained *nifH* genes with a sequence similar to those of *Frankia*, strongly suggests that *Micromonospora* may also possess the ability to fix nitrogen (Valdés et al., 2005).

In this study we describe the isolation, diversity and phylogenetic relationship of *Micromonospora* populations isolated from the root nodules of naturally occurring *L. angustifolius* plants collected in various geographical regions in Spain. In addition, we studied the capacity of some of these strains to grow in nitrogen-free media and determined the presence of *nifH*-like sequences in their genomes. In addition, we used fluorescent *in situ* hybridization (FISH) to detect the presence of *Micromonospora* inside the root nodules. Finally, a 500 bp DNA fragment corresponding to the *gyrase B* gene of *Micromonospora* was detected by direct amplification of DNA isolated from nodules. These findings suggest that *Micromonospora* is part of the indigenous bacterial community inhabiting legume nitrogen-fixing nodules, which is still poorly understood.

Materials and methods

Plant sampling and soil characterization

Naturally occurring plants of *L. angustifolius* were collected from the regions of Castilla y León (Cabrerizos, 40°58'43" N–5°36'46" W, and Saelices 40°40'06" N–6°38'02" W) and Extremadura

(Plasencia, 39°58'38" N–6°2'26" W) located in the mid and southwest Spain, respectively, and characterized by a Mediterranean climate. Different types of soils have developed from the parent materials of the area. There are acid soils on granites (Saelices) and slates (Plasencia) whereas basic soils over sandstones and conglomerates are found in Cabrerizos. The soil attached to the roots (rhizosphere and rhizoplane) was used to determine various physico-chemical parameters. Soil samples were air-dried and screened to 2 mm and analyses were performed with the fine-earth fraction (<2 mm). The pH was measured potentiometrically in a 1:1 soil-water suspension, in a CRISON digit, micropH 2001 instrument. Organic matter was determined by potassium-dichromate oxidation as described by Walkley (1947) and total organic N analyses were conducted by the Kjeldahl method (Bremner and Mulvaney, 1982). Available Ca, Mg and K were extracted with 1M ammonium acetate and determined by plasma ICP-OES ULTIMA-2 Jovin-Yvon mod. In addition, available P was determined by the Bray II method, modified from Bray and Kurtz (1945).

Isolation of microorganisms

Four to five plants were collected from each site for three consecutive years; sterile pots were used to transfer the samples to the laboratory for immediate isolation of the microorganisms. Root systems were thoroughly washed with sterile distilled water to remove attached soil, followed by sonication to dislodge any possibly trapped soil pockets. Clean root nodules were either used for bacterial isolation or stored in glycerol (20% v v⁻¹) at –80 °C for *in situ* detection studies. Nodules for isolation of microorganisms (2–4) were selected mainly on the basis that they were effective nodules, that is, a pink–red colour indicating the presence of nitrogen fixation activity. Isolations were made according to the method of Vincent (1970) for the recovery of nitrogen-fixing bacteria. Briefly, the washed nodules were surface sterilized with HgCl₂ (2.5%, w/v) for 2 min, rinsed five times with sterile distilled water and aseptically crushed using a sterile glass rod. The macerate was inoculated on yeast mannitol agar (YMA, Vincent, 1970) under aseptic conditions and the plates were incubated for 3 weeks at 28 °C. No antibiotics or antifungals were added to the isolation medium. For each isolation experiment a sterile, non-crushed nodule was rolled over YMA agar and incubated under the same conditions as the homogenized samples. A washed non-sterile nodule was also included as control. Sixty-four nodules were processed: 29 originated from nine plants collected in Cabrerizos; 32 nodules from 12 plants collected in Plasencia and 10 nodules from four plants collected in Saelices. Colonies on isolation plates were selected on the basis of their morphological appearance as described for *Micromonospora*

(Kawamoto, 1989) and picked under a stereoscopic microscope, checked for purity and streaked on YMA to obtain pure cultures. In addition, the strains were Gram-stained using the procedure of Doetsch (1981).

DNA isolation

All bacterial strains isolated were grown on International *Streptomyces* Project 2 agar (Shirling and Gottlieb, 1966) for 5 days at 28 °C. A single colony from each culture was washed in Tris-EDTA (50 mM Tris-HCl pH 8.0, 10 mM EDTA) followed by centrifugation at 10 000 g for 5 min. DNA extraction was performed using the REExtract-N-Amp Plant PCR kit (Sigma, Steinheim, Germany) following the manufacturer's instructions but with an additional purification step using phenol/chloroform.

BOX-PCR profiling

BOX-PCR fingerprinting profiles from bacterial genomic DNA were generated using the BOXA1R primer for the 136 isolates (Versalovic *et al.*, 1994). PCR amplifications for each strain were performed using the PCR mixture included in the REExtract-N-Amp Plant PCR kit (Sigma) in a final volume of 20 µl per reaction and following the manufacturer's recommendations. Thermal cycling parameters were as follows: 7 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 3 min at 72 °C, followed by 10 min final extension at 72 °C. Ten microliters of each PCR product were loaded on a 2% agarose gel (15 cm × 20 cm) containing 0.5 µg ml⁻¹ ethidium bromide. Electrophoresis was run at 75 V for 3 h in freshly prepared 1X TBE-EDTA buffer at pH 8.0 using a Bio-Rad powerPac 300 power supply. DNA molecular weight marker XIV (Roche, Mannheim, Germany) was used as a molecular size standard. After electrophoresis gels were photographed, stored on disk as TIFF files and imported into the software package BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity matrices of densitometric curves of the gel tracks were calculated using the Pearson Product Moment Correlation Coefficient followed by tree construction using the UPGMA algorithm. Strain clusters were defined at the 60% level of similarity.

Multi-dimensional scaling analysis

All BOX-PCR fingerprints were also compared by scoring similarities using the Pearson's moment coefficient and then building a multidimensional scaling map from the resulting similarity matrix to obtain information about the relationships among the microorganisms isolated from the three different locations.

Amplification and sequencing of the 16S rRNA gene

The 16S rRNA gene of 64 strains selected from the 37 BOX clusters (1–3 strains for cluster) were amplified using the REExtract-N-Amp Plant PCR

kit (Sigma) in 20 µl reactions using the universal primer set 27f and 1522r (Lane, 1991). Amplification and sequencing conditions were as follows: 9 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C, followed by 7 min final extension at 72 °C. PCR products were electrophoresed in 1% agarose gels containing ethidium bromide (0.5 µg ml⁻¹) using modified Tris-acetate EDTA buffer (Millipore, Cork, Ireland). The amplified bands were excised and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The sequence reactions were performed on an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye terminator v3.0 cycle sequencing kit as supplied by the manufacturer and using the primers described previously (Rivas *et al.*, 2003). The sequences were manually aligned using clustal X (Thompson *et al.*, 1997) and compared with other sequences deposited in public databases (GenBank/EMBL) and the Eztaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic distances were calculated with the Kimura 2-parameter model (Kimura, 1980) and the tree topologies were inferred using the neighbour-joining (Saitou and Nei, 1987) method using 1000 bootstrap replications. All analyses were carried out using the MEGA 4 program (Tamura *et al.*, 2007).

Growth in nitrogen-free media

Thirty-seven strains (one representative from each cluster) were grown in nitrogen-free semisolid agar to determine their potential nitrogen-fixing activity. *Bradyrhizobium* sp. ISLU 65 (Jarabo-Lorenzo *et al.*, 2003) was used as a strain control. Strains grown on ISP 2 agar were point inoculated in the bottom of test tubes with 10 ml semi-solid agar, which contained 1% Yeast Carbon Base (Difco, Sparks, MD, USA) and 1% Noble Agar (Difco). The strains were incubated in the dark at 28 °C for 3 weeks. After the first incubation period, the strains were transferred to fresh media using the first tubes as inoculating source and incubated for an additional 3-week period. The above medium supplemented with (NH₄)₂SO₄ (2 g l⁻¹) was used as a positive control.

Amplification and sequencing of the nifH gene

A combination of touchdown and nested PCR was used to screen for the presence of *nifH* genes in the strains that grew in nitrogen-free media. Different protocols were assayed to successfully amplify the *nifH* from 7 strains. In the case of isolate Lupac 08, a *nifH*-like fragment was amplified and sequenced using the primers and procedure described by Valdés *et al.* (2005). The remaining six isolates were amplified using a two-step PCR. The first fragment of ~650 bp was amplified from genomic DNA using the REExtract-N-Amp Plant PCR kit mixture (Sigma) as described before, the primers used were

Mgf (5'-CACGGATCCGCAAGGGTGGTATT-3') and MGr2 (5'-TGAAGCTTCTCGATGACCGTCATCCG-3') (Normand *et al.*, 1988). Thermal cycler conditions for touchdown PCR program were: initial step of 3 min at 95 °C, 10 cycles of 25 s at 94 °C, 30 s at 64 °C and 40 s at 72 °C, with a 0.4 °C decrease of the annealing temperature every cycle. After completion of the touchdown program, 30 additional cycles were performed under the following conditions: 25 s at 94 °C, 30 s at 57 °C and 40 s at 72 °C ending with a final extension of 5 min at 72 °C.

