# Biodiversity of populations of phosphate solubilizing rhizobia that nodulates chickpea in different Spanish soils

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Received: 31 May 2006

*Key words:* Phosphate solubilizing bacteria, rhizobia, *Mesorhizobium*, chickpea

**Abstract**

Within rhizobia, two species nodulating chickpea, *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*, are known as good phosphate solubilizers. For this reason, we have analysed the ability to solubilize phosphate of a wide number of strains isolated from *Cicer arietinum* growing in several soils in Spain. The aim of this work was to analyse microbial populations nodulating chickpea, that are able to solubilize phosphates, using molecular techniques. In the present work we analyzed 19 strains isolated from effective nodules of *C. arietinum* growing in three soils from the North of Spain. Nineteen strains showed ability to solubilize phosphate in YED-P medium. These strains were separated into 4 groups according to the results obtained by 879F-RAPD fingerprinting. The 16S rDNA sequencing of a representative strain from each group allowed the identification of strains as belonging to the genus *Mesorhizobium*. Strains from groups I and II showed a 99.4% and 99.2% similarity with *M. mediterraneum* UPM-CA142$^T$, respectively. The strains from group III were related to *M. tianshanense* USDA 3592$^T$ at a 99.4% similarity level. Finally, the strain from group IV was related to *M. ciceri* USDA 3383$^T$ with a 99.3% similarity. The LMW RNA profiles confirmed these results. Strains from groups I and II showed an identical LMW RNA profile to that of *M. mediterraneum* UPM-CA142$^T$; the profile of strains from group III was identical to that of *M. tianshanense* USDA 3592$^T$ and the profile of strains from group IV was identical to that of *M. ciceri* USDA 3383$^T$. Different 879F-RAPD patterns were obtained for strains of the group I, group II and the *M. mediterraneum* type strain (UPM-CA142$^T$). The 879-RAPD patterns obtained for group III also differed from the pattern shown by *M. tianshanense* USDA 3592$^T$. Finally, the patterns between group IV and *M. ciceri* USDA 3383$^T$ were also different. These results suggest that groups I and II may be subspecies of *M. mediterraneum*, group III a subspecies of *M. tianshanense* and group IV a subspecies of *M. ciceri*. Nevertheless, more studies are needed to establish the taxonomic status of strains isolated in this study.

## Introduction

The solubilization of phosphates has been found in several species of rhizobia that nodulate different legumes. Within them, species nodulating chickpea are the most powerful P solubilizers (Halder et al. 1990, Peix et al. 2001). Currently these species, that were previously classified in the genus *Rhizobium*, are included in the genus *Mesorhizobium* (Jarvis et al. 1998). The species from this genus have a lower growth rate than those of genus *Rhizobium* and form a group phylogenetically separated from this genus. Currently the

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E. Velázquez and C. Rodríguez-Barrueco (eds.),
First International Meeting on Microbial Phosphate Solubilization, 23–33

genus *Mesorhizobium* includes several species, many of them recently described. *Mesorhizobium mediterraneum* and *Mesorhizobium ciceri* have been described as chickpea endosymbionts (Nour et al. 1994, 1995), separating them from the species *Mesorhizobium loti* that initially included the strains nodulating chickpea (Jarvis et al. 1982). The species *M. loti* is a very complex group of strains, and many of them are currently included in other species. *Mesorhizobium huakuii* nodulates *Astragalus* (Chen et al. 1991), *M. tianshanense* nodulates *Glycyrrhiza pallidiflora* (Chen et al. 1995), *M. amorpha* nodulates *Amorpha fruticosa* (Wang et al. 1999), *M. plurifarium* nodulates tropical trees (de Lajudie et al. 1998) and *M. chacoense*, isolated in the Chaco Arido (Argentina), nodulates *Prosopis* (Velázquez et al. 2001a).

Nevertheless, it is not possible to relate an exclusive bacterial species with a legume species, because most of the rhizobial endosymbionts and legumes are promiscuous and even non-rhizobial species have been recently described as endosymbionts of several legumes. Some of them belong to alpha subclass of Proteobacteria as *Methylobacterium* (Sy et al. 2001) or *Devosia* (Rivas et al. 2002b) and other to beta subclass of Proteobacteria as *Burkholderia* (Moulin et al. 2001) and *Ralstonia* (Chen et al. 2001). Nevertheless, all species of legumes nodulated by non-rhizobial strains are tropical legumes and only few studies have been made on biodiversity of species nodulating temperate legumes. Within them, the chickpea endosymbionts have not been exhaustively studied, except when *M. ciceri* and *M. mediterraneum* were described.

