Revision of the taxonomic status of the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889\(^\text{AL}\), *Rhizobium phaseoli* Dangeard 1926\(^\text{AL}\) and *Rhizobium trifolii* Dangeard 1926\(^\text{AL}\). *R. trifolii* is a later synonym of *R. leguminosarum*.

Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov.

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The taxonomic status of the species *Rhizobium leguminosarum*, *Rhizobium trifolii* and *Rhizobium phaseoli* was analysed in this study on the basis of their molecular and phenotypic characteristics. According to the results, the type strain *R. phaseoli* ATCC 14482\(^\text{T}\) does not belong to any of the already described species of the genus *Rhizobium* and it should therefore be considered as a different species. In contrast, the strains of *R. trifolii* examined belonged to *R. leguminosarum* and thus *R. trifolii* is a later synonym of *R. leguminosarum*. The results of the analysis of 16S–23S intergenic spacer region and *rrs*, *recA* and *atpD* gene sequences as well as those of DNA–DNA hybridization experiments and phenotypic characterizations showed that the type strains *R. leguminosarum* USDA 2370\(^\text{T}\) and *R. leguminosarum* DSM 30132 do not belong to the same species. Taking into account that strain USDA 2370\(^\text{T}\) corresponds to the original strain of this species, 3Hoq18\(^\text{T}\), this strain should be considered as the true type strain of *R. leguminosarum* whereas strain DSM 30132 should be reclassified as a different species, for which the name *Rhizobium pisi* sp. nov. is proposed (type strain, DSM 30132\(^\text{T}\) = NCIMB 11478\(^\text{T}\)).

Until 1984, *Rhizobium leguminosarum*, *Rhizobium trifolii* and *Rhizobium phaseoli* were considered to be separate species. In 1984, Jordan (1984) recorded a proposal in Bergey’s *Manual of Systematic Bacteriology* for the reclassification of *R. trifolii* and *R. phaseoli* as two biovars of *R. leguminosarum*. Since then, most rhizobiologists have accepted this proposal, although no formal proposal has been published. In the second edition of Bergey’s *Manual*, the description of the species *R. leguminosarum* is included but its re-examination is recommended (Kuykendall, 2005). In order to analyse the taxonomic status of these species by the molecular approaches currently used for bacterial species definition, we sequenced the 16S rRNA, *atpD* and *recA* genes and the 16S–23S intergenic spacer (ITS) regions of the type strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* according to previously described methods (Gaunt et al., 2001; Kwon et al., 2005; Rivas et al., 2002). These sequences were compared with those held in GenBank using the BLASTN program (Altschul et al., 1990). The sequences were aligned using CLUSTAL W software (Thompson et al., 1994) and the distances were calculated according to the model of Kimura (1980). Phylogenetic trees were inferred using the neighbour-joining method.
Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar et al., 2001) was used for all analyses. The DNA–DNA hybridization experiments were performed according to the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001).

When the 16S rRNA gene sequence (Fig. 1) of *R. leguminosarum* ATCC 10004T (held in our lab since 1990) was compared with the sequences held in the GenBank database, it was surprisingly different (99.2 % sequence similarity) from that of the *R. leguminosarum* USDA 2370T (GenBank accession no. U29386) sequenced by van Berkum et al. (1996). The sequences of the recA and atpD genes of *R. leguminosarum* ATCC 10004T held in our lab and those of *R. leguminosarum* USDA 2370T showed differences that suggested they did not belong to the same species (see Figs 2 and 3). The sequences of the 16S–23S ITS confirmed these results (Fig. 4) since our *R. leguminosarum* ATCC 10004T showed relevant differences with respect to the 16S–23S ITS sequence of *R. leguminosarum* LMG 14904T sequenced by Kwon et al. (2005).

From this point, assuming there was obviously a problem with our ATCC type strain, we investigated the history of the original strain of *R. leguminosarum* named 3Hoq18T in the collections from which sequence data are available. According to the information recorded in their respective web pages, the USDA collection has the original deposit of *R. leguminosarum* 3Hoq18T and the LMG collection received the strain directly from the ATCC collection in 1994. Therefore it seems that the ATCC collection has
distributed different type strains of *R. leguminosarum* with the same accession number before and after 1994. In order to confirm this hypothesis, we analysed the type strains deposited in other relevant collections such as DSMZ and NCIMB which had received the strains from ATCC before 1990. The results obtained on the basis of *atpD*, *recA* and ITS sequences confirmed that the old strain ATCC 10004^T^, that we received in 1990, was identical to strains DSM 30132 and NCIMB 11478 and was different from strains LMG 14904^T^ and USDA 2370^T^ (which were identical to...