The second (nested) PCR amplified an internal region of ~350-bp fragment using the primers ELA1 (5'-ATGGCKGCCATGGCCGAG-3') (Gtari *et al.*, 2007) and PolR (5'-ATSGCCATCATYTCRCCGGA-3') (Poly *et al.*, 2001). The template for the second PCR was 1 µl of the touchdown PCR product. Reactions were carried out in 50 µl reactions, each containing 5 µl 10 × buffer (Applied Biosystems), 4 µl 25 mM MgCl₂ (Applied Biosystems), 2 µl 0.1% seroalbumin (Sigma), 1.25 µl dNTPs (10 mM each, Applied Biosystems), 0.5 µl each primer (20 µM), 0.4 µl (5U/µl) Taq Gold DNA polymerase. PCR conditions were: 9 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 57 °C, 2 min at 72 °C and a final extension of 7 min at 72 °C. PCR product purification and sequencing were performed as described above using the PolR primer. A third PCR product was obtained for strain Lupac 09 using primers ELA1 and MGr2 under the following conditions: 9 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 64 °C and 2 min at 72 °C ending with a final extension of 7 min at 72 °C. This fragment was subsequently sequenced using primer NF462 (5'-AGGCCGGCCGTACGAGGAC-3') designed in this study. The sequenced fragment, which overlapped with the 350 pb obtained before was used to construct a single contig of 619 bp.

Design and labelling of probes for FISH

Two probes complementary to the 16S rRNA gene of *Micromonospora* and *Bradyrhizobium* were designed after sequence alignment of the 16S rRNA gene sequences of available *Micromonospora* and *Bradyrhizobium* type strains and the sequences obtained in this study. The probes MicromCy5.5 (5'-CTGGAGGTTTTGCGGCCA-3', *E. coli* position 1000) and IsluCy5.5 (5'-AGGGTTGCGCCCATTGTCC-3', *E. coli* position 369) were checked for possible targets against the Genbank (Blast) and the ribosomal database project II databases (Probe Match program; <http://rdp.cme.msu.edu/probematch/search.jsp>). In both cases only hits identified as *Micromonospora* or *Bradyrhizobium* sequences were obtained. The oligonucleotides were labelled with the fluorochrome Cy5.5 (indodicarbocyanine) and were synthesized by Isogen (De Meern, Netherlands). The universal probe EU338 5'-GCTGCCTCCCGTAGAGT-3' (Amann *et al.*, 1995) labelled with FITC was used to optimize permeabilization and hybridization parameters.

Pretreatment and sample fixation

To evaluate the specificity and binding of the probes, *in situ* hybridization was performed using pure cultures of various *Micromonospora* and *Bradyrhizobium* type strains as targets. In addition, *Streptomyces coelicolor* A3(2) was used as a negative control. Cells were grown in ISP 2 broth (YMA for *Bradyrhizobium*) for 3–4 days at 28 °C, harvested and washed several times in phosphate-buffered saline 1X (PBS, 130 mM Na₂HPO₄, 30 mM NaH₂PO₄, pH 7.2). Three volumes of fresh fixation buffer (4% paraformaldehyde in PBS, pH 7.2) were added to one volume of sample (concentration adjusted to 10⁹ cells per ml) and incubated for 3 h at 4 °C. Following fixation, cells were centrifuged and washed in PBS, harvested by centrifugation, resuspended in 1:1 (v v⁻¹) PBS/ethanol solution. Five microliters of each fixed sample was applied to 10-well poly-L-lysine covered slides (Erie Scientific Company) and dried at 37 °C for 20 min.

Nodules were fixed in sterile 4% paraformaldehyde and 0.5% glutaraldehyde in PBS and vacuum was applied for 20 min (Speed vac, Savant) and incubated at 4 °C overnight. After washing several times with PBS 1X, the nodules were embedded in paraffin (Sakura, Tissue Tek) and sections of 2 µm were cut and mounted on 10-well poly-L-lysine coated slides and air dried. Before permeabilization, nodule sections were incubated at 60 °C for 15 min and then deparaffinized in a xylene bath (2 times, 10 min) followed by successive passages through 100, 90, 70 and 50% ethanol washes (5 min each) ending with a final wash in distilled water.

Permeabilization

To facilitate probe penetration into the cells, pure cultures and nodule sections were treated with lysozyme (5 mg ml⁻¹) followed by proteinase K (5 µg ml⁻¹) in 0.1M Tris-HCl, 5 mM EDTA (pH 8) for 10 min at 25 °C for each enzyme. All samples were washed in Millipore MilliQ water and dehydrated by successive passages through 50, 70, and 100% ethanol for 3 min each, air dried and processed for hybridization.

Fluorescent *in situ* Hybridization

For hybridization, 9 µl of hybridization solution (NaCl 0.9M, 20 mM Tris-HCl, 0.1% SDS, pH 7.4) were added to each well that contained sample (pure culture or nodule tissue), followed by 1 µl of the test probe (50 ng). Slides were placed in a chamber humidified with distilled water and incubated for 2–4 h at 48 °C. After hybridization, samples were placed in washing solution (0.05M NaCl, 0.01M Tris-HCl, 0.01% SDS, 0.05M EDTA, pH 7.4) for 20 min at 48 °C and rinsed 3 times with distilled water and air-dried.

The slides were mounted with polyvinyl alcohol mounting medium with DABCO, antifading solution (Fluka BioChemica, Steinheim, Germany) and

then observed with a confocal laser scanning microscope equipped with a neon laser Axiovert 100M). Excitation at 633 nm with emission collected between 675 and 690 nm were used to localize Cy5.5. Despite the greatly reduced sample auto-fluorescence with red excitation, control samples carried through the hybridization procedures without probes still had a few fluorescing spots. To eliminate this noise, these fluorescence counts were subtracted from samples with probes. Confocal settings were chosen to optimize contrast and minimize background.

Probe EU338 labelled with FITC (488 nm excitation) was observed with an epifluorescence microscope (Leica, DMRXA) to define conditions for FISH experiments.

Amplification, cloning and sequencing of the gyrB gene of Micromonospora

Total DNA was extracted from four surface sterilized, peeled lupin root nodules (each separately) using a combination of CTAB, liquid nitrogen and grinding as described by Ramírez-Saad *et al.* (1996). All working reagent solutions were freshly prepared and great care was taken to avoid contamination of the samples with exogenous DNA. Amplification of the *gyrB* gene was performed using the GYF1 (5'-TCCGGYGGYCTGCACGGCGT-3'; position 19–38) and GYR1B (5'-CGGAAGCCCTCYTCGTGSGT-3'; position 548–567) primers designed for the specific amplification of *Micromonospora* strains (Garcia *et al.*, 2010). PCR amplification of a 500 bp fragment was obtained in a final volume of 20 µl using the REDExtract-N-Amp Plant PCR kit (Sigma) as described before. PCR conditions were an initial denaturation at 95 °C for 9 min, followed by 35 cycles with denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 2 min, followed by a step at 72 °C for 7 min. DNA from pure cultures of *Micromonospora pisi* DSM 45175^T (Garcia *et al.*, 2010) and *Bradyrhizobium canariense* BTA-1^T (Rivas *et al.*, 2009) were used as positive and negative controls, respectively. PCR products were purified using the QIAquick kit (Qiagen), ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and cloned in *Escherichia coli* JM109. Plasmid purification was done with the Wizard kit (Promega) following the manufacturer's instructions. Clones containing the expected

size fragment were sequenced using an automatic sequencer ABI 373A.

Microscopy

For transmission electron microscopy, nodules were cut in half along their length from the root downwards, fixed overnight in 2.5% (vol vol⁻¹) glutaraldehyde in 0.05 M sodium cacodylate and vacuum infiltrated to remove air bubbles. The samples were subsequently stained with osmic acid, and embedded in LR White resin according to the manufacturer's instructions (The London Resin Co., Loughborough, England). Sections were cut on a Leica UC6 microtome (Leica, Milton Keynes, UK) and examined in an FEI Tecnai 20 transmission electron microscope (FEI, Eindhoven, The Netherlands) at 200 kV. For scanning electron microscopy, nodules were cut in half and fixed as described above. Samples were then dehydrated through a graded ethanol series, critical-point dried and sputter-coated with gold and examined in a Zeiss, DSM 940 scanning electron microscope.