To perform taxonomic studies of endosymbiont populations of legumes we have already applied the LMW RNA profiles obtained by using staircase electrophoresis (Cruz-Sánchez et al. 1997) which comprise 5S rRNA and class 1 and 2 tRNA in bacteria. These profiles can be applied to a large number of isolates and allowed the differentiation among microbial genera, based on the 5S rRNA zone, and species, based on tRNA profiles. The LMW RNA profiles have been used to differentiate species of rhizobia (Velázquez et al. 1998b), to detect new species of *Mesorhizobium* (Velázquez et al. 2001a) and to identify strains isolated from several legumes, including chickpea, in diverse geographical locations (Peix et al. 2001; Velázquez et al. 2001b; Jarabo-Lorenzo et al. 2000). These profiles have also been applied to Gram positive bacteria (Palomo et al. 2000) including endosymbionts of non-legumes as *Frankia* (Velázquez et al. 1998a) and eukaryotic microorganisms (Velázquez et al. 2000). From all these works it is possible to conclude that LMW RNA are molecular signatures of both prokaryotic and eukaryotic microorganisms (Velázquez et al. 2001c).

Therefore, we have used LMW RNA profiling to identify the phosphate solubilizing strains isolated from chickpea in different geographical locations in Spain and the biodiversity within each species was analysed using RAPD patterns. Finally, we have compared the ability of these strains to solubilize phosphate and to movilize it to chickpea plants.

## Material and methods

### *Bacterial strains*

The reference strains, the new isolates and their host plants are listed in Table 1. A total of 19 new rhizobial isolates were obtained from effective nodules of chickpea growing in different soils from Spain. Isolations were made according to Vincent (1970) using yeast manitol agar, YMA (Bergersen 1961). The same medium was used to grow all strains tested.

### *Evaluation of bicalcium phosphate solubilization of rhizobial strains*

The ability to solubilize bicalcium phosphate of the type strains of species from genus *Mesorhizobium* and those of isolates from this study was tested in Petri dishes containing YED (yeast extract 0.5%; glucose 1% and agar 2%) supplemented with a 0.2% of bicalcium phosphate (YED-P). The inoculated plates were incubated for 7 days until the solubilization zone surrounding the colonies was observed.

### *Analysis of 879F-RAPD and RAPD patterns*

Total genomic DNA from the isolates was extracted according to the method employed by Rivas et al. (2001). The primer 879F (5′-GCC TGGGGAGTACGGCCGCA-3′) was used to

*Table 1.* Characteristics of strains used in this study

| Strain | Host | Soil | Phylogenetic group (16S rRNA sequence) | LMW RNA[1] | 879F-RAPD pattern[2] | RAPD pattern[3] |
|---|---|---|---|---|---|---|
| PECA03 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ia |
| PECA11 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ib |
| PECA12 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ib |
| PECA15 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ib |
| PECA19 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ic |
| PECA21 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ib |
| PECA23 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | I | Ib |
| PECA09 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIa |
| PECA10 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIb |
| PECA13 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIa |
| PECA14 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIa |
| PECA16 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIa |
| PECA18 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIa |
| PECA20 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIb |
| PECA22 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIb |
| PECA30 | *Cicer arietinum* (var. "pedrosillano") | Spain (Salamanca) | *Mesorhizobium mediterraneum* | A | II | IIb |
| RCAN03 | *Cicer arietinum* (var. "pedrosillano") | Spain (León) | *Mesorhizobium tianshanense* | B | III | IIIa |
| RCAN08 | *Cicer arietinum* (var. "pedrosillano") | Spain (León) | *Mesorhizobium tianshanense* | B | III | IIIa |
| FCA08 | *Cicer arietinum* (var. "fuentesauco") | Spain (Salamanca) | *Mesorhizobium ciceri* | C | IV | IVa |
| *Mesorhizobium mediterraneum* UPM-CA142$^T$ | *Cicer arietinum* | Spain | *Mesorhizobium mediterraneum* | A | V | Va |
| *Mesorhizobium tianshanense* USDA 3592$^T$ | *Glyzyrrhiza pallidiflora* | China | *Mesorhizobium tianshanense* | B | VI | VIa |
| *Mesorhizobium ciceri USDA 3383$^T$* | *Cicer arietinum* | Spain | *Mesorhizobium ciceri* | C | VII | VIIa |

[1]Each letter corresponds to a different group of strains which present a different 16S rRNA sequence.
[2]Each roman number corresponds to a different pattern obtained using 879F-RAPD. Strains from the same 16SrRNA sequence group may belong to different 879F-RAPD groups.
[3]A combination of roman numbers and letters indicates that within a 879F-RAPD pattern group there are different RAPD patterns.

