The common bean (Phaseolus vulgaris) is a legume indigenous to Mesoamerica and the Andean region of South America that is extensively cultivated throughout the world (Martínez-Romero, 2003). It constitutes a staple cultivation in many developing countries. This legume is considered as a promiscuous host, since it can be nodulated by several rhizobial species. To date, six species belonging to the genus Rhizobium have been identified as endosymbionts of common bean (Amarger et al., 1997; Jordan, 1984; Martínez-Romero et al., 1991; Segovia et al., 1993; Velázquez et al., 2001b) but some other species, from this and other rhizobial genera, have been also described as infective under experimental conditions (Martínez-Romero, 2003). The diversity of rhizobia nodulating P. vulgaris has been widely studied, but, because of its promiscuous nature, novel endosymbionts of this legume should be expected as more ecological niches are studied. In the present work, we analysed several strains nodulating common bean in several soils from the region of Arcos de Valdevez, in the north-west of Portugal, where a traditional mixed farming system, with livestock and crops integrated into a single ecosystem, and local varieties of common beans have been used for centuries. A polyphasic study of these strains, including phenotypic and molecular taxonomic

Rhizobium lusitanum sp. nov. a bacterium that nodulates Phaseolus vulgaris

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The species Phaseolus vulgaris is a promiscuous legume nodulated by several species of the family Rhizobiaceae. During a study of rhizobia nodulating this legume in Portugal, we isolated several strains that nodulate P. vulgaris effectively and also Macroptilium atropurpureum and Leucaena leucocephala, but they form ineffective nodules in Medicago sativa. According to phylogenetic analysis of the 16S rRNA gene sequence, the strains from this study belong to the genus Rhizobium, with Rhizobium rhizogenes and Rhizobium tropici as the closest related species, with 99-9 and 99-2 % similarity, respectively, between the type strains of these species and strain P1-7T. The nodD and nifH genes carried by strain P1-7T are phylogenetically related to those of other species nodulating Phaseolus. This strain does not carry virulence genes present in the type strain of R. tropici, ATCC 11325T. Analysis of the recA and atpD genes confirms this phylogenetic arrangement, showing low similarity with respect to those of R. rhizogenes ATCC 11325T (91-9 and 94-1 % similarity, respectively) and R. tropici IIB CIAT 899T (90-6 % and 91-8 % similarity, respectively). The intergenic spacer (ITS) of the strains from this study is phylogenetically divergent from those of R. rhizogenes ATCC 11235T and R. tropici CIAT 899T, with 85-9 and 82-8 % similarity, respectively, with respect to strain P1-7T. The tRNA profile and two-primer random amplified polymorphic DNA pattern of strain P1-7T are also different from those of R. rhizogenes and R. tropici by several phenotypic characteristics. The results of DNA–DNA hybridization showed means of 28 and 25 % similarity between strain P1-7T and R. rhizogenes ATCC 11235T and R. tropici CIAT 899T, respectively. All these data showed that the strains isolated in this study belong to a novel species of the genus Rhizobium, for which we propose the name Rhizobium lusitanum sp. nov.; the type strain is P1-7T (=LMG 22705T = CECT 7016T).
approaches, showed that they belong to a novel species of the genus *Rhizobium* phylogenetically close to *Rhizobium rhizogenes* and *Rhizobium tropici*.

In this study, 22 strains (Table 1) were isolated from *P. vulgaris* plants growing in three soils from the north-west of Portugal (Arcos de Valdevez region) with different chemical characteristics (data not shown). For isolation of bacterial strains, nodules present in *P. vulgaris* roots were surface sterilized using a 2-5% aqueous solution of HgCl2 for 2 min. The nodules were then washed ten times with sterile water, disrupted in sterile water and cultivated in YMA medium (Bergersen, 1961) at 28°C for 4 days. To test the symbiotic characteristics of the new bacterial isolates, *P. vulgaris, Leucaena leucocephala, Macroptilium atropurpureum* and *Medicago sativa* plants were inoculated as described by Velázquez et al. (2001b), using *R. tropici* CIAT 899T as a control. All the novel strains were able to elicit effective nodules in *P. vulgaris*, *Macroptilium atropurpureum* and *L. leucocephala* and ineffective nodules in *Medicago sativa*. The ability to induce the production of roots on discs of *Daucus carota* was examined as described previously (Moore et al., 1979). The strains isolated in this study were unable to produce these symptoms.