Re-infection tests

Surface-sterilized seeds of *Lupinus albus* were germinated axenically in petri dishes. Seedlings were transferred to pots with sterile vermiculite and watered with nitrogen-free Rigaud and Puppo nutrient solution (Rigaud and Puppo, 1975). Fifteen plantelets (five per treatment) were inoculated with 1 ml each of a bacterial suspension (10⁸ cells per ml) of strains *Micromonospora* Lupac 08 and *Micromonospora* Lupac 09T. The inoculated plants were placed for 10 weeks in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m⁻² s⁻¹; 400 to 700 nm), programmed for a 16-h photoperiod, day–night cycle, with a constant temperature in the range of 25–27 °C and 50–60% relative humidity. Plants inoculated with *Bradyrhizobium* sp. ISLU 65 served as positive controls and uninoculated *L. albus* plants watered with nitrogen-free Rigaud and Puppo solution were used as negative controls.

Results

Soil characterization

The physico–chemical characteristics of the five soil samples are shown in Table 1. With respect to

Table 1 Physico–chemical characteristics of soils where the plants were collected

Soil sample	pH	Organic matter (%)	N (%)	Ca (mg kg ⁻¹)	Mg (mg kg ⁻¹)	K (mg kg ⁻¹)	P (mg kg ⁻¹)
Cabrerizos site 1	7.99	0.40	0.055	144	52	59	7.92
Cabrerizos site 2	7.34	0.20	0.025	42	50	28	8.46
Plasencia site 1	5.91	1.31	0.098	17	37	68	6.59
Plasencia site 2	5.44	1.92	0.121	13	31	42	4.54
Saelices	5.23	1.87	0.11	245	51	31	8.7

the texture, all samples were characterized by a high percentage of a sand fraction, especially coarse sand, resulting in the presence of sandy loam textures, which would favour drainage, aeration and biological activity. The pH of the soils ranged from 5.23 (Saelices soil) to 7.99 (Cabrerizos soil). The organic matter varied from a low of 0.20–0.40% for the two soil samples from Cabrerizos to a 1.92% content for one of the soils from Plasencia. The nitrogen values ranged from a low of 0.025% (Cabrerizos) to 0.121% (Plasencia). The inorganic phosphate content ranged from 4.54 to 8.7 mg kg⁻¹. Available K and Mg were present in low amounts, however, important differences were found in the amount of Ca present in Plasencia (13–17 mg kg⁻¹) and Saelices (245 mg kg⁻¹).

Bacterial isolation and morphological characterization

One hundred and thirty-six orange pigmented, Gram-positive actinomycete-like colonies were isolated from surface sterilized root nodules on yeast mannitol agar along with *Rhizobium*-like bacteria after a 3-week period of incubation at 28 °C (Supplementary Figure S1). A total of 136 strains were isolated from the sampling sites: 74 colonies were counted on the isolation plates from the nodules collected in Cabrerizos. In this case nine nodules from three plants did not yield any *Micromonospora* strains. The final number of isolates from the remaining 20 nodules was 61 as some of the strains were lost during the purification process. Twenty-one of the 32 nodules from the plants collected in Plasencia yielded 67 *Micromonospora* colonies and 61 strains were obtained in pure culture. In this case no isolates were obtained from four plants. Fourteen strains were recovered from the 10 nodules collected in Saelices; four nodules from two different plants did not contain any *Micromonospora*. Thus, the distribution of *Micromonospora* in the 64 nodules sampled varied from no isolates in plants collected from the three different sampling sites to as many as 15, 22 or up to 29 colonies from a single nodule (Cabrerizos); however, in most cases, 1 to 4 colonies were obtained. Therefore *Micromonospora* strains were recovered in 73% of the nodules sampled.

All 136 actinobacterial strains showed a morphology described for the genus *Micromonospora*, they were orange, filamentous, lacked aerial mycelium and turned dark after 3 weeks upon sporulation. Fast growing microorganisms such as *Aspergillus*, *Penicillium*, *Bacillus* and *Streptomyces* were rarely observed on the isolation plates.

BOX-PCR profiles

High resolution fingerprints were obtained for the 136 actinomycetes isolated from the nitrogen-fixing root nodules of *L. angustifolius*. The band fragments ranged from 0.1 to 2.2 kb. Clusters based on

the Pearson's moment coefficient and UPGMA algorithm were defined at the 60% similarity level, yielding 37 clusters and revealing a significant genomic diversity of the isolates. Figure 1 shows the diversity of the genetic profiles for representative strains, the complete data set is available as Supplementary Material (Supplementary Figure S2). One hundred and eighteen strains were distributed in 19 clusters containing 2–15 strains; the remaining 18 isolates had a unique profile. With respect to the isolation site, the strains isolated from Plasencia (61 strains) were included in 12 clusters; the 61 strains recovered from Cabrerizos were found in 20 clusters and the 14 strains from Saelices formed 6 clusters. In one case strains from Plasencia and Cabrerizos were recovered in the same cluster (cluster 12); however, none of the 37 defined groups contained strains from the three different sampling sites. The composition of each cluster including number of strains, isolation site and code is given in Table 2.

Multidimensional scaling analysis

Statistical multi-dimensional scaling was performed on BOX patterns from each strain. A distribution of the isolates marked according to their geographical origin is given in Figure 2. Groups A, B, C and D contained 72% of the strains isolated from the plants collected in Cabrerizos (green dots), except for strain SB1-28, (in group D), which originated from Plasencia. A significant number of the Cabrerizos strains (21%) did not show any relationship to any of the remaining strains and appeared scattered on the plot area. The isolates from Plasencia (in red) formed three groups (E, G and I) and were well separated from the four previous groups. However, three isolates from Saelices (Lupac 02, and Lupac 04A and Lupac 04B) joined groups G and I. Six strains from Plasencia (SB1-35, SB1-32, SB1-24, SB1-13, SB1-39 and SB1-14) did not relate to any other strains and were loosely scattered. Only 5 of the 14 strains from Saelices were recovered as one moderately related group (J). Finally groups F and H contained strains isolated from the three different regions. These results point to the high diversity of the strains isolated from the nodules based on their BOX-PCR fingerprints and indicate the complexity of the *Micromonospora* populations isolated. In some cases, strains obtained from the same nodule did not group together (for example, MIA65 and MIA74). In other cases, strains isolated from nodules of different plants showed a close relationship (for example, groups G, H and I).

16S rRNA Phylogeny

Sixty-four isolates representing the 37 BOX-PCR clusters were selected for 16S rRNA gene sequencing. Nearly complete 16S rRNA gene sequences (~1450 nt) were obtained for all strains. NCBI and