obtain groups within the species isolated in this study. The M13 primer (5′-GAGGGTGGCGGTTCT-3′) was used to differentiate among the strains from the same subspecies. Crude DNA (2 $\mu$l) was used as template for PCR amplifications. PCR was performed using AmpliTaq Gold reagent kit (Perkin-Elmer Biosystems, California, USA) following the manufacturer's instructions: 2.5 $\mu$l of GeneAmp 10× buffer, 1 $\mu$l of BSA 0.1%, 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP, 2 U of AmpliTaq Gold DNA polymerase, and 2 $\mu$M of primer for 25 $\mu$l of final reaction volume. PCR conditions were as follow: pre-heating at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min; annealing at 50°C for 1 min and extension at 75°C for 2 min, and a final extension at 72°C for 7 min. Eight microliters of amplified PCR product was separated by electrophoresis on 1.5% agarose gels, in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA; pH 8.5) for 2 h at 6 V $cm^{-1}$, stained in a solution containing 0.5 $\mu$l of ethidium bromide $ml^{-1}$, and photographed on a UV transilluminator. Standard VI (Boehringer-Roche, Indianapolis, IN, USA) was used as a size marker.

*Amplification and determination of nucleotide sequences of the 16S rRNA gene and analysis of the sequence data*

DNA extraction was carried out as previously described (Rivas et al. 2001). PCR was performed using an AmpliTaq reagent kit (Perkin-Elmer Biosystems, California, USA) following the manufacturer's instructions (1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP and 2 U of Taq polymerase for 25 $\mu$g final volume of reaction). The PCR amplification of 16S rDNA was carried out using the following primers: 5′-AGAGTTTGATCTGGCTCAG-3′ (*Escherichia coli* positions 8–27) and 5′-AAGGAGGTGATCCANCCRCA-3′ (*Escherichia coli* positions 1509–1522) at a final concentration of 0.2 $\mu$M. PCR conditions were as follows: pre-heating at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min; annealing at 59°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR product (25 $\mu$g) was electrophoresed on 1% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH: 8.5) at 6 V $cm^{-1}$, stained in a solution containing 0.5 $\mu$l ethidium bromide $ml^{-1}$. Standard VI (Boehringer-Roche, USA) was used as a size marker. Three microliters of 6× loading solution (40% sucrose and 0.25% bromophenol blue) were added to each sample. The band corresponding to the 16S rDNA was purified directly from the gel by centrifugation in Eppendorff tubes with a special filter (Millipore Co., Illinois, USA) for 10 min at $5000 \times g$ at room temperature according to the manufacturer's instructions.

The sequence reaction was performed on an ABI377 sequencer (Applied Biosystems Inc.) using a BigDye terminator v3.0 cycle sequencing kit as supplied by the manufacturer. The following primers were used: 5′-AACGCTGGCGGCRKGCYTAA-3′, 5′-ACTCCTACGGGAGGCAGCAG-3′, 5′-CTGCTGCCTCCCGTAGGAGT-3′, 5′-CGTGCCAGCAGCCGCGGTAA-3′, 5′-CAGGATTAGATACCCTGGTAG-3′ and 5′-GAGGAAGGTGGGGATGACGTC-3′, which correspond to *E. coli* small-subunit rDNA sequence positions 32–52, 336–356, 356–336, 512–532, 782–803 and 1173–1194, respectively. The sequence obtained was compared with those from the GenBank using the FASTA program (Pearson and Lipman 1988).

Sequences were aligned using the Clustal W software (Thompson et al. 1997). The distances were calculated according to Kimura's two-parameter method (Kimura 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar et al. 2001) was used for all analyses. The trees were rooted using *Bradyrhizobium japonicum* as outgroup.

*LMW RNA extraction and SCE LMW RNA profiling*

LMW RNA extraction was accomplished following the phenol/chloroform method described by Höfle (1988), using cells grew in tryptone-yeast agar, TY (Beringer 1974). The following commercial molecules from Boehringer Manheim (Manheim, Germany) and Sigma (St. Louis, MO, USA) were used as reference: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (Bidle and Fletcher 1995), tRNA specific for tyrosine from *E. coli* (85 nucleotides) and tRNA specific for valine from *E. coli* (77 nucleotides)

