Mesorhizobium acaciae sp. nov., isolated from root nodules of Acacia melanoxylon R. Br.

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Three novel strains, RITF741T, RITF1220 and RITF909, isolated from root nodules of Acacia melanoxylon in Guangdong Province of China, have been previously identified as members of the genus Mesorhizobium, displaying the same 16S rRNA gene RFLP pattern. Phylogenetic analysis of 16S rRNA gene sequences indicated that the three strains belong to the genus Mesorhizobium and had highest similarity (100.0 %) to Mesorhizobium plurifarium LMG 11892T. Phylogenetic analyses of housekeeping genes recA, atpD and glnII revealed that these strains represented a distinct evolutionary lineage within the genus Mesorhizobium. Strain RITF741T showed >73 % DNA–DNA relatedness with strains RITF1220 and RITF909, but <60 % DNA–DNA relatedness with the closest type strains of recognized species of the genus Mesorhizobium. They differed from each other and from their closest phylogenetic neighbours by presence/absence of several fatty acids, or by large differences in the relative amounts of particular fatty acids. While showing distinctive features, they were generally able to utilize a wide range of substrates as sole carbon sources based on API 50CH and API 20NE tests. The three strains were able to form nodules with the original host Acacia melanoxylon and other woody legumes such as Acacia aneura, Albizia falcataria and Leucaena leucocephala. In conclusion, these strains represent a novel species belonging to the genus Mesorhizobium based on the data obtained in the present and previous studies, for which the name Mesorhizobium acaciae sp. nov. is proposed. The type strain is RITF741T (=CCBAU 101090T =JCM 30534T), the DNA G + C content of which is 64.1 mol% (Tm).

Australian blackwood, Acacia melanoxylon R. Br., is a native and adaptable tree species in southern Australia and has been introduced to Africa, Asia, Europe, South America and other regions (Hausen & Schmalle, 1981; Calviño-Cancela et al., 2014). Acacia melanoxylon is cold/drought resistant, and is variably used for erosion control and landscaping and its beautiful heartwood is used in cabinet-making (Searle, 2000). It has therefore been grown in China since the late nineteenth century and now has been extensively cultivated in Fujian, Guangdong, Guangxi, Hainan and Jiangxi Provinces in southern China. In addition, several species of rhizobia form microsymbionts with Acacia melanoxylon, providing nitrogen to host plants and also improving soil fertility (Dou et al., 2012).

In a previous study, we isolated 174 strains from the root nodules of Acacia melanoxylon plants which were classified into nine types based on 16S rRNA gene RFLP analysis (Dou et al., 2012). Three representative strains belonging to 16S rRNA PCR-RFLP type B were further studied to determine their taxonomic status using a polyphasic approach.

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The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains RITF741T, RITF1220 and RITF909 are J0697665, J0697677 and J0697670, respectively, and those for the partial recA, atpD, glnII and nifH gene sequences of strain RITF741T are KM358158, KM358143, KM358151 and KP193087, respectively.

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.
The three strains from this study were collected from Huizhou Xiangtoushan Forest Farm (23° 19' 59.90"N 114° 27' 37.43"E) and Zengcheng Forest Farm (23° 17' 41.63"N 113° 48’ 59.81"E), Guangdong Province. The tested strains were maintained on yeast mannitol agar (YMA) medium at 4 °C (Vincent, 1970) in a laboratory of the Research Institute of Tropical Forestry. For experimental analyses, the three strains and reference strains were incubated at 28 °C in a constant temperature incubator, unless specifically noted otherwise.