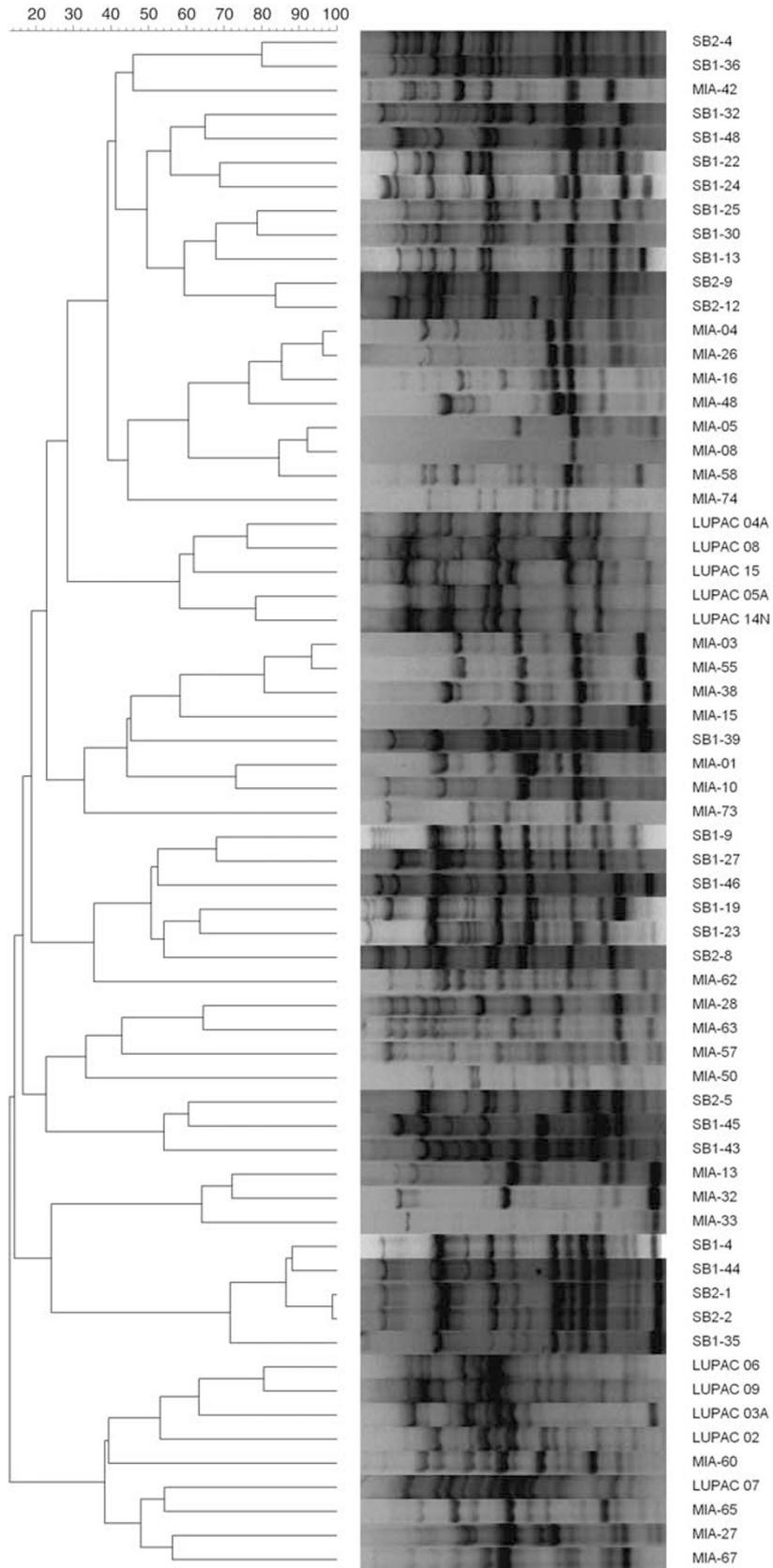


Figure 1 Dendrogram showing genetic relatedness of 64 *Micromonospora* strains isolated from *L. angustifolius* determined by analysis of BOX-PCR fingerprints using the Pearson's coefficient and UPGMA cluster methods.

Table 2 Strains used in this study

BOX group	Strains	No. strains	Origin	16S RNA Identification	Percentage of similarity
1	SB1-36 , SB1-41, SB2-4	3	Plasencia	<i>M. olivasterospora</i>	98.4–98.9
2	MIA-42 , MIA-43	2	Cabrerizos	<i>M. olivasterospora</i>	99.0
3	MIA-62*	1	Cabrerizos	<i>M. coxensis</i>	98.9
4	SB1-13 , SB1-14, SB1-17, SB1-18, SB1-20, SB1-21, SB1-22 , SB1-15, SB1-16	9	Plasencia	<i>M. olivasterospora</i>	98.9
5	SB1-38, SB1-48 , SB2-9 , SB2-10, SB2-7, SB2-12 , SB1-2, SB1-8, SB1-29, SB1-30 , SB1-42, SB2-3, SB2-11, SB1-40, SB1-25	15	Plasencia	<i>M. olivasterospora</i>	98.4–99.0
6	SB1-28, SB1-32 , SB1-24	3	Plasencia	<i>M. olivasterospora</i>	98.0
7	LUPAC: 08 , 12, 04A , 04B	4	Saelices	<i>M. lupini</i>	99.6
8	LUPAC 15	1	Saelices	<i>M. lupini</i>	99.3
9	LUPAC 05A , 05B , 14N ^T	3	Saelices	<i>M. lupini</i>	100
10	MIA-53, MIA-54, MIA-16 , MIA-20, MIA-04* , MIA-26* , MIA-11, MIA-31, MIA-48 , MIA-59, MIA-12, MIA-17, MIA-68	13	Cabrerizos	<i>M. coriariae</i> (MIA-16, MIA-48) <i>M. Aurantiaca</i> (MIA-04, MIA-26)	99.3–99.4 99.5–99.6
11	MIA-74*	1	Cabrerizos	<i>M. coxensis</i>	98.6
12	SB1-35 , MIA-49, SB1-44 , SB1-47, SB1-3, SB1-4 , SB1-10, SB2-1 , SB2-2 , SB2-14, SB2-16, SB2-13	12	Plasencia, Cabrerizos	<i>M. fulviviridis</i>	98.9–99.3
13	SB1-1, SB1-9 , SB1-5, SB1-33, SB1-34, SB1-37, SB1-27 , SB1-11	8	Plasencia	<i>M. chaiyapumensis</i>	99.5
14	SB1-23	1	Plasencia	<i>M. chaiyapumensis</i>	99.7%
15	SB1-26, SB1-31, SB1-6, SB1-46 , SB1-12, SB1-19	6	Plasencia	<i>M. chaiyapumensis</i>	99.3
16	SB2-8	1	Plasencia	<i>M. olivasterospora</i>	99.0
17	SB2-5 , SB1-45	2	Plasencia	<i>M. echinospora</i>	98.5–99.4
18	SB1-43	1	Plasencia	<i>M. sagamiensis</i>	99.4%
19	MIA-60	1	Cabrerizos	<i>M. coxensis</i>	98.8
20	MIA-66, MIA-72, MIA-34, MIA-15	4	Cabrerizos	<i>M. coxensis</i>	99.2
21	SB1-39	1	Plasencia	<i>M. echinospora</i>	98.9
22	MIA-06, MIA-10 , MIA-47, MIA-56, MIA-35, MIA-41, MIA-01* ,	7	Cabrerizos	<i>M. echinospora</i>	99.5
23	MIA-08* , MIA-24, MIA-05 , MIA-71, MIA-58* ,	5	Cabrerizos	<i>M. echinospora</i> <i>M. narathiwatensis</i> (MIA-58)	99.6 99.1
24	MIA-03* , MIA-25*, MIA-23, MIA-46*, MIA-55* , MIA-22*, MIA-38*	7	Cabrerizos	<i>M. echinospora</i>	99.6
25	MIA-73	1	Cabrerizos	<i>M. saelicesensis</i>	99.6
26	MIA-29, MIA-30, MIA-09, MIA-70, MIA-61, MIA-32 , MIA-13 , MIA-14, MIA-19	9	Cabrerizos	<i>M. saelicesensis</i>	99.1
27	MIA-33	1	Cabrerizos	<i>M. coriariae</i>	99.3
28	LUPAC 07	1	Saelices	<i>M. saelicesensis</i>	99.6
29	MIA-65*	1	Cabrerizos	<i>M. auratinigra</i>	98.6
30	MIA-27	1	Cabrerizos	<i>M. saelicesensis</i>	99.5
31	MIA-67	1	Cabrerizos	<i>M. saelicesensis</i>	99.4
32	LUPAC: 06 , 09T , 03A , 03B	4	Saelices	<i>M. saelicesensis</i>	99.6
33	LUPAC 02	1	Saelices	<i>M. saelicesensis</i>	99.5
34	MIA-18, MIA-63	2	Cabrerizos	<i>M. saelicesensis</i>	99.1
35	MIA-28	1	Cabrerizos	<i>M. saelicesensis</i>	99.2
36	MIA-57	1	Cabrerizos	<i>M. saelicesensis</i>	99.4
37	MIA-50*	1	Cabrerizos	<i>M. chaiyapumensis</i>	99.2

In bold: strains sequenced.

SB1 and SB2: strains isolated from Plasencia (sites 1 and 2, respectively).

MIA*: strains isolated from Cabrerizos (site 1).

MIA: strains isolated from Cabrerizos (site 2).

Lupac: strains isolated from Saelices.