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**Fig. 2.** Neighbour-joining tree based on partial *recA* gene sequences of species of the genus *Rhizobium* and other related organisms. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

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**Fig. 3.** Neighbour-joining tree based on partial *atpD* gene sequences of species of the genus *Rhizobium* and other related organisms. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.
each other). Finally, we analysed the strain that is currently provided by the ATCC under the designation of ATCC 10004¹ and the results showed that it was identical to strains LMG 14904¹ and USDA 2370¹.

All these results suggest that there are currently two different type strains of *R. leguminosarum* deposited in culture collections (see Figs 1, 2, 3 and 4 and Supplementary Table S1 in IJSEM Online) and that these two strains do not belong to the same species. Therefore it is necessary to choose one of the two strains as the type strain of *R. leguminosarum* and to establish the taxonomic status of the other strain. There is also the need to take a decision about the status of the names *R. trifolii* and *R. phaseoli* according to the current taxonomic criteria used to define bacterial species.

Since strain USDA 2370¹ is the original deposit corresponding to strain 3Hoq18¹, this strain should have preference for retaining the name *R. leguminosarum* and should be considered as the true type strain of this species. Strains ATCC 10004¹ (the currently available strain) and LMG 14904¹ which show 100 % sequence similarity in the 16S rRNA, recA and atpD genes and in the 16S–23S ITS region are equivalent to strain USDA 2370¹ (Figs 1, 2, 3 and 4, respectively). Therefore, strain DSM 30132 (=NCIMB 11478) should no longer be considered as the type strain of *R. leguminosarum* and should be regarded as belonging to a separate species (see below).

The type strain of *R. trifolii* ATCC 14480¹ (=DSM 30137¹=LMG 8819¹), showed 99.2 % and 87.6 % similarity in the 16S rRNA gene and ITS sequences, respectively, with the type strain of *R. leguminosarum* USDA 2370¹ and 91.2 % and 91.5 % similarity to the recA and atpD genes, respectively (Supplementary Table S1, Figs 2, 3 and 4). DNA–DNA hybridization between *R. trifolii* ATCC 14480¹ and *R. leguminosarum* USDA 2370¹ showed 51 % relatedness (Supplementary Tables S2 and S3). When compared with *R. etli* CFN 42¹, strain ATCC 14482¹ showed 99.3, 93.8, 90.7 and 94.1 % sequence similarity for the 16S rRNA gene, ITS region, recA gene and atpD gene, respectively. *R. etli* CFN 42¹ and strain ATCC 14482¹ showed 52 % DNA–DNA relatedness values. Although further studies on the variability of the recA and atpD genes will be necessary to establish the sequence similarity range

![Diagram](http://ijs.sgmjournals.org/2487)

**Fig. 4.** Neighbour-joining tree based on 16S–23S rRNA gene intergenic sequences of species of the genus *Rhizobium* and phylogenetically related species. Bootstrap values calculated for 1000 replications are indicated. Gaps were not considered. Bar, 5 nt substitutions per 100 nt.
between strains from the same species and those of different species from the genus *Rhizobium*, similarities ranging from approximately 90 to 95 % have been found in species of this genus that showed DNA–DNA hybridization values lower than 50 % (Valverde et al., 2006). Therefore the results of analyses of these housekeeping genes and DNA–DNA hybridization studies, together with the phenotypic differences between *R. phaseoli* and the remaining species of the same phylogenetic group (Table 1) showed that *R. phaseoli* should be retained as a separate species according to the criteria currently used for the definition of bacterial species.

Strain DSM 30132 (=NCIMB 11478), deposited in the DSMZ culture collection as the type strain of *R. leguminosarum*, was analysed and compared with strains *R. leguminosarum* USDA 2370T and *R. phaseoli* ATCC 14482T by sequence comparisons of different genes and by DNA–DNA hybridization experiments and the results of these investigations are recorded in Supplementary Tables S2 and S3. When strain DSM 30132 was compared with *R. etli* CFN 42T, the sequence similarity values were 99.5, 87.9, 91.6 and 91.4 % for the 16S rRNA gene, ITS region, *recA* gene and *atpD* gene, respectively. The DNA–DNA hybridization value between strain DSM 30132 and strain CFN 42T was 52 %. The sequence similarity values obtained from the analyses of the ITS region and the *recA* and *atpD* genes in addition to the values obtained in DNA–DNA hybridization experiments showed that strain DSM 30132 does not belong to *R. leguminosarum*, *R. phaseoli* or *R. etli*.