(Sprinzl et al. 1985). Samples containing 3 $\mu$g were added to 5 $\mu$g of loading solution (300 mg/ml of sucrose, 460 mg/ml of urea, 10 $\mu$l/ml 20% SDS, 1 mg/ml xylene cyanol) and, after 10 min of heating at 70°C, applied to each well. LMW RNA profiles were obtained using staircase electrophoresis (Cruz-Sánchez et al. 1997) which was performed in $400 \times 360 \times 0.4$ mm gels in a vertical slab unit (Poker Face SE 1500 Sequencer, Hoeffer Scientific Instruments, San Francisco, CA, USA). The separating gel contained 14% acrylamide/Bis (acrylamide: *N*, *N*-methylene bisacrylamide 29:1 (w/w), 7 M urea in TBE buffer: 100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH: 8.5) in TBE buffer, pH: 8.5. Before running the pre-electrophoresis (30 min at 100 V), the system was stabilized at 50°C. The running buffer (TBE, x1.2) was recycled at a flow rate of 300 ml/min with a peristaltic pump (MasterFlex, Cole Parmer Instruments, Chicago, Illinois, USA). After electrophoresis, gels were silver-stained according to Haas et al. (1994).

*Mobilization of phosphorous in plants*

Experiments for studying the phosphorous mobilization in plants were made on chickpea and were conducted in pots containing vermiculite as sterile support amended with 0.2% (w/w) bicalcium phosphate. The pots were placed in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins $m^{-2}$ $s^{-1}$; 400–700 nm), programmed for a 16 h photoperiod, day–night cycle, with a constant temperature varying from 15–27° C (night–day), and 50–60% relative humidity. Fifteen pots were used for each treatment. The seeds were placed in each pot at a depth of 2 cm.

For inoculation, each strain was grown in Petri dishes with YMA (Bergersen 1961) for 7 days. After that, sterile water was added to the plates to obtain a suspension with ca. $10^8$ cells $ml^{-1}$. For inoculation we added 1 ml of the suspension of each strain to each seed placed in Petri dishes. The seeds were dried overnight at room temperature.

At harvest (30 days) the dry weight of the aerial part of the inoculated plants was determined. Plant nitrogen, phosphorous, potassium, calcium and magnesium content was measured according to the A.O.A.C. methods (Johnson 1990). The data obtained were analyzed by one-way analysis of variance, with the mean values compared using the Fisher's Protected LSD (Low Significative Differences) ($P = 0.05$).

## Results and discussion

*Analysis of 879-F patterns*

According to the results obtained in other microbial groups, primers targeting 16S rDNA sequences, when used at relatively high annealing temperatures (typically 50°C or 55°C), yield DNA patterns that allow to discriminate at species or subspecies levels (Igual et al. in press). For that reason we have used this primer to obtain groups within the isolates of this study. Figure 1 shows that these strains are separated in four groups. The groups I (lanes 4–10) and II (lanes 11–19) include strains isolated in a soil from Salamanca (Spain). The group III (lanes 20 and 21) includes strains isolated from a soil in León (Spain) and the group IV (lane 22) a strain isolated in the soil of Salamanca (Spain). In lanes 1, 2 and 3 the 879-F patterns of *M. mediterraenum* UPM-CA142$^T$ , *M. ciceri* USDA 3383$^T$ and *M. tianshanense* USDA 3592$^T$ are shown.

*16S rDNA sequencing and analysis*

The complete sequences of 16S rDNA genes from strains PECA03 (group I), PECA20 (group II), RCAN03 (group III) and FCA08 (group IV) were obtained and compared with those from databanks using the FASTA program (Pearson and Lipman 1988). Strain PECA03 sequence showed a 99.4% similarity with that of *M. mediterraneum* UPM-CA142$^T$. Strain PECA20 sequence showed a 99.2% similarity with that of *M. mediterraneum* UPM-CA142$^T$. The sequence of strain RCAN03 showed a 99.4% similarity with that of *M. tianshanense* USDA 3592$^T$. Finally, the strain FCA08 sequence showed a 99.3% similarity with that of *M. ciceri* USDA 3383$^T$. Therefore, the phylogenetic analysis (Figure 2) of 16S rRNA sequences places the strains from this study in the genus *Mesorhizobium*.