Eztaxon nucleotide blast searches revealed that 100% of the sequenced microorganisms were identified with the genus *Micromonospora* as suggested by their morphological characteristics. Sequence similarities between the new isolates and currently described *Micromonospora* species ranged from 98.0 to 100%. The population evaluated showed moderate phylogenetic relationship with 11 different *Micromonospora* species: *Micromonospora*

olivasterospora (clusters 1, 2, 4, 5, 6, 16), *Micromonospora coxensis* (clusters 3, 11, 19, 20), *Micromonospora lupini* (clusters 7, 8, 9), *Micromonospora coriariae* (clusters 10, 27), *Micromonospora aurantiaca* (cluster 10), *Micromonospora fulviviridis* (cluster 12), *Micromonospora chaiyapumensis* (clusters 13, 14, 15, 37), *Micromonospora echinospora* (clusters 17, 21, 22, 23, 24), *Micromonospora narathiwatensis* (cluster 23), *Micromonospora*

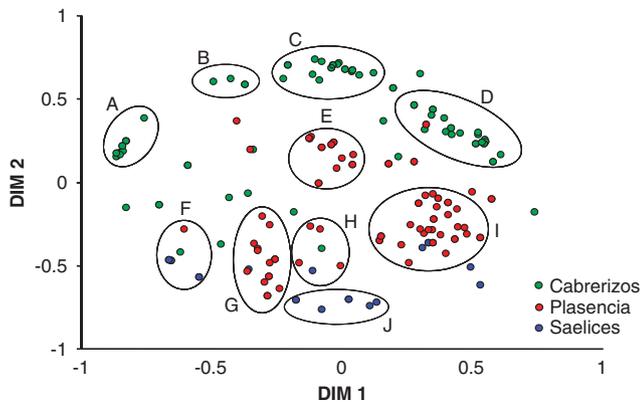


Figure 2 Multi-dimensional scaling analysis based on BOX-PCR fingerprints of the 136 strains isolated from root nodules collected in Cabrerizos (green), Plasencia (red) and Saelices (blue).

saelicesensis (clusters 28, 30, 31, 32, 33, 34, 35, 36), *Micromonospora auratinigra* (cluster 29) and *Micromonospora sagamiensis* (cluster 18). Similarity values of the sequenced strains and identification of the closest phylogenetic neighbours are given in Table 2.

The phylogenetic tree based on the neighbour-joining method (Figure 3) shows the distribution of the 64 isolates and the most closely related *Micromonospora* species. A tree using all presently described *Micromonospora* species was also constructed (not shown). In most cases, the strains grouped in the same branch as the type strains to which they were most closely related, however, a significant number of the strains did not group with any of the currently recognized species.

A significant number of the sequenced isolates (~40%) showed <99% sequence similarity with any of the 37 *Micromonospora* species with validly published names. For example, isolates MIA60, MIA62 and MIA74 showed similarity values of 98.6–98.8% to *M. coxensis*; the similarity between the three strains was also below 99%. The branch grouping strains MIA01, MIA10, MIA03, MIA08, MIA38, MIA05 and MIA55 with sequence similarity values of 99.8–100% among them shared less than 99% similarity with *M. echinospora*. Isolate SB1-39, recovered in a single branch had a similarity of 98.9% with *M. echinospora* its closest phylogenetic neighbour. A group of 13 strains isolated from Plasencia (except MIA42), formed a complex group with sequence similarities ranging from 98.6–100%. Moderate phylogenetic relation was found with two species *M. olivasteraspora* and *M. auratinigra* (97.4–99%). This cluster grouped strains isolated from two different collection sites, Plasencia and Cabrerizos. Finally, MIA65 isolated from Cabrerizos formed a branch by itself and had a 98.6% sequence similarity with *M. auratinigra*. The above groups probably represent new taxa given their phylogenetic position with respect to the 37 described *Micromonospora* species.

Twenty-five percent of the strains isolated showed a relationship between 99–99.4% with described *Micromonospora* species (Table 2). These strains formed a complex branch and grouped with *M. coriariae* (MIA16 and MIA48), *M. narathiwatensis* (MIA58), and *M. fulviviridis* (SB1-35, SB2-2, SB2-1, SB1-4, SB1-44). However, isolates MIA50, SB2-5 and SB1-45 did not cluster with any type strain. Furthermore, taxonomic work will be necessary to determine the status of these strains.

The remaining strains (35%) had sequence similarity values higher than 99.4% with recognized species including *M. chaiyaphumensis*, recently described; (Jongrungruangchok et al., 2008); *M. echinospora*, *M. coriariae*, *M. lupini*, and *M. saelicesensis*; the latter three isolated from nitrogen-fixing nodules (Trujillo et al., 2006, 2007). These results strongly suggest that a significant number of different *Micromonospora* species are able to establish a relationship with nitrogen-fixing nodules.

Growth in nitrogen-free media

Thirty-seven strains representing all BOX-PCR clusters were chosen to determine their ability to grow in nitrogen-free media as a quick screen to select strains for *nifH* gene detection. Twenty-eight of the 37 strains showed good growth in semi-solid agar after a 3-week period (see Supplementary Figure S1). In some cases, the growth was similar to the growth in the control medium supplemented with ammonium sulphate.

Amplification and sequencing of the *nifH* gene

Based on the capacity to grow in nitrogen-free medium, representative strains were further screened for the presence of *nifH* genes.

Using various primer combinations PCR fragments of 337–613 bp were obtained for *M. lupini* Lupac 08 and *M. saelicesensis* Lupac 09^T (Saelices); isolates SB1-39 and SB1-46 (Plasencia); and MIA32, MIA38 and MIA57 (Cabrerizos); thus, *nifH*-like gene sequences were obtained for representative strains isolated from the three collection sites under study. The corresponding *nifH* gene fragment was also amplified for the positive control, *F. alni* ACN14a, but not for *S. coelicolor* A3(2), the negative control. The sequenced fragments corresponded to the *nifH* gene of *Frankia alni* (Table 3); specifically, the strains showed a sequence similarity of 98–99% with *Frankia alni* ACN14a and the translated nucleotides indicated that the sequences corresponded to the dinitrogenase reductase protein (nitrogenase iron protein component II). The phylogenetic tree constructed with the neighbour-joining algorithm (Figure 4) shows the relationship of the sequences of the seven strains in this study, the sequences of the non-*Frankia* strains isolated from *Casuarina equisetifolia* (Valdés et al., 2005), and the corresponding sequences of *Frankia alni* ACN14a

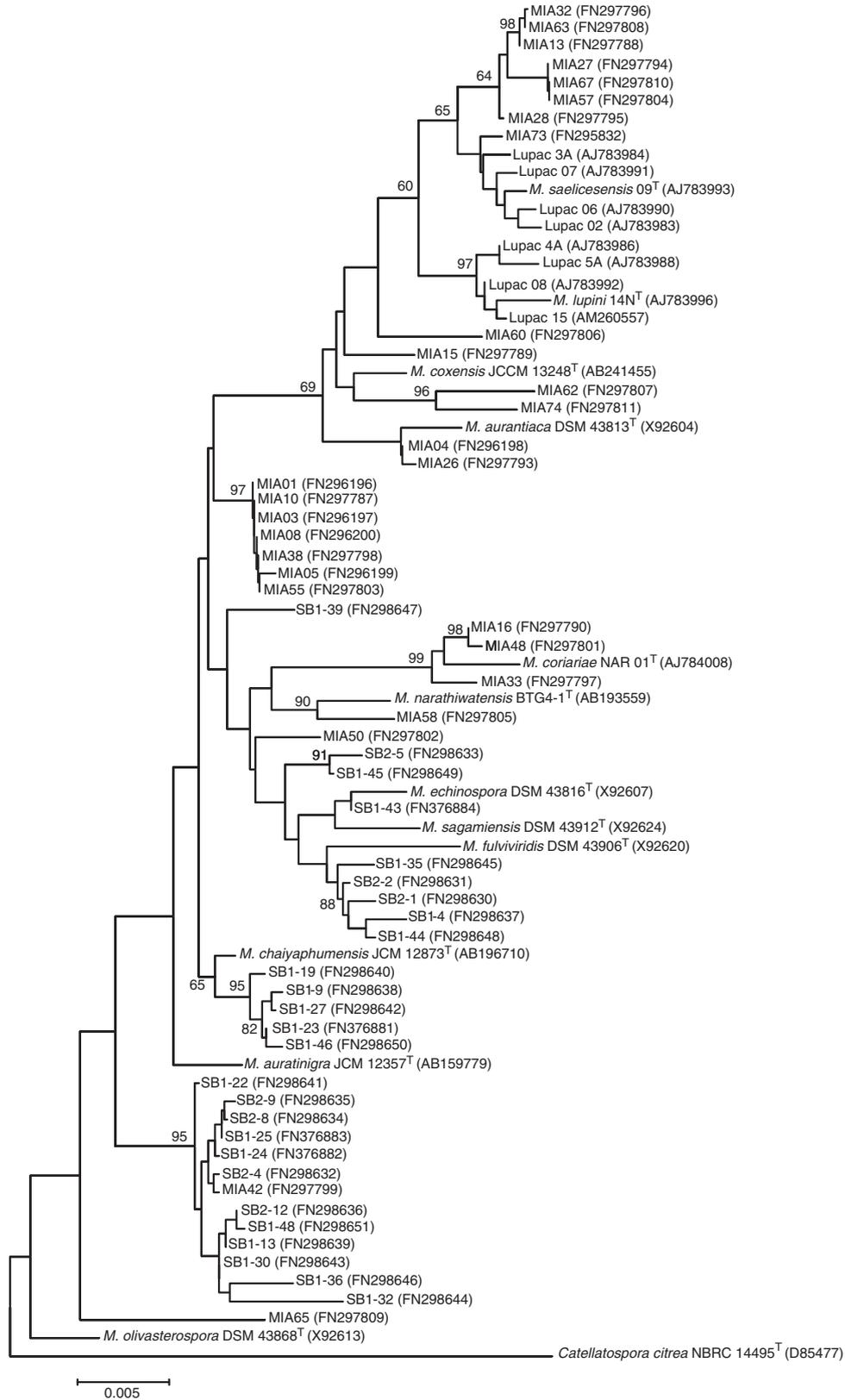


Figure 3 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships among the 64 strains sequenced and the phylogenetically closest *Micromonospora* species. Bootstrap percentages (from 1000 replicates) above 50% are shown at nodes. Bar, 0.005 substitutions per nucleotide position.