Phenotypic characterization of the strains from this study was based on growth with different carbon and nitrogen sources, the production of exoenzymes (on PNP-substrates) and resistance to different antibiotics as has been previously described (Velázquez et al., 2001a, b; Zurdo-Piñeiro et al., 2004; Valverde et al., 2006) and by using API 20NE tests according to the manufacturer’s instructions. The temperature range for growth was determined by incubating cultures in YMA medium (Bergersen, 1961) between 4 and 45 °C. The pH range was determined in the same medium with a final pH from 4.0 to 10.0. Salt tolerance was tested in YMA medium containing 0–5 % (w/v) NaCl. For testing antibiotic resistance, the following antibiotics were used (µg per disc): ampicillin (2 µg), erythromycin (2 µg), ciprofloxacin (5 µg), penicillin (10 U), polymyxin (300 U), cloxacillin (1 µg), oxytetracycline (30 µg), gentamicin (10 µg), cefuroxime (30 µg) and neomycin (5 µg) (Becton Dickinson, USA). The basal medium was YMA (Vincent, 1970) supplemented with 10 g l−1 of yeast extract.

The phenotypic differences between strain DSM 30132 and the remaining species of the phylogenetic group (*R. leguminosarum*, *R. phaseoli* and *R. etli*) are shown in Table 1. Taken together, these genotypic and phenotypic data show that strain DSM 30132 does not belong to *R. leguminosarum*, *R. phaseoli* or *R. etli* but to a novel species, for which we propose the name *Rhizobium pisi* sp. nov.

**Description of *Rhizobium pisi* sp. nov.**

*Rhizobium pisi* (pi’si. L. gen. neut. n. *pisi* of the pea, referring to the isolation source of this micro-organism, nodules of *Pisum sativum*).

Gram-negative rods as found for the other species of the genus. Colonies are small and pearl white coloured in YMA at 28 °C, the optimal growth temperature. The optimum pH for growth is 7–7.5. Growth is observed between 10 and 37 °C, at pH 5 to 8 and at up to 1 % (w/v) NaCl. Nitrate reduction is negative. Positive for the production of β-galactosidase and urease and for the hydrolysis of aesculin in the API 20NE system. Positive for the production of α- and β-arabinosidases, α- and β-glucosidases, α- and

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**Table 1. Phenotypic differences among the recognized species of the phylogenetic group of *R. leguminosarum***

<table>
<thead>
<tr>
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<td>Urea (in API 20NE)</td>
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<td>+</td>
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*Weakly positive result in some strains.
β-galactosidas, β-glucosaminidase, β-galactosaminidase, β-cellobiase, α- and β-fucosidas, α- and β-mannosidas, α-maltosidase, α- and β-xilosidas, α-rhamnosidas, lactosidas and acid and alkaline phosphatas using chromogenic paranimotrophin substrates. The production of β-maltosidase is weak. The production of indole, arginine dehydrolyse aesculin in the API 20NE system. Glucose, l-arabinose, fructose, galactose, l-rhamnose, xylose, melibiose, lactose, cellobiose, mannose, adenitol, mannitol, sorbitol, inositol, xylitol, N-acetylglucosamine, maltose, raffinose, sucrose, trehalose, salicin, pyruvate, gluconate, l-malate, l-alanine, l-histidine, l-serine, aspartate, glutamate, betaine and sarcosine are used as sole carbon sources, whereas l-sorbose, melezitose, erythritol, citrate, caproate, adipate, propionate, phenylacetate, l-arginine, l-lysine and l-valine are not used as sole carbon sources. Sensitive to ciprofloxacin, polymyxin B, oxytetracycline, ampicillin and gentamicin. Weakly sensitive to neomycin and erythromycin. Resistant to penicillin, cefuroxime and cloxacillin.

The type strain, DSM 30132T (=NCIMB 11478T), was isolated from effective nodules of Pisum sativum and also nodulate Trifolium repens and Phaseolus vulgaris. The DNA G+C content of the type strain is 61.5 mol%.