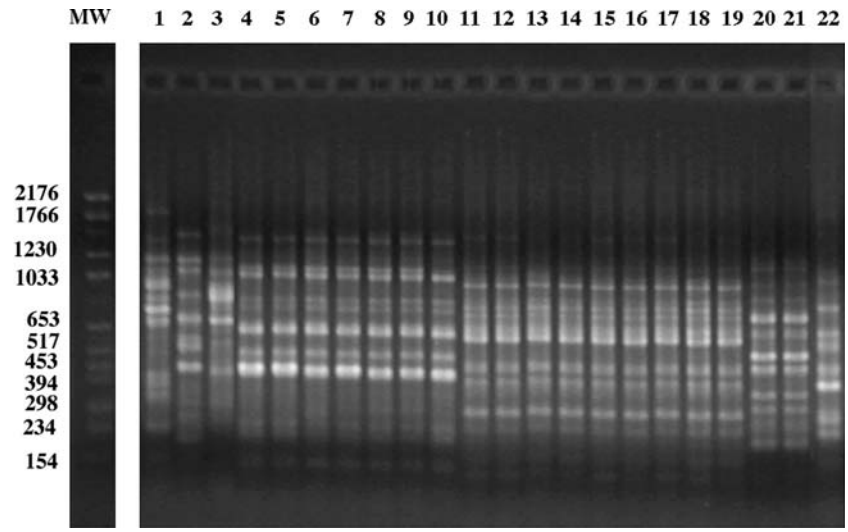
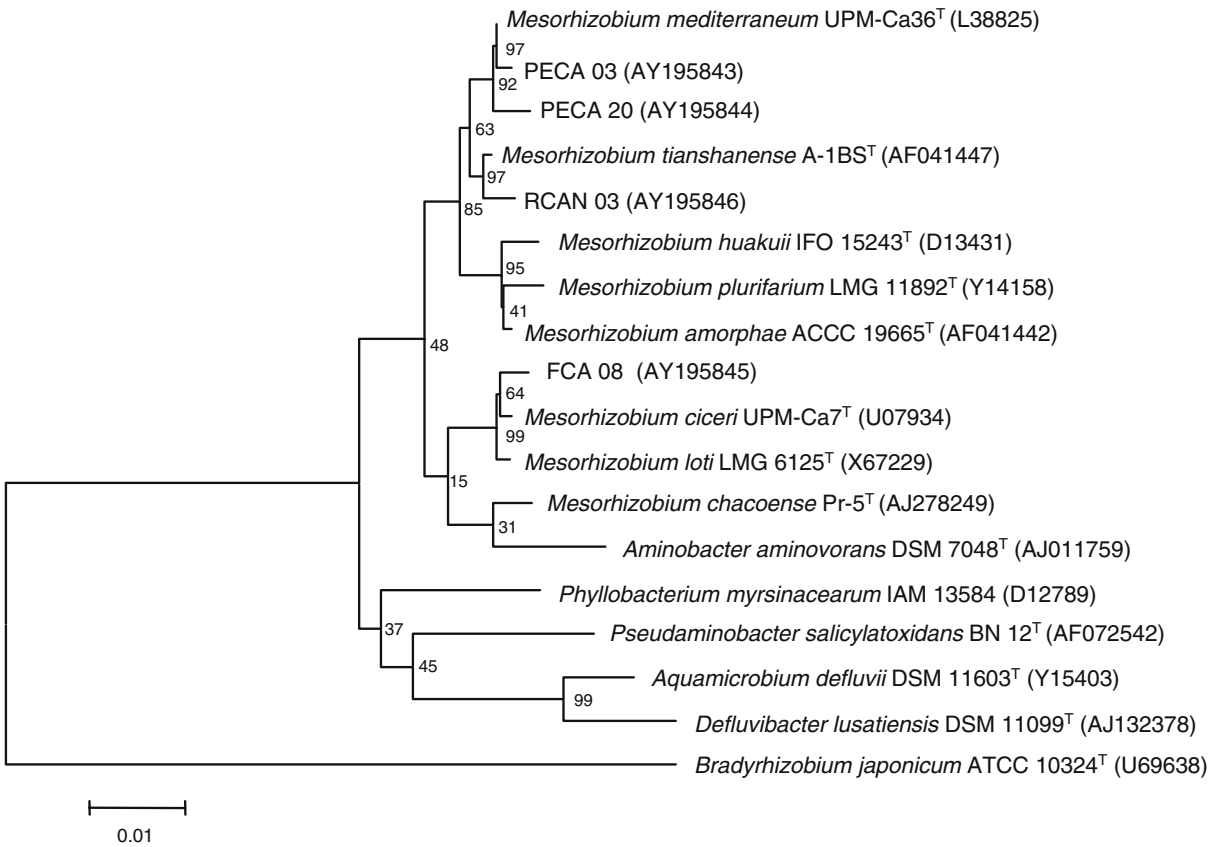



*Figure 1*. Patterns obtained using the primer 879-F: *M. ciceri* USDA 3383$^T$ (lane 1), *M. mediterraneum* UPM-CA142$^T$ (lane 2), *M. tianshanense* USDA 3592$^T$ (lane 3), PECA03 (lane 4), PECA11 (lane 5), PECA12 (lane 6), PECA15 (lane 7), PECA19 (lane 8), PECA21 (lane 9), PECA23 (lane 10), PECA09 (lane 11), PECA10 (lane 12), PECA13 (lane 13), PECA14 (lane 14), PECA16 (lane 15), PECA18 (lane 16), PECA20 (lane 17), PECA22 (lane 18), PECA30 (lane 19), RCAN03 (lane 20), RCAN08 (lane 21) and FCA08 (lane 22).