Table 3 *nifH* gene sequences determined for the study strains

Strain	Sequence length	Closest match	Identities	Translated nucleotides
Lupac 08	454 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(433/435)	Nitrogenase iron protein
Lupac 09 ^T	613 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(606/610)	Nitrogenase iron protein
MIA32	367 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(365/366)	Nitrogenase iron protein
MIA38	367 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(365/366)	Nitrogenase iron protein
MIA57	367 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(365/366)	Nitrogenase iron protein
SB1-39	337 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (99%)	(335/337)	Nitrogenase iron protein
SB1-46	338 bp	<i>F. alni</i> ACN14a <i>nifH</i> gene (98%)	(334/338)	Nitrogenase iron protein

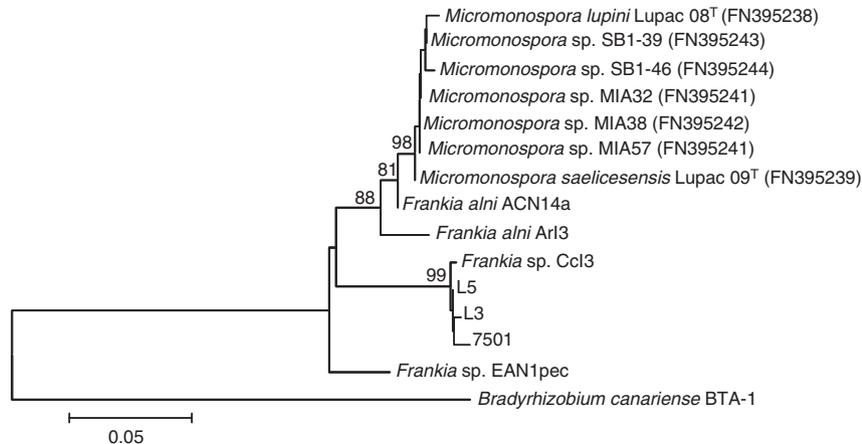


Figure 4 Neighbour-joining analysis of *nifH* gene sequences showing the relationship among the seven isolates obtained in this study and related strains retrieved from the public databases. The significance of each branch is indicated by a bootstrap value for 1000 subsets. Bar, 0.05 substitutions per nucleotide position.

and ArI3, *Frankia* sp. CcI3 and *Frankia* sp. EAN1pec. Three groups were distinguished: group 1 formed a tight clade containing the *Micromonospora* strains isolated from the nodules of *L. angustifolius* and the two *F. alni* representatives ACN14a and ArI3; this branch was supported by a bootstrap value of 88% based on 1000 replicates. Group 2 supported by a bootstrap of 99% included isolates L5, L3, 7501 and *Frankia* sp. CcI3, isolated from *Casuarina*. Group 3 contained the *nifH* sequence of strain EAN1pec isolated from *Elaeagnus*.

Fluorescence in situ Hybridization

Whole cell hybridizations performed with pure cultures of *M. lupini* Lupac 08 and *Bradyrhizobium* sp. ISLU 65 were carried out to show the specificities of the probes designed. The probes MicromCy5.5 and IsluCy5.5 hybridized only with *Micromonospora* and *Bradyrhizobium*, respectively. Additional FISH experiments carried out to test probe IsluCy5.5 against *Micromonospora* and MicromCy5.5 against *Bradyrhizobium* were negative (Figure 5). These results confirmed *in silico* simulation using the Probe Match Program (<http://rdp.cme.msu.edu/probematch/search.jsp>).

Nodule sections without any probe were scanned between 420 and 620 nm to determine plant tissue autofluorescence. The fluorochrome Cy5.5 was

selected over other molecules because reliable detection of bacteria on the plant tissue was only achieved when samples were excited at 633 nm, where nodule tissue autofluorescence was significantly less compared with lower excitation wavelengths. (Supplementary Figure S3)

Histological nodule sections of 2 µm were used to hybridize with probe MicromCy5.5 to detect *Micromonospora* inside the plant cells. Figure 6 shows bacteria-infected plant cells where intercellular spaces are also clearly seen. No fluorescence signal was detected in infected areas where presumably *Bradyrhizobium* is present showing specific hybridization as previously determined by the pure culture experiments. Image analysis and confocal settings allowed elimination of autofluorescence counts originating from the plant tissue to ensure that fluorescence was due to specific hybridization with the bacteria inside the nodules. Our results indeed, indicate the presence of *Micromonospora* in the wild nodule tissues of *L. angustifolius*.

Amplification, cloning and sequencing of the *gyrB* gene of *Micromonospora*

To support the idea that *Micromonospora* strains were isolated from the interior of the root nodules, direct amplification of a 500 bp corresponding to an internal fragment of the *gyrB* gene (accession no.