**Emended description of Rhizobium leguminosarum** (Frank 1879) Frank 1889

*Rhizobium leguminosarum* [le.gu.mi.no.sa’rum. N.L. gen. pl. fem. n. *leguminosarum* of Leguminosae (the former systematic family name of the legumes)].

Gram-negative rods as characteristic for the other species of the genus. Colonies are small, pearl white coloured in YMA at 28 °C, the optimal growth temperature. The optimum pH for growth is 7–7.5. Growth occurs at 10 to 35 °C and at pH 5 or in the presence of 1% (w/v) NaCl. Nitrate reduction is negative. Positive for the production of β-galactosidase and urease; aesculin hydrolysis is variable in the API 20NE system. Positives for the production of α- and β-arabinosidas, α- and β-glucosidas, α- and β-galactosidas, α-glucosaminidase, α- and β-fucosidas, α- and β-mannosidas, α- and β-xilosidas, α-rhamnosidas and acid and alkaline phosphatas using chromogenic paranimotrophin substrates. The production of β-glucosaminidase and β-cellobiase was variable. The production of lactosidas and α- and β-maltosidas is negative for most strains. The production of indole, arginine dehydrolyse and gelatinase is negative in the API 20NE tests. Glucose, l-arabinose, fructose, galactose, l-rhamnose, xylose, melibiose, cellobiose, mannose, mannitol, sorbitol, inositol, xylitol, N-acetylglucosamine, maltose, raffinose, sucrose, trehalose, salicin, l-alanine, l-histidine, aspartate, glutamate, betaine and sarcosine are used as the sole carbon source, whereas l-sorbose, melezitose, caproate, adipate, citrate, pyruvate, propionate, phenylacetate, l-serine, l-lysine and l-valine are not utilized as a sole carbon source. Erythritol is used as a carbon source by most strains. l-Arginine is weakly utilized by some strains as a carbon source. Assimilation of l-malate and gluconate is variable. Sensitive to ciprofloxacin, polymyxin B, oxytetracycline, cefuroxime and gentamicin. Weakly sensitive to neomycin and erythromycin. Resistant to penicillin and cloxacillin. Resistance to ampicillin is variable.

The type strain, USDA 2370T (=ATCC 10004T=LMG 14904T), was isolated from effective nodules of *Pisum sativum* and is also known to nodulate *Trifolium repens* and *Phaseolus vulgaris*. The DNA G+C content of the type strain is 62.5 mol%.

**Emended description of Rhizobium phaseoli** Dangeard 1926

Gram-negative rods as characteristic for the other species of the genus. Colonies are small, pearl white coloured in YMA at 28 °C, the optimal growth temperature. The optimum pH for growth is 7–7.5. Growth occurs between 10 and 35 °C and at between pH 6 and 8. Growth at 37 °C is weak. Growth at pH 5 and in the presence of 1% (w/v) NaCl is negative. Nitrate reduction is negative. Positive for the production of β-galactosidase and urease and is able to hydrolyse carboxylases in the API 20NE system. Produces α- and β-arabinosidas, α- and β-glucosidas, α- and β-galactosidas, β-glucosaminidase, β-galactosaminidase, α- and β-fucosidas, α- and β-mannosidas, α- and β-xilosidas, rhamnosidas and acid and alkaline phosphatas using chromogenic paranimotrophin substrates. The production of β-cellobiase and lactosidas is weak and tests for the production of α- and β-maltosidas are negative using the same substrates. The production of indole, arginine dehydrolyse and gelatinase is negative in the API 20NE system. Glucose, l-arabinose, fructose, galactose, l-rhamnose, xylose, melibiose, cellobiose, mannose, mannitol, sorbitol, inositol, xylitol, N-acetylglucosamine, maltose, raffinose, sucrose, trehalose, salicin, l-histidine, aspartate, glutamate, betaine and sarcosine are used as the sole carbon source, whereas l-sorbose, melezitose, erythritol, citrate, caproate, adipate, propionate, phenylacetate, l-arginine, l-lysine and l-valine are not utilized as the sole carbon source. Pyruvate assimilation is weak. Sensitive to ciprofloxacin, polymyxin B, oxytetracycline, cefuroxime and gentamicin. Weakly sensitive to neomycin and erythromycin. Resistant to ampicillin, penicillin and cloxacillin.

The type strain, ATCC 14482T (=DSM 30137T), was isolated from effective nodules of *Pisum sativum* and also nodulate *Trifolium repens*, but not *Pisum sativum*. The G+C content of the type strain is 59.1 mol%.

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