Analysis of 16S rRNA gene sequences is a widely used method to determine the phylogenetic relationships among bacteria (So et al., 1994). As the 16S rRNA gene of rhizobia displays little divergence between species, analyses of housekeeping gene sequences are required to assign and identify rhizobial species. The housekeeping genes recA, atpD and glnII used as phylogenetic markers are sufficiently conserved, and show greater sequence divergence than the 16S rRNA gene (Stackebrandt et al., 2002; Xu et al., 2013). In this study, the 16S rRNA gene and partial sequences of the above three housekeeping genes and symbiotic gene nifH were amplified from the test strains. The length of 16S rRNA gene PCR products was ~1450 bp with the universal primers 27F and 1492R (DeLong, 1992); primers for the housekeeping genes recA (~600 bp), atpD (~530 bp) and glnII (~680 bp) are described by Islam et al. (2008). PCR primers nifHF and nifHI were used to amplify partial sequences of the symbiotic nifH gene (~800 bp) and PCR conditions were similar to those reported by Laguerre et al. (2001). All target genes were amplified using a GeneAmp PCR system 9700 (ABI) and sequenced with a BigDye terminator v3.1 kit using an ABI-PRISM 3730 Genetic Analyzer (ABI) with protocols recommended by the manufacturer. The corresponding sequences of recognized species of the genus Mesorhizobium were obtained from the GenBank database. The program MEGA 5.2 (Tamura et al., 2011) was employed to reconstruct phylogenetic trees based on the three housekeeping genes and symbiotic gene nifH sequences, used to characterize and identify unknown strains of rhizobia (Jarvis et al., 2000) reports that cellular fatty acid profiles can be used to characterize and identify unknown strains of rhizobia and for establishing taxonomic relationships between species. The three novel strains had nearly identical sequences for the nifH gene (Fig. 2). High-molecular-mass DNA for DNA–DNA hybridization studies and DNA base composition determination was extracted according to the method of Marmur (1961). The DNA–DNA hybridizations were carried out with strains RITF741T, RITF1220 and RITF909 together with Mesorhizobium septentrenale SDW014T, Mesorhizobium silamarinense CCBAU01550T, M. hawassense AC99bT, M. shonenese AC39aT and Mesorhizobium abyssinicae AC98cT using the spectrophotometric method (De Ley et al., 1970). The G+C content of the DNA was measured by the thermal denaturation method of Marmur & Doty (1962) using Escherichia coli K-12 as the standard. The DNA–DNA relatedness values among the representative strains of the novel group were always higher than 73 %, while the values between strain RITF741T and the type strains of the reference species were lower than 60 % (ranging from 21.4 to 59.8 %, Table S2). Based on the recommended threshold value of 70 % DNA–DNA relatedness (Wayne et al., 1987), the results indicate that the strains should be considered as representing a novel species. The DNA G+C contents of strains RITF741T, RITF1220 and RITF909 were 64.1, 64.3 and 64.1 mol%, respectively (Table S2), which are similar to the values of other previously reported species of the genus Mesorhizobium (Jarvis et al., 1997).

Symbiotic genes are often located in transferable elements, such as plasmids or symbiotic islands, and phylogenetic analyses may reveal events of lateral gene transfer among rhizobial symbiotic genes (Haukka et al., 1998). According to nifH gene sequence analysis (Fig. S2), the three novel strains had identical sequences, and shared highest similarity (91.3 %) with Mesorhizobium amorphae ACCC 19665T and Mesorhizobium huakuii CCBAU 25056, respectively isolated from Amorpha fruticosa (Wang et al., 1999) and Lespedeza inschanica (Gu et al., 2007) grown in China.

DNA–DNA hybridization is a standard approach for defining novel bacterial species (Wayne et al., 1987; Zhou et al., 2010). In this study, the DNA–DNA hybridization experiment was conducted based on the results of multilocus sequence analysis (Fig. 2). Phylogenetic analyses of the 16S rRNA gene showed that similarity among the representative strain RITF741T and the type strains of recognized species of the genus Mesorhizobium was more than 97.6 %, suggesting that this novel isolate belonged to the genus Mesorhizobium. Strains RITF741T, RITF909 and RITF1220 had the same 16S rRNA gene sequences, and they shared highest similarity (100.0 %) with Mesorhizobium plurifarium LMG 11892T (Fig. 1; Table S1, available in the online Supplementary Material).

The three novel strains had nearly identical sequences for the three housekeeping genes recA, atpD and glnII, and they shared <95.5 % similarity with the type strains of all recognized Mesorhizobium species (Table S1 and Fig. S1). In the phylogenetic trees based on recA and atpD gene sequences, strain RITF741T was most similar to Mesorhizobium muleiense CCBAU 83963T (93.8 % similarity) and Mesorhizobium hawassense AC99bT (95.5 % similarity), respectively. The atpD and glnII gene sequences of the three novel strains were most closely related to that of Mesorhizobium shonense AC39aT (94.0 % similarity). Nonetheless, the novel strains showed different close phylogenetic neighbours depending on the housekeeping gene analysed. Multilocus sequence analysis was used based on the three housekeeping genes recA, atpD and glnII. The strains of the novel group in the concatenated tree grouped in a monophyletic clade with other previously defined species (Fig. 2).
The cellular fatty acid profiles of the strains of the novel group and their closest phylogenetic neighbours in the genus *Mesorhizobium* are shown in Table 1. The three strains (RITF741\(^T\), RITF1220 and RITF909) were assigned to the genus *Mesorhizobium* as they all possessed C\(_{16}:0\), iso-C\(_{17}:0\) and C\(_{18}:0\) fatty acids, but lacked C\(_{20}:3\)\(\omega_6,9,12\) and summed feature 2 (including C\(_{12}:0\), unknown ECL 10.928, iso-C\(_{16}:1\) I and/or C\(_{14}:0\) 3-OH), in accordance with previous reports (Tighe et al., 2000; Zhao et al., 2012). However, the novel strains could be differentiated from their closest phylogenetic neighbours based on differences in the relative concentration of particular fatty acids such as C\(_{19}:0\) cyclo \(\omega 8\)c (Table 1).

