Bradyrhizobium lablabi sp. nov., isolated from effective nodules of *Lablab purpureus* and *Arachis hypogaea*

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Five strains isolated from root nodules of *Lablab purpureus* and *Arachis hypogaea* grown in the Anhui and Sichuan provinces of China were classified as members of the genus *Bradyrhizobium*. These strains had identical 16S rRNA gene sequences which shared 99.48 %, 99.48 % and 99.22 % similarity with the most closely related strains of *Bradyrhizobium jicamae* PAC68^T, *Bradyrhizobium pachyrhizi* PAC48^T and *Bradyrhizobium elkanii* USDA 76^T, respectively. A study using a polyphasic approach, including 16S rRNA gene RFLP, IGS-RFLP, BOX-PCR, comparative sequence analysis of the 16S–23S rRNA intergenic spacer (IGS) and the *recA*, *atpD* and *glnII* genes, DNA–DNA hybridization and phenotypic tests, showed that the five strains clustered into a coherent group that differentiated them from all recognized species of the genus *Bradyrhizobium*. Sequencing of *nifH* and *nodC* genes and cross-nodulation tests showed that the representative strains CCBAU 23086^T, CCBAU 23160 and CCBAU 61434, isolated from different plants, had identical *nifH* and *nodC* gene sequences and were all able to nodulate *Lablab purpureus*, *Arachis hypogaea* and *Vigna unguiculata*. Based upon these results, the name *Bradyrhizobium lablabi* sp. nov. is proposed for this novel species and strain CCBAU 23086^T (=LMG 25572^T=HAMBI 3052^T) is designated as the type strain. The DNA G+C mol% is 60.14 (T_m).

Arachis hypogaea L. (peanut or groundnut) is an important legume crop that provides food and edible oil for direct human consumption. It plays a significant part in the economy of many countries in the world (El-Akhal *et al.*, 2008). Most rhizobial isolates nodulating peanut belong to the genus *Bradyrhizobium*, such as *Bradyrhizobium japonicum* (Chen *et al.*, 2003; El-Akhal *et al.*, 2009; Taurian *et al.*, 2006), although some other effective fast-growing rhizobia have also been described (El-Akhal *et al.*, 2008; Taurian *et al.*, 2006).

Lablab purpureus originated in Africa and has been widely distributed in many tropical and subtropical countries where it has become naturalized. In China, *L. purpureus* is grown as an annual or a short-lived perennial in different regions. In these areas, the seeds and immature pods are used for human consumption while the herbage is used as a green manure (Murphy & Colucci, 1999). To date, only a few rhizobial strains isolated from *L. purpureus* nodules have been studied and most of these have been identified as members of the genus *Bradyrhizobium* (Morrison *et al.*, 1986), except for the broad host range strain NGR 234 (Trinick, 1980).

During a study of rhizobia nodulating *A. hypogaea* and *L. purpureus* grown in southern China, 73 isolates (of a

Abbreviations: BOX-PCR, BOX-repeat-based PCR; IGS, 16S–23S rRNA intergenic spacer; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are GU433448, GU433456 and GU433471 for *B. lablabi* sp. nov. strains CCBAU 23086^T, CCBAU 23160 and CCBAU 61434, respectively. The accession numbers for the partial IGS, *atpD*, *recA*, *glnII*, *nifH* and *nodC* gene sequences of the three strains, CCBAU 23086^T, CCBAU 23160 and CCBAU 61434, are as follows: GU433583, GU433599 and GU433589 (IGS); GU433473, GU433482 and GU433495 (*atpD*); GU433522, GU433530 and GU433544 (*recA*); GU433498, GU433505 and GU433519 (*glnII*); GU433546, GU433553 and GU433563 (*nifH*); GU433565, GU433571 and GU433581 (*nodC*).