The plasmid content was determined by the method of Plazinski et al. (1985), with the modifications described by Rivas et al. (2002b), using *Ensifer meliloti* GR4 as a reference (Toro & Olivares, 1986). The results obtained (see Supplementary Fig. S1 in IJSEM Online) showed that the strains from this study present seven different plasmid profiles (see Table 1) containing between one and four plasmids, the sizes of which ranged from approximately 90 to 1700 kb, some of which are megaplasmids (>1000 kb).

The nodD and *nifH* genes harbouring by strain P1-7T were amplified and sequenced as described previously (Rivas et al., 2002b). According to nodD and *nifH* gene sequences, the closest relative of strain P1-7T is *Devosia neptuniae* J1T, showing 99.6 and 99.5% similarity, respectively (Supplementary Figs S2 and S3). The same genes from *R. tropici* CIAT 899T showed 99.2 and 99.5% similarity, respectively. In a previous work (Rivas et al., 2002b), we have already shown the high similarity of the nodD and *nifH* genes carried by *D. neptuniae* J1T and those of *R. tropici* CIAT 899T. These results are in agreement with those obtained in the analysis of the host range of strain P1-7T, because it was able to nodulate *Phaseolus* and *Leucaena*, two common hosts for *R. tropici* (Martínez-Romero et al., 1991). Nevertheless, the nodD and *nifH* genes recently sequenced in *R. rhizogenes* ATCC 11325T are phylogenetically distant from those of strain P1-7T, strongly supporting our previous hypothesis that *P. vulgaris* was not the ancestral host of *R. rhizogenes* (Velázquez et al., 2005).

The presence of the *virA* gene in strain P1-7T was analysed using the primers and PCR conditions described previously (Velázquez et al., 2005). The results were negative.

**Table 1.** Differential genotypic characteristics of the novel strains (*Rhizobium lusitanum* sp. nov.) and phylogenetically closely related *Rhizobium* species

<table>
<thead>
<tr>
<th>Strain</th>
<th>TP-RAPD type</th>
<th>RAPD profile</th>
<th>ITS profile</th>
<th>LMW RNA profile</th>
<th>Sequence identity to strain P1-7T*</th>
<th>Plasmid profile</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium lusitanum</em> sp. nov.</td>
<td></td>
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<tr>
<td>P1-7T, P6-18, P6-20</td>
<td>A</td>
<td>I</td>
<td>a</td>
<td>1</td>
<td>100, 100, 100, 100</td>
<td>A</td>
</tr>
<tr>
<td>P1-16, P1-17, P1-18, P1-19, P1-21, P1-22</td>
<td>A</td>
<td>I</td>
<td>a</td>
<td>1</td>
<td>100, 100, 100, 100</td>
<td>B</td>
</tr>
<tr>
<td>P3-12, P3-13, P3-15, P3-16, P3-17, P3-18, P3-19</td>
<td>A</td>
<td>II</td>
<td>a</td>
<td>1</td>
<td>100, 99-5, 98-5, 98-5</td>
<td>C</td>
</tr>
<tr>
<td>P3-20, P3-21, P3-25</td>
<td>A</td>
<td>I</td>
<td>a</td>
<td>1</td>
<td>100, 100, 100, 100</td>
<td>D</td>
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<tr>
<td>P3-24</td>
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<td>I</td>
<td>a</td>
<td>1</td>
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<tr>
<td>P6-7</td>
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<td>a</td>
<td>1</td>
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<td>a</td>
<td>1</td>
<td>100, 100, 100, 100</td>
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<td>CFN 299 (IIA)</td>
<td>B</td>
<td>VIII</td>
<td>b</td>
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<td>99-6, 84-3, 92-3, 92-0</td>
<td>Acosta-Durán &amp; Martínez-Romero (2002)</td>
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<tr>
<td>CIAT 899T (IIB)</td>
<td>D</td>
<td>VI</td>
<td>c</td>
<td>3</td>
<td>99-2, 82-8, 91-8, 90-6</td>
<td>Acosta-Durán &amp; Martínez-Romero (2002)</td>
</tr>
<tr>
<td>Br859 (IIB)</td>
<td>D</td>
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<td>c</td>
<td>3</td>
<td>ND, 83-9, 92-4, 90-7</td>
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<td>ND</td>
</tr>
<tr>
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<td>C</td>
<td>IV</td>
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<td>2</td>
<td>99-9, 89-8, 93-4, 91-8</td>
<td>Velázquez et al. (2005)</td>
</tr>
<tr>
<td>ATCC 11325T</td>
<td>C</td>
<td>V</td>
<td>a</td>
<td>2</td>
<td>99-9, 85-9, 94-1, 91-9</td>
<td>Velázquez et al. (2005)</td>
</tr>
</tbody>
</table>

ND, No data available.