*LMW RNA profiles*

In order to identify bacteria at the species level, 16S rDNA sequences were complemented with the LMW RNA profile analysis of our strains comparing them with those of the type strains *M. mediterraneum* UPM-CA142$^T$, *M. tianshanense* USDA 3592$^T$ and *M. ciceri* USDA 3383$^T$. The LMW RNA profile of *M. mediterraneum* UPM-CA142$^T$ (Figure 3, lane 1) is identical to that of strains from groups I and II (represented in Figure 3, lane 2), *M. ciceri* USDA 3383$^T$ (Figure 3, lane 3) shows the same LMW RNA profile than strains from group IV (represented in Figure 3, lane 4) and *M. tianshanense* USDA 3592$^T$ shows the same LMW RNA profile (Figure 3, lane 5) than strains from group III (represented in lane 6, Figure 3). In previous studies, we have demonstrated that LMW RNA profiles are molecular signatures of eukaryotic and prokaryotic microorganisms at genus and at species level (Velázquez et al. 2001c). Therefore, strains from groups I and II belong to *M. mediterraneum* species. Group III strains belong to *M. tianshanense* species and group IV belong to species *M. ciceri*. These results confirm the identification obtained by means of 16S rDNA sequence.

According to the results the 879F-RAPD pattern of strains from group I and II do not coincide between them and neither with that of type strain of *M. mediterraneum* UPM-CA142$^T$. The 879F-RAPD pattern of strains from group III do not coincide with the type strain of *M. tianshanense* USDA 3592$^T$ and the pattern of strains from group IV does not coincide with the type strain of *M. ciceri* USDA 3383$^T$. Therefore, these results point out the existence of more than one genomic group within the three species of this study. These results confirm those obtained in *C. michiganensis* subspecies using primers targeting 16S rDNA sequence (Rivas et al. 2002a). The taxonomic status of the strains isolated in this study must be established in further studies, but it is possible that the species *M. mediterraneum*, *M. ciceri* and *M. tianshanense* contain several subspecies. These results coincide with those obtained by other authors that have recently described new subspecies in the species *Bacillus subtilis* (Nakamura et al. 1999) and *Photorhabdus* (Fischer-Le Saux et al. 1999). Moreover, our results are in agreement with those of other authors that have proposed a new subspecies within *M. huakuii* (Nuswantara et al. 1999).




*Figrue 2.* Comparative sequence analysis of 16S rDNA from the strains PECA03, PECA20, FCA08 and RCN03 and representative strains from the GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Bar, 1 nt substitutions per 100 nt.

*Analysis of the intraspecific biodiversity using RAPD patterns*

To analyse the intraspecific biodiversity from species of this study we also used the primer M13 to detect strain specific patterns (Table 1). The results are shown in Figure 4. Within strains from group I of 879-F patterns, three RAPD patterns have been found using M13 primer: Ia (lane 4), Ib (lanes 5, 6, 7, 9 and 10) and Ic (lane 8). Most of the strains from this group showed the RAPD pattern type Ib. The strains from group II of 879-F patterns showed two types of RAPD pattern: IIa (lanes 11, 13, 14, 15, 16, 18 and 19) and IIb (lanes 12 and 17). The strains from group III showed identical RAPD pattern (lanes 18 and 19). The RAPD pattern of strain from group IV is shown in lane 20. Finally, the type strains of *M. mediterraneum* UPM-CA142$^T$ (lane 1), *M. tianshanense* USDA 3592$^T$ (lane 2) and *M. ciceri* USDA 3383$^T$ (lane 3) showed different RAPD patterns among them and with respect to the other strains from this study.

The conventional RAPD patterns are strain dependent and usually vary among the strains from the same subspecies (de la Puente-Redondo et al. 2000; Wieser and Busse 2000). The results of this work are in agreement with those reported in the literature and confirm that the 879F-RAPD patterns are strain non-dependent and that probably the strains showing different pattern belong to different subspecies. Nevertheless, to demonstrate that a taxonomic polyphasic study must be performed on the strains isolated in this study.