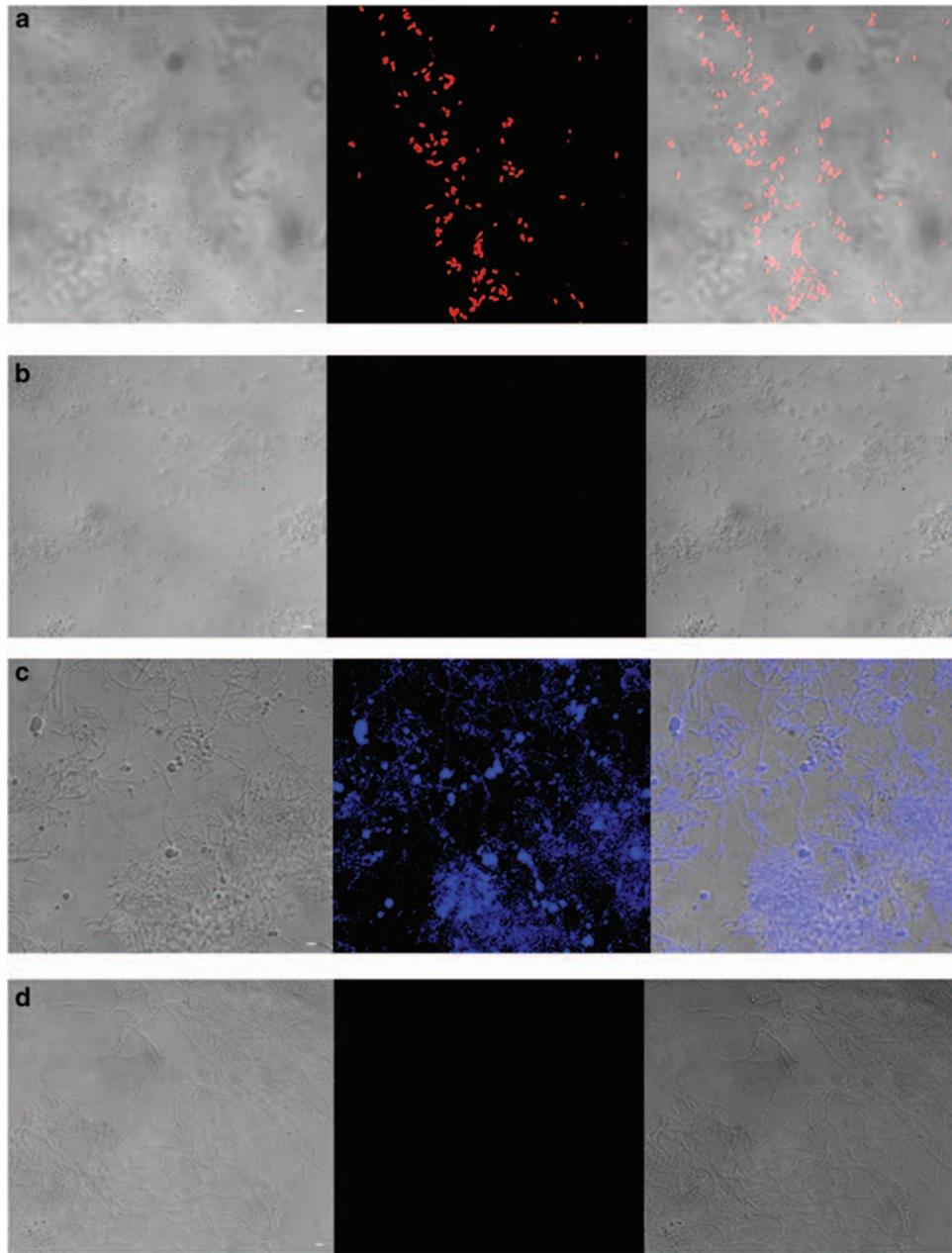


Figure 5 Pure cultures of *Bradyrhizobium* sp. ISLU 65 and *M. lupini* Lupac 08 hybridized with probes Islu Cy5.5 and Microm Cy5.5. (a) *Bradyrhizobium* sp. ISLU 65 hybridized with probe IsluCy5.5. Left, phase contrast; middle, hybridization signal; right, superimposed images. (b) *Bradyrhizobium* sp. ISLU 65 hybridized with probe MicromCy5.5. Left, phase contrast image; middle, hybridization signal; right, superimposed images. (c) *M. lupini* Lupac 08 hybridized with Microm Cy5.5. Left, phase contrast image; middle, hybridization signal; right, superimposed images. (d) *M. lupini* Lupac 08 hybridized with Islu Cy5.5. Left, phase contrast image; middle, hybridization signal; right, superimposed images. Bar, 2 μ m.

FN645943) was obtained from the pooled DNA isolated from the nodules. Blast comparison of the sequenced cloned amplicons indicated that two of these fragments corresponded to a *gyrB* gene sequence with a similarity of 98% to the environmental isolate *Micromonospora* sp. 37 (de Menezes *et al.*, 2008). The next 50 sequences in the Blast list corresponded to additional *gyrB* gene sequences of other *Micromonospora* strains including validly described *Micromonospora* species.

Therefore, correct identification was assumed. In the case of the DNA controls from *Micromonospora pisi* DSM 45175^T and *Bradyrhizobium canariense* BTA-1^T positive and negative results were obtained, respectively.

Microscopy

To search for the presence of *Micromonospora* in nodule tissues, scanning and transmission electron

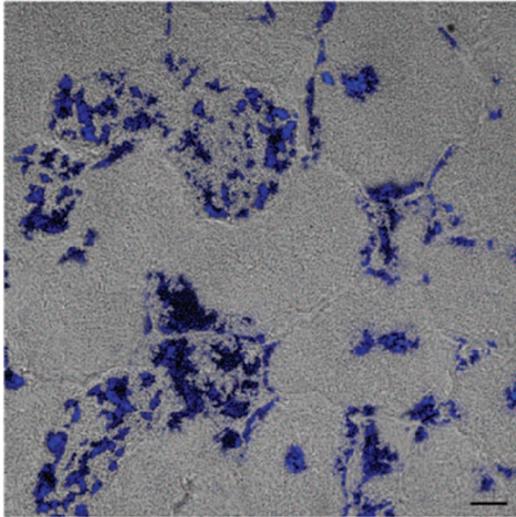


Figure 6 Detection of *Micromonospora* inside nitrogen-fixing nodules of the wild legume *Lupinus angustifolius*. Overlay of DIC and fluorescence image. Bar, 10 μm .

microscopy was done. The presence of filamentous bacteria in different parts of the nodule structure can be seen in Figure 7. The filamentous microorganism shown in Figure 7b was located underneath the first cell layers of a host nodule (Figure 7a) suggesting entrance of the bacterium. Hyphal size (0.5–0.9 μm) corresponds to bacterial not fungal hyphae. Figure 7c shows an infected nodule cell containing bacteroids and a possible hyphal structure.

Different plant cell organelles can be seen in all TEM photographs, in addition, the structures indicated by an asterisk (Figures 7d and f) suggest filamentous bacterial structure; they are definitely not bacteroids. These hyphal structures were located in the nodule cortex region. The density of the cytoplasm in these structures is different than that of the host cell suggesting they are not cell wall or membrane invaginations. Figure 7e shows what appears to be a septum in the filamentous structure.

Re-infection tests

Ten plants were inoculated with bacterial suspensions of *Micromonospora* strains Lupac 08 and Lupac 09^T. After 10 weeks incubation none of the 10 plants inoculated with the two *Micromonospora* strains developed nodules; uninoculated control plants did not form nodules either while the plants inoculated with the *Bradyrhizobium* sp. ISLU 65 were nodulated.

Discussion

Bradyrhizobium is a symbiotic nitrogen-fixing soil microorganism that has the ability to form root nodules on diverse genistoid legumes. Plants of the genus *Lupinus*, which accommodates around 250 species of herbs and shrubs distributed in the

Mediterranean area and in the American continent, are nodulated by *Bradyrhizobium* spp. (Jarabo-Lorenzo et al., 2003; Vinuesa et al., 2005; Stepkowski et al., 2007).

In this study, the number of *Micromonospora* strains recovered from the root nodules of the legumes sampled in our laboratory strongly suggests that large populations of *Micromonospora* inhabit the nodule tissues of healthy leguminous plants. Isolation of *Micromonospora* strains was successful in 73% of the nodules sampled supporting the observation that *Micromonospora* is a common inhabitant of nodule tissues. The distribution pattern was very complex ranging from no isolates for some nodules to as many as 29 colonies arising from a single nodule. Variation could be observed from plant to plant or even from different nodules of the same plant. Further work will be required to understand the distribution of *Micromonospora* in nodules and what factors determine its presence in the host.

The fact that no other microorganisms were isolated on the plates except for the nitrogen-fixing bacteria and the *Micromonospora* strains, indicate that the sterilization protocol was efficient as shown by the control nodules (Supplementary Figure S1).

The differences found in the physico-chemical characteristics of the rhizosphere soils where the plants developed, did not appear to determine the presence of *Micromonospora* in the nodules. These results suggest that the recovery of *Micromonospora* was limited only by the ability of the plants to develop nodules.

The presence of *Micromonospora* colonies already after 7–10 days incubation might indicate that colonies did not originate from surface spores, which survived the sterilization procedure; longer incubation times are usually necessary for the isolation of *Micromonospora* from other substrates such as soil (Kawamoto, 1989; Thawai et al., 2005). The absence of fast-growing fungi on the isolation plates was also an indication of the effectiveness of the isolation protocol considering that antifungal compounds were not added to the isolation agar. Furthermore, it would be expected that other spore-forming, faster growing microorganisms such as *Bacillus* or *Streptomyces* would appear earlier than *Micromonospora*, which was not the case.

BOX-PCR fingerprinting has been shown to be a useful tool to discriminate highly related strains and has been applied to study the genetic diversity of different bacterial taxa including *Micromonospora* (Lanoot et al., 2004; Maldonado et al., 2008; Torres et al., 2008). To get more insight into the genetic diversity of the *Micromonospora* strains isolated, they were analysed by BOX-PCR fingerprinting. A high degree of genetic variation was observed indicating that the strains isolated from the nodules were not clones but represented different bacterial genotypes. The 37 groups obtained provided a useful background to determine the taxonomic