*Values respectively represent similarity for the 16S rRNA gene, ITS and *atpD* and *recA* genes.
coinciding with the inability of our strains to induce pathogenicity symptoms in plants.

The diversity of strains isolated in this study was assessed by randomly amplified polymorphic DNA (RAPD) pattern analysis using the primer M13 (5′-GAGGTTGGCG-GTTCTC-3′) as described previously (Igual et al., 2003). Two different RAPD patterns were found among the 22 strains isolated from the three studied soils (Supplementary Fig. S4). Most of them showed RAPD pattern I (lanes 1–15), whereas seven strains showed pattern II (lanes 16–22). The remaining strains used in this study (lanes 23–28) presented different RAPD patterns (see Table 1).

The RAPD patterns are strain dependent and therefore they are useful in analysing the intraspecific diversity of a rhizobial population (Corich et al., 2001; Moschetti et al., 2005). Other PCR-based profiles, such as two-primer RAPD (TP-RAPD) patterns, are not strain dependent and, according to our previous studies (Rivas et al., 2001, 2004; Zurdo-Piñeiro et al., 2004), are useful in differentiating among rhizobial species. TP-RAPD patterns were analysed according to the method described by Rivas et al. (2002a) using the primers 879F (5′-GCCTGGAGGATCCGGCGCA-3′) and 1522R (5′-AAGGAGTGATC-CANCCRA-3′), which correspond to Escherichia coli positions 879–898 and 1509–1522, respectively. The DNA patterns obtained contain a band that corresponded to the fragment of the 16S rRNA gene amplified with these two primers and several others produced by random amplification on the total DNA (Rivas et al., 2001). In a previous work, we demonstrated that all strains that show identical TP-RAPD pattern belong to the same species. No variations were observed in these patterns in strains from the same species with different plasmid profiles, and they do not vary with the growth phase (Rivas et al., 2001). All the strains isolated in this study presented the same TP-RAPD pattern (see Table 1 and Supplementary Fig. S5) and, therefore, all of them belong to the same bacterial species. This pattern (lanes 1–22) was different from those from strains of R. tropici IIB (lanes 25 and 28), R. tropici IIA (lane 23) and R. rhizogenes (lanes 24, 26 and 27), which are the closest phylogenetically related species on the basis of the 16S rRNA, recA and atpD gene sequences as well as the 16S–23S intergenic spacer (ITS) sequences (see below).

Strains P1-7T and P3-13, which presented different RAPD patterns, were selected for analysis of 16S rRNA, atpD and recA gene sequences, analysis of the ITS sequence and DNA–DNA hybridization experiments.

Nearly complete 16S rRNA gene sequences were obtained in this study according to the method described previously by Rivas et al. (2002b). Sequences of the recA and atpD genes were obtained according to Gaunt et al. (2001). The ITS region was amplified and sequenced as described by Kwon et al. (2005). These sequences were compared with those held in GenBank using the BLASTN program (Altschul et al., 1990). 16S rRNA gene sequences were aligned using the CLUSTAL W software (Thompson et al., 1997) and distances were calculated according to the models of Jukes & Cantor (1969), Kimura (1980), Tajima & Nei (1984) and Tamura & Nei (1993). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987), minimum evolution (Rzhetsky & Nei, 1993) and parsimony analysis (Felsenstein, 1983). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar et al., 2001) was used for all analyses. As no significant topological differences were found among the phylogenetic trees obtained by the different methods assayed, only those trees constructed by using the neighbour-joining method after distance analysis of aligned sequences according to Kimura’s two parameters (ITS and 16S rRNA gene) or Tamura–Nei (recA and atpD genes) models are shown.

The 16S rRNA gene sequences of strains P1-7T and P3-13 exhibit 100 % similarity and, thus, only the type strain was included in the phylogenetic analysis. The resulting neighbour-joining tree corresponding to 16S rRNA gene sequences is shown in Fig. 1 (a full phylogenetic tree is available as Supplementary Fig. S6). The results of the phylogenetic analysis indicate that the strains from this study are related to members of the genus Rhizobium within the family Rhizobiaceae. According to the 16S rRNA gene sequences, the closest relative to strain P1-7T is R. rhizogenes ATCC 11325T, showing 99·9 % similarity, followed by R. tropici IIB CIAT 899T, with 99·2 % similarity. R. tropici IIA CFN 299 showed 99·6 % similarity (95·0 % when an insertion of 72 nucleotides at the beginning of the 16S rRNA gene is considered).

![Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain P1-7T (Rhizobium lusitanum sp. nov.) and closely related species within the genus Rhizobium. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 5 substitutions per 1000 nt.](http://ijs.sgmjournals.org)
According to the results of Gaunt et al. (2001), recA and atpD gene-based phylogenies of members of the family Rhizobiaceae are congruent with the 16S rRNA gene-based phylogeny. Although more studies on the variability of these genes will be necessary to establish the similarity range between strains from the same species and those of different species from the same genus within the family Rhizobiaceae, it seems that they are very useful in the classification and identification of rhizobial isolates (Vinueza et al., 2005a, b). Thus, to confirm the phylogenetic position of the strains isolated in this study, we sequenced their recA and atpD genes (Supplementary Figs S7 and S8). The results obtained completely confirm the phylogenetic position of these strains within the group R. rhizogenes–R. tropici and, moreover, the recA and atpD sequence similarities between strains P1-7T and P3-13 were near to 98.5 % for both genes, demonstrating that these two strains are not clones. The sequence similarities between strain P1-7T and R. rhizogenes ATCC 11325T, R. tropici IIA CFN 299 and R. tropici IIB CIAT 899T were, respectively, 91-9, 92-0 and 90-6 % for recA and 94-1, 92-3 and 91-8 % for atpD. These values are similar to those found between other phylogenetically close species of the genera Rhizobium and Ensifer, such as between Rhizobium leguminosarum and Rhizobium etli or between E. meliloti and Ensifer medicae (Gaunt et al., 2001). These similarity values with respect to R. rhizogenes and R. tropici suggest that the strains isolated in this study may belong to a novel species.

Comparison of 16S–23S rRNA ITS regions provides a fast tool to assess relatedness between closely related rhizobial strains (Kwon et al., 2005). This region was sequenced in the strains P1-7T and P3-13, representative of each of the two RAPD groups. The length of the fragment obtained in both strains was 1281 bp and their sequence similarity was 99.5 %. The same length was obtained for R. rhizogenes ATCC 11325T; however, the ITS regions of R. tropici IIA CFN 299 and R. tropici IIB CIAT 899T were, respectively, 130 and 190 bp shorter (Supplementary Fig. S9). After a pairwise analysis, the ITS sequence of strain P1-7T showed 85-9 % (73-0 % including gaps), 84-3 % (70-9 % including gaps) and 82-8 % (66-3 % including gaps) similarity with respect to those of R. rhizogenes ATCC 11325T, R. tropici IIA CFN 299 and R. tropici IIB CIAT 899T, respectively. In agreement with the phylogenetic analyses based on the 16S rRNA, atpD and recA gene sequences, phylogenetic analysis of ITS sequences also demonstrated that the strains from this study belong to the R. rhizogenes–R. tropici cluster (Supplementary Fig. S10). However, differences in the nucleotide sequence between the type strains of these two species and strain P1-7T suggest that the strains isolated in this study belong to a different species, which is in concordance with other results from this work.

Although discordant phylogenies have been reported within the rrn (16S rRNA) locus in some rhizobial species (van Berkum et al., 2003), the results here obtained on the basis of rrn, recA and atpD sequence analyses show a complete concordance in the phylogenetic location of the strains isolated in this study within the cluster R. rhizogenes–R. tropici. In order to confirm these phylogenetic data we also analysed the low-molecular-weight (LMW) RNA profiles of the isolated strains (Supplementary Fig. S11), as was described recently (Velázquez et al., 2006). These profiles contain three clearly distinguishable zones. The 5S rRNA zone is identical in species from the same genus, as occurs in the case of strain P1-7T, R. rhizogenes ATCC 11325T, R. tropici IIB CIAT 899T and R. tropici IIA CFN 299 (Supplementary Fig. S11, lanes 1–4, respectively). The class 1 and 2 tRNAs are different in different species from the same or different genera and the differences are related to phylogenetic distances on basis of the 16S rRNA gene sequences (Velázquez et al., 2001d). As expected, the tRNA profiles of all of the novel strains were identical (data not shown). As can be seen in Supplementary Fig. S11, the tRNA profile of strain P1-7T (lane 1) has only one band different from R. rhizogenes ATCC 11325T (lane 2), whereas many differences are observed with respect to the tRNA profiles of R. tropici IIB CIAT 899T (lane 3) and R. tropici IIA CFN 299 (lane 4). These results confirmed the placement of the strains from this study in the group of R. rhizogenes and R. tropici, being the most closely related species to the novel strains. The existence of a different tRNA band between the novel strains and R. rhizogenes supports the hypothesis that the strains isolated in this study do not belong to R. rhizogenes, since a single difference in tRNA profiles has been proposed to be diagnostic in bacterial species differentiation (Höfle, 1990). Moreover, the different LMW RNA profiles showed by the strains belonging to the subgroups IIA and IIB of R. tropici, in addition to the results from ITS, 16S rRNA, recA and atpD sequence analyses, as well as the DNA–DNA hybridization results (see below), indicate that they belong to different species.