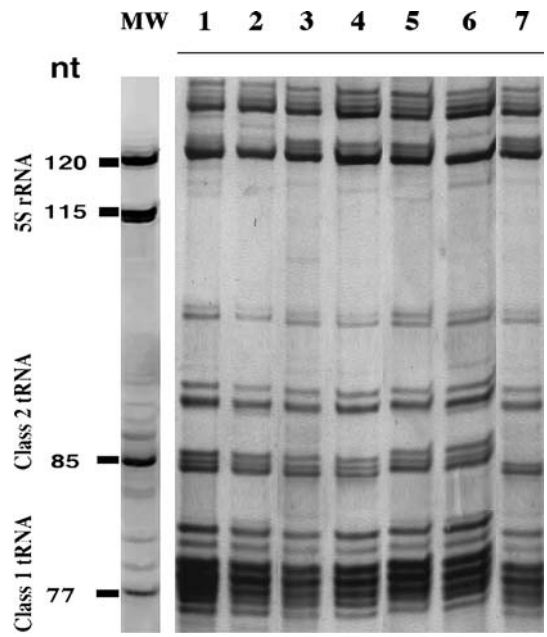



*Figure 3*. LMW RNA profiles displayed by the strains of this study. Lane 1, *M. mediterraneum* UPM-CA142$^T$. Lane 2 shows the profile of strains PECA03, PECA11, PECA12, PECA15, PECA19, PECA21, PECA23, PECA09, PECA10, PECA13, PECA14, PECA16, PECA18, PECA20, PECA22, PECA30. Lane 3, *M. ciceri* USDA 3383$^T$. Lane 4, strain FCA08. Lane 5, *M. tianshanense* USDA 3592$^T$. Lane 6 shows the profile of strains RCAN03, RCAN08. Lane 7, *M. loti* DSM 2626$^T$.

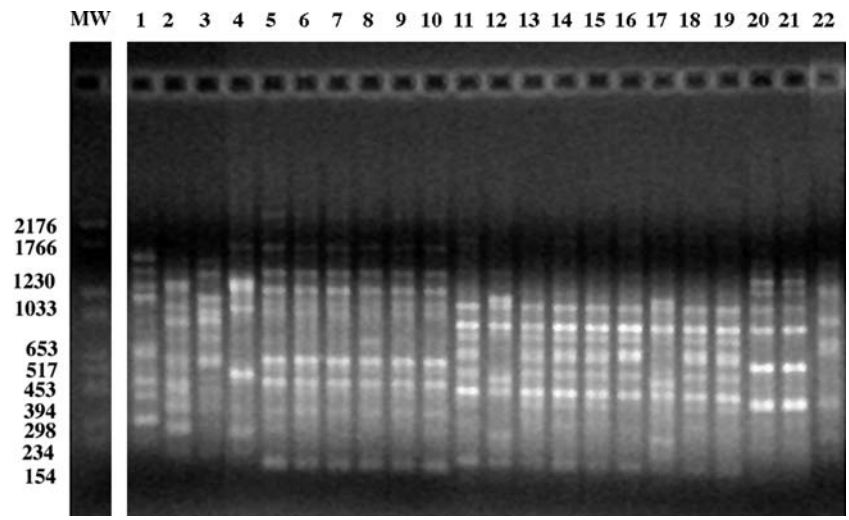



*Figure 4*. RAPD patterns obtained using the primer M13: *M. ciceri* USDA 3383$^T$ (lane 1), *M. mediterraneum* UPM-CA142$^T$ (lane 2), *M. tianshanense* USDA 3592$^T$ (lane 3), PECA03 (lane 4), PECA11 (lane 5), PECA12 (lane 6), PECA15 (lane 7), PECA19 (lane 8), PECA21 (lane 9), PECA23 (lane 10), PECA09 (lane 11), PECA10 (lane 12), PECA13 (lane 13), PECA14 (lane 14), PECA16 (lane 15), PECA18 (lane 16), PECA20 (lane 17), PECA22 (lane 18), PECA30 (lane 19), RCAN03 (lane 20), RCAN08 (lane 21) and FCA08 (lane 22).

### *Evaluation of bicalcium phosphate solubilization of rhizobial strains*

The strains isolated in this study showed differences in their ability to solubilize phosphate in plates (Table 2). All strains nodulating chickpea in the soils studied were able to solubilize phosphate. The strains belonging to species *M. ciceri* presented the smallest clearing halo on YED-P plates followed by those of *M. mediterraneum*