Phenotypic features of representative strain RITF741\(^T\) were determined and compared with those of the type strains of some of the closely related species of the genus *Mesorhizobium* using the API 50CH and API 20NE kits (bioMerieux) following the manufacturer’s instructions. The tested strains were cultivated on YM minus-mannitol medium as described by Lu et al. (2014). Distinctive phenotypic/physiological features of strain RITF741\(^T\) were identified demonstrating that it
was able to utilize a wide range of substrates as sole carbon sources. In addition, strain RITF741\textsuperscript{T} differed from the reference species based on their negative or weak use of substrates as sole carbon sources, such as methyl-β-D-xylopyranoside, salicin and raffinose (Table 2).

It is generally acknowledged that the ability of a rhizobium to nodulate with a range of leguminous plants, as a practical characteristic, is an essential determinant for the description of a novel rhizobial species (Graham et al., 1991; Wang et al., 2007). Strain RITF741\textsuperscript{T}, representing the novel Mesorhizobium group, was inoculated onto four legume species. Tissue cultured seedlings of *Acacia melanoxylon* were used in this study. Seeds of *Leucaena leucocephala*, *Albizia falcataria* and *Acacia aneura* were scarified and surface-sterilized using concentrated sulfuric acid (20 min), and then washed with sterilized water until no trace of acid remained (de Lajudie et al., 1998). Both inoculated and uninoculated seedlings were grown in a greenhouse for 2 months. The results showed good nodulation of strain RITF741\textsuperscript{T} on the roots of all the four woody legume species (Fig. S3).

The distinct genotypic and phenotypic characteristics of strains RITF741\textsuperscript{T}, RITF1220 and RITF909 suggest that they represent a novel species of the genus *Mesorhizobium*, for which we propose the name *Mesorhizobium acaciae* sp. nov.

**Description of *Mesorhizobium acaciae* sp. nov.**

*Mesorhizobium acaciae* sp. nov. (a.ca’ci.ae. L. gen. n. aca’ciae of *Acacia*, a genus of leguminous plants, referring to the host from which the type strain was isolated).

Cells are Gram-negative, non-spore-forming, flagellated rods. Colonies on YMA plates are circular, convex, white.
and opaque, usually 1–2 mm in diameter within 5–7 days of incubation at 28 °C. Optimum growth conditions on YMA medium are pH 7.0 and 28 °C. Positive reactions for nitrate reduction, β-galactosidase, oxidase and catalase production. Negative for indole production (tryptophan), glucose fermentation, arginine dihydrolase reaction and hydrolysis of gelatin. Production of urease is variable, being positive for the type strain. Produces acid in bromothymol blue.

In API 50 CH and API 20 NE kits, the following substrates are used as sole carbohydrate source: glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, arbutin, aesculin, cellobiose, maltose, sucrose, trehalose, xylitol, turanose, D-lyxose, D-fucose, L-fucose and D-arabitol; grows slowly using L-sorbose, methyl α-D-glucopyranoside, N-acetylglucosamine, salicin, gentiobiose, potassium 5-ketogluconate; but cannot grow with methyl β-D-xylopyranoside, methyl β-D-mannopyranoside, amygdalin, inulin, melezitose, starch, glycojen, potassium gluconate, potassium 2-ketogluconate, capric acid, adipic acid, trisodium citrate and phenyl-lactic acid. Variable results for growth with erythritol, dulcitol, lactose, melibiose, raffinose, D-tagatose, L-arabitol and malic acid. Summed feature 8 (C_{18:1} \ varepsilon_6c/C_{18:1} \ varepsilon_7) and C_{19:0} \ varepsilon_8c are abundant fatty acids. Effective nodulation is observed with the original host Acacia melanoxylon and other woody legumes such as Acacia aneura, Albizia falcataria and Leucaena leucocephala.

The type strain, RITF741^T (=CCBAU 101090^T=JCM 30534^T), was isolated from active root nodules of Acacia melanoxylon.
R. Br. collected from Guangdong Province, southern China. The DNA G+C content of the type strain is 64.1 mol% \((T_m)\). RITF1220 and RITP909 are additional strains of the species.

Acknowledgements

We thank Dr Yanming Jin and students of China Agricultural University for their valuable suggestions and experimental assistance. This study was supported by the Special Fund for Forest Scientific Research in the Public Welfare, China (Grant no. 201004075) and the Fundamental Research Funds for the Central Non-profit Research Institution of RITF (Grant no. RITFYWZX201207).

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