DNA–DNA hybridization was carried out by using the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001). Strains P1-7T and P3-13 were hybridized with two strains from the subgroup IIB of R. tropici, CIAT 899T and Br859 (Martínez-Romero et al., 1991), which presented the same TP-RAPD pattern (Supplementary Fig. S5, lanes 25 and 28) but different RAPD pattern (Supplementary Fig. S4, lanes 26 and 27), with R. tropici IIA CFN 299 (Supplementary Figs S5 and S4, lanes 23 and 28, respectively) and with three strains of R. rhizogenes, IAM 13571 (de Oliveira et al., 1999), ATCC 11325T and 163C (Velázquez et al., 2005), which presented the same TP-RAPD pattern (Supplementary Fig. S5, lanes 24, 26 and 27) but different RAPD patterns (Supplementary Fig. S4, lanes 23–25). Strains P1-7T and P3-13 showed hybridization values of 90–100 % (Supplementary Table S1). DNA–DNA hybridization between either of these strains and strains belonging to the species R. rhizogenes or R. tropici always yielded values of 43 % or lower. These results indicate that the strains from this study do not belong to either of these recognized species when the recommendation of a threshold value of 70 % DNA–DNA
relatedness for species definition is considered (Wayne et al., 1987).

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The G+C content of DNA of strain P1-7T was determined using the thermal denaturation method (Mandel & Marmur, 1968) as 65±15 mol%. This value is similar to those obtained for other Rhizobium species (Jordan, 1984).

Phenotypic characterization of the strains was based on growth with different carbon and nitrogen sources, the production of exoenzymes and resistance to different antibiotics as described previously (Kersters & De Ley, 1984, Velázquez et al., 2001a, b; Zurdo-Piñeiro et al., 2004) and by using an API 20NE kit according to the manufacturer’s instructions (bioMérieux). For testing antibiotic resistance, the following antibiotics were used: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1 μg), oxytetracycline (30 μg), gentamicin (10 μg), cefuroxime (30 μg) and neomycin (5 μg) (Becton Dickinson). The basal medium was YMA (Vincent, 1970) supplemented with 10 g yeast extract l

* Data from Zurdo-Piñeiro et al. (2004) confirmed in this study.
† Data from this study for R. tropici IIA CFN 299.
‡ Data from this study for several strains included in Velázquez et al. (2001c).

Strains may grow between 10 and 37 °C, pH 5 and 8 and weakly up to 1 % (w/v) NaCl. Denitrification is carried out by the strains from this study. The strains produce β-galactosidase and urease and hydrolyse aesculin in the API 20NE system. They also produce α- and β-arabinosidases, α- and β-galactosidases, β-galactosaminidases, β-cellobiases, α-galactosidases and β-maltosidases and resistance to erythromycin, oxytetracycline and cefuroxime.

Therefore, the novel group can be differentiated genotypically and phenotypically from previously described species and we therefore propose to name it *Rhizobium lusitanum* sp. nov.

**Description of Rhizobium lusitanum sp. nov.**

*Rhizobium lusitanum* (lu.si.ta’num. L. neut. adj. *lusitanum* of *Lusitania*, the Roman name of Portugal, where the strains reported in this study were isolated).

Gram-negative rods, as for the other species of the genus. Colonies are small, pearl white on YMA at 28 °C, which is the optimal growth temperature. The optimum pH is 7–7.5.
in the presence of neomycin. The G + C content of strain P1-7T is 65-15 mol%.

The type strain, P1-7T (= LMG 22705T = CECT 7016), was isolated from effective nodules of Phaseolus vulgaris in Portugal.

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