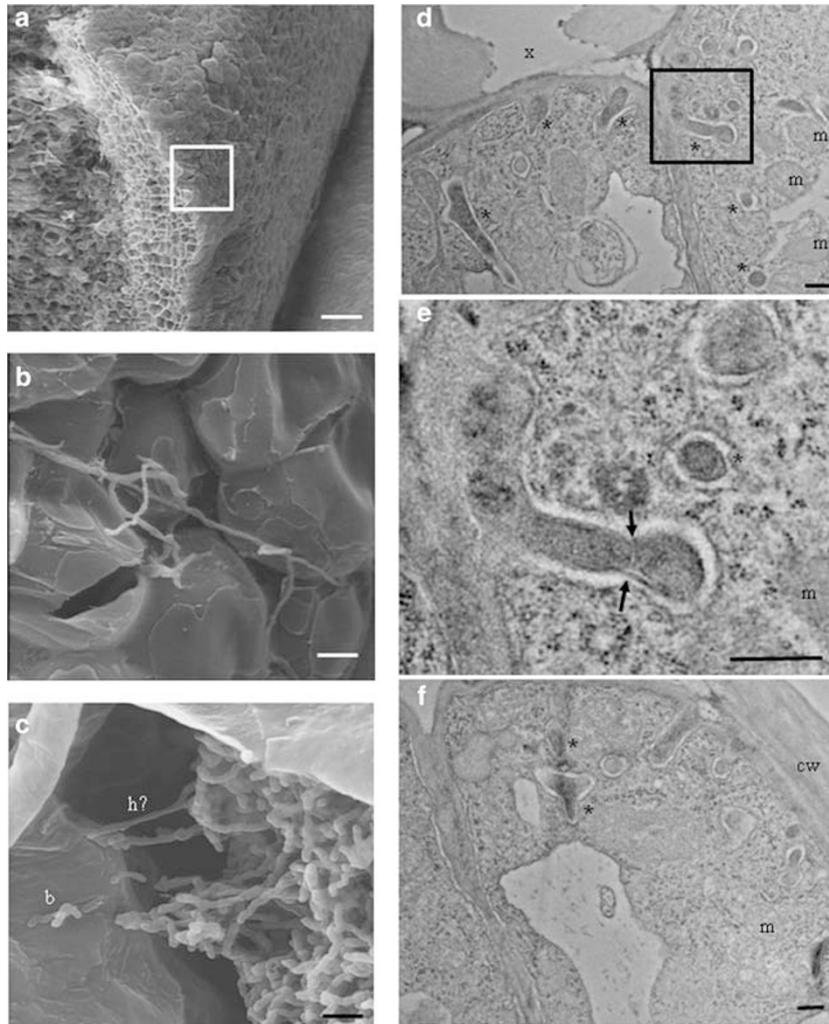


Figure 7 s.e.m. of longitudinal section of a wild lupine nodule (**a**, **b**, **c**). Localization of hyphal growth on the internal tissue layers (**a**); amplification of a zone from the previous panel where a filamentous bacterium is near an open host cell (**b**), possible hyphal structure (**c**). TEM of filamentous-like bacterial structures entering or inside the host cells (**d** and **f**). A septum, indicated by arrows can be seen in the filamentous structure in (**e**). The bars in panels **a**, **b** and **c** are 100, 10 and 2 μm , respectively whereas those in panels **d**, **e** and **f** are 500 nm. x, xylem; m, mitochondria; cw, host cell wall; b, bacteroids; h?, possible hyphae; *structures which may correspond to *Micromonospora*.

relationship of the strains isolated as these groups served to select strains for 16S rRNA gene sequencing.

Multi-dimensional scale analysis was used to obtain information about the diversity of the strains isolated in relation to their geographical origin and also get a better picture of the relationship among the 136 isolates based on their BOX-PCR profiles. These analyses showed that the samples collected in Cabrerizos revealed an unexpected degree of diversity as indicated by the fact that 13 strains were recovered as single member groups. These results underpin the unexpected genomic diversity of *Micromonospora* populations inhabiting the nodules of *L. angustifolius*. Although the strains isolated from Plasencia showed a lower diversity, the strains from each sampling site formed unique populations. Only a moderate relationship was

found among the three sampling sites with a few exceptions. Future taxonomic studies should help to get a better picture about the number of species isolated from each site and those found in common.

16S rRNA gene sequencing and phylogenetic analyses carried out for 64 selected strains and presently described *Micromonospora* species pointed out that some of these strains could not be related to any species and probably represent new taxa. There is indeed strong evidence suggesting that *Micromonospora* type strains, genomically defined by lower than about 70% DNA-DNA reassociation values, are delineated by threshold values of almost 99.3% 16S rRNA gene sequence similarities (Kasai *et al.*, 2000; Thawai *et al.*, 2007; Trujillo *et al.*, 2006, 2007; Jongrungruangchok *et al.*, 2008; Maldonado *et al.*, 2008). In this study, many of the strains sequenced were found in this range.

Furthermore, an important number of strains had 16S rRNA gene sequence similarities below 99%. Many of these strains very likely represent new species. Indeed, further taxonomic characterization of some of these strains has resulted in the description of two new species: *M. lupini* and *M. saelicesensis* (Trujillo *et al.*, 2007). Therefore, good congruence was found between the 16S rRNA gene data and the BOX-PCR fingerprints to select hitherto undescribed taxa in the genus *Micromonospora*. At this time, *M. saelicesensis* seems to be the most abundant species present in the nodules; however, statistical analyses and diversity indices should be applied to confirm this observation.

In the last 4 years our research group has isolated many *Micromonospora* strains from nitrogen-fixing nodules of different legume species. Indeed, we have found *Micromonospora* strains in the root nodules of every legume species sampled so far. The significant numbers of *Micromonospora* populations in the root nodules suggest that the presence of this microorganism is not casual, but indicates that *Micromonospora* has an important ecological role, which is not restricted to a single plant species. Thus, the question is: what exactly is *Micromonospora* doing in the legume root nodules? The presence of *nifH*-like gene sequences obtained from some of these strains showed a close relationship with *nifH* gene sequences from *Frankia* ACN14a. It is possible that *Micromonospora* also fixes nitrogen in symbiosis with its host. Whether these strains contain the complete set of genes responsible for nitrogen fixation in legumes also remains to be studied. As suggested by Valdés *et al.* (2005), the similarity of the *nifH* gene sequences of *Micromonospora* and *Frankia* suggest a lateral gene transfer between these microorganisms, which deserves further investigation as it may shed light on our understanding of their evolutionary history. In the case of the 7 *Micromonospora* strains in this study, the gene seems to have been transferred from a different *Frankia* lineage than that of strain L5. *Micromonospora* L5 shows a closer relationship to the *nifH* gene of *Frankia* IPNCe16 isolated from *Casuarina*, the plant from where strain L5 was recovered.

As *Micromonospora* is a sporulating microorganism, and considering the possibility that surface spores had survived the sterilization protocol, we designed specific 16S rRNA probes to determine the presence of this organism inside the nodules by fluorescent *in situ* hybridization. These studies showed the specificity of the probe used to detect *Micromonospora* as shown by the experiments carried out with pure cultures. Furthermore, an efficient protocol was developed to apply this technique successfully to plant tissues that are usually problematic due to the presence of internal structures that show fluorescence across a wide range of wavelengths. In this study, this problem was overcome by using the fluorescent molecule Cy5.5 with

emission wavelengths that coincided with low levels of autofluorescence in the plant tissue.

Simultaneous detection of *Micromonospora* and *Bradyrhizobium* in the same sample was not possible because it is essential to use fluorochromes with fluorescent spectra that can be clearly differentiated to create unique signals, in our case, the wavelength working window was very narrow due to the plant autofluorescence. Nevertheless, when both probes were applied to the same sample, the fluorescence signal was stronger, filling 'empty spaces' that did not show a fluorescence signal when either of the two probes were used alone, suggesting the presence of *Bradyrhizobium* and *Micromonospora* together inside the infected cells.

The presence of *Micromonospora* within the nodule tissues of lupin is strongly supported by TEM studies, suggesting a close interaction between the host plant and the bacterium. Long filamentous and branched structures were also observed in infected cells; however, it was not possible by visual inspection to determine whether these structures were hyphae or pleomorphic bacterioids which, in many cases, enlarge up to seven times from their original bacterial rod-shape size (Oke and Long, 1999; de Maria *et al.*, 2005). Future studies are needed to determine how *Micromonospora* relates to *Bradyrhizobium* in a tripartite interaction; a synergistic effect of both bacteria on the plant is possible (Tokala *et al.*, 2002).

The application of specific primers to amplify a fragment that corresponded to a *gyrB* gene sequence of *Micromonospora* provided additional evidence that this bacterium inhabits the internal tissues of legume nitrogen-fixing nodules. Selection of the *gyrB* gene over the 16S rRNA gene was to avoid amplification of other bacterial DNA present in the nodules, that is, *Bradyrhizobium* or chloroplast DNA.

Re-infection experiments in our laboratory indicate that *Micromonospora* is not able to induce nodules on its own and that the presence of *Bradyrhizobium* is probably necessary for *Micromonospora* to penetrate the tissues; future studies will be designed to determine how *Micromonospora* penetrates the plant tissues, extracellular enzymes such as cellulases and xylanases produced by many of our isolates may have an important role.

The ability to produce bioactive secondary metabolites is well documented in actinobacteria and *Micromonospora* is no exception. Some of these novel strains were screened for new bioactive secondary metabolites, and the results obtained confirmed the production of previously undescribed molecules, designated lupinacidins A and B (Igarashi *et al.*, 2007). These strains may have the potential to produce other bioactive molecules, which may benefit its host plant.

In summary, our studies strongly suggest that the genus *Micromonospora* maintains a close interaction with leguminous plants as shown by the

high numbers of strains isolated. Furthermore, the presence of this microorganism does not appear to be restricted to a single legume plant species, as our results indicate that it is widely distributed (Trujillo *et al.*, 2006, 2007; Cerda, 2008; Rodríguez, 2008; Carro, 2009; Garcia *et al.*, 2010). This study, therefore, shows that large *Micromonospora* populations have been largely overlooked in this niche, the potential ecological role played by this actinobacterium in both actinorhizal and legume root nodules is yet to be unraveled.

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