*Table 2.* Symbiotic characteristics of strains nodulating chickpea used in this study

| Strain | Solubilization "halo" (mm)* | Number of nodules | Dry weight per plant (mg) | Total N (mg) | Total P (mg) | Total Ca ($\mu$g) | Total Mg ($\mu$g) | Total K (mg) |
|---|---|---|---|---|---|---|---|---|
| PECA03 | 10 | $8^{a}$ | $120^{a}$ | $2.4^{ab}$ | $0.20^{a}$ | $43.5^{c}$ | $86.0^{b}$ | $0.48^{b}$ |
| PECA20 | 5 | $5^{a}$ | $90^{a}$ | $1.8^{a}$ | $0.15^{a}$ | $32.6^{b}$ | $89.0^{b}$ | $0.34^{a}$ |
| RCAN03 | 15 | $7^{a}$ | $120^{a}$ | $3.0^{c}$ | $0.6^{b}$ | $50.0^{d}$ | $106.2^{c}$ | $1.30^{c}$ |
| FCA08 | 2 | $9^{a}$ | $120^{a}$ | $2.5^{ab}$ | $0.1^{a}$ | $22.0^{a}$ | $76.3^{a}$ | $0.25^{a}$ |

Values followed by the same letter are no significantly different from each other at $P = 0.05$ according to Fisher's Protected LSD (Least Significant Differences). * After seven days of incubation at 28°C in YED-P plates with bicalcium phosphate as P source.

(Table 2). As we show in the present study some strains nodulating chickpea were identified as *M. tianshanense* and to our knowledge, this is the first report of the nodulation of *C. arietinum* by this species. The type strain of *M. tianshanense* USDA $3592^{T}$ showed low ability (solubilization halo lower than 2 cm) to solubilize phosphate but the strain RCAN03 belonging to this species showed the highest clearing halo on YED-P plates (15 cm). These results are in agreement with those obtained in previous studies (Halder et al. 1990; Peix et al. 2001) in which it is shown that strains nodulating chickpea are the best phosphate solubilizers. In this work we also report for the first time phosphate solubilization produced by strains of *M. tianshanense*.

*Mobilization of phosphorous in plants*

We analysed the content of P, N, Ca, Mg and K in plants inoculated with representative strains of each group of 879F-RAPD (Table 2). According to our results the ability to mobilize phosphorous to plants is directly related to that to solubilize phosphates in vitro. In this way, the highest P content was measured in the plants inoculated with the strain RCAN03 belonging to *M. tianshanense* and the lowest content in the plants inoculated with the strain FCA08 belonging to *M. ciceri*. The effect of the inoculation with different strains on the dry matter and nitrogen content was not related to the ability to solubilize phosphate. The content in K, Ca and Mg was the highest in the plants inoculated with the strain RCAN03 which is the best phosphate solubilizer and the lowest in plants inoculated with the strain FCA08 that showed a low ability to solubilize phosphate. The phosphate solubilizing strains form low number of nodules per plant and this fact is related to the N content and dry weight. This fact was already observed for the strain PECA21 belonging to *M. mediterraneum* (Peix et al. 2001). Although this strain was able to promote the growth of chickpea, the dry matter and fixed nitrogen were less increased than the phosphorous content. Therefore, to obtain an optimal growth promotion of chickpea it could be necessary to inoculate with strains that show a great ability to solubilize phosphate but also a high effectiveness in nodulation and nitrogen fixation. Taking into account that the type strain of *M. tianshanense* was not able to nodulate chickpea and it shows a low ability to solubilize phosphate it is possible that both nodulation and phosphate solubilization are related to determinate subspecies within the same species. Nevertheless, more strains of different subspecies, species and genera must be analyzed to confirm this hypothesis.

In conclusion, this study shows that chickpea can be nodulated by several species, not only by *M. ciceri* and *M. mediterraneum* but also species that were originally described as endosymbionts of other legumes such as *M. tianshanense*. These results support the findings of several authors concerning the nodulation of the same host by several species of rhizobia (Herrera Cervera et al. 1999; Velázquez et al. 2001b). Nevertheless, until the moment rhizobia associated with plants from tribe *Cicerae* were thought to belong to concrete rhizobial species (Perret et al. 2000). In this way, until now only two species have been described as effective endosymbionts of *Cicer arietinum*, *M. ciceri* (Nour et al. 1994) and *M. mediterraneum* (Nour et al. 1995). However, in this work it has been demonstrated that *M. tianshanense* can nodulate either species from tribe *Cicerae* as chickpea and species from other tribes as *Galegae* (*Glycyrrhiza pallidiflora*). Moreover, in this study it is shown that the chickpea isolates from

*M. tianshanense* have a higher ability to solubilize phosphate and mobilize phosphorous to the plants than those of *M. ciceri* and *M. mediterraneum* that are reported at present as the most powerful phosphate solubilizing rhizobia (Peix et al. 2001).

## Acknowledgements

This work was supported by the Junta de Castilla y León and the DGICYT (Dirección General de Investigación Científica y Técnica).

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