Supplementary figures and tables are available with the online version of this paper.

total of 93) were characterized and classified in the genus *Bradyrhizobium* based on 16S rRNA gene RFLP, 16S–23S rRNA intergenic spacer (IGS) RFLP and sequence analyses of *rrs*, IGS, *atpD*, *recA*, *nodC* and *nifH* genes (unpublished data). Within these bradyrhizobia, five isolates (Table 1) exhibited distinct characteristics and differed from recognized species of the genus *Bradyrhizobium*. For 16S rRNA gene RFLP, the gene was amplified with primers P1 and P6 by the PCR protocol of Tan *et al.* (1997) from DNA extracted from each strain by a routine method (Terefework *et al.*, 2001). The amplified fragments were digested with *Msp*I, *Hin*fI, *Alu*I and *Hae*III as specified by the manufacturer. The five strains had identical patterns and were defined as the same rRNA type, which grouped together with *Bradyrhizobium jicamae* PAC68^T, *Bradyrhizobium pachyrhizi* PAC48^T and *Bradyrhizobium elkanii* USDA 76^T (see Supplementary Fig. S1 in IJSEM Online) in the cluster analysis using the DICE coefficient and the UPGMA method.

In IGS-RFLP fingerprints, the DNA fragments were amplified with the primers FGPS1490 and FGPL132 (Laguerre *et al.*, 1996) as described by Kwon *et al.* (2005). The fragments were digested with *Msp*I, *Hha*I and *Hae*III. As found in the 16S rRNA gene analysis, the five strains shared the same pattern, which showed the highest similarity (65 %) with *B. jicamae* PAC68^T and *B. pachyrhizi* PAC48^T (Supplementary Fig. S2), indicating that the five strains might represent a novel species.

BOX-PCR fingerprinting is a powerful tool to estimate the genomic diversity of bacteria and to identify strains (de Bruijn, 1992; Nick & Lindstrom, 1994; Nick *et al.*, 1999). In the present study, BOX-PCR was performed to characterize the five strains by using the primer BOXAIR (5′-CTACGGCAAGGCGACGCTGACG-3′) and the procedure of Versalovic *et al.* (1994). The amplified products were subjected to electrophoresis in 1.5 % (w/v) agarose gels stained with ethidium bromide. The electrophoretic patterns were analysed by the Gelcompar II program and a UPGMA dendrogram was constructed. Three BOX-PCR fingerprints were obtained, indicating that the strains were not members of the same clone. The strains formed a cluster at 75 % similarity (see Supplementary Fig. S3), which was greater than the similarities obtained among the three recognized species of the genus *Bradyrhizobium*.

Currently, 16S rRNA gene sequencing is used as the principal method to define a bacterial genus, but it is not sufficiently sensitive to distinguish between closely related species. Due to this limitation, analysis of the IGS region has become a useful tool for determining relatedness among closely related bacteria, including bradyrhizobial strains (Willems *et al.*, 2001, 2003). In addition to the IGS region, several other phylogenetic markers, such as the *atpD*, *recA* and *glnII* genes, have been proposed for species and genospecies descriptions within the genus *Bradyrhizobium* (Vinuesa *et al.*, 2005). In accordance with previous studies and the RFLP results in the present study, three strains (CCBAU 23086^T, CCBAU 23160 and CCBAU 61434) representing different BOX types and different host origins were chosen for sequence analyses of the IGS and 16S rRNA, *atpD*, *recA*, *glnII*, *nodC* and *nifH* genes, as well as for DNA–DNA hybridization.

The 16S rRNA gene and IGS region of representative strains were amplified in the same manner as in the RFLP analysis. The *atpD*, *recA* and *glnII* genes were amplified and sequenced by using the method of Vinuesa *et al.* (2005), with primer sets atpD255F/atpD782R, recA41F/recA640R and glnII12F/glnII689R, respectively. The amplification and sequencing of partial *nodC* and *nifH* genes was performed with the primers nodC540/nodC1160 (Sarita *et al.*, 2005) and nifH1F/nifH1R (Laguerre *et al.*, 2001), respectively. All the amplified fragments were directly sequenced as described by Hurek *et al.* (1997). The sequences were aligned with those of related species of the genus *Bradyrhizobium* using the CLUSTAL W program in the MEGA 4.0 software package (Kumar *et al.*, 1994, 2008). Aligned sequences were analysed by using MEGA 4.0 software to produce a Jukes–Cantor distance (Jukes & Cantor, 1969) and to construct an optimal unrooted tree using the neighbour-joining (NJ) (Laguerre *et al.*, 1996; Saitou & Nei, 1987) method. Bootstrap analysis was based on 1000 replications (Felsenstein, 1985). Maximum-likelihood (ML) trees were constructed as unrooted trees using PhyML 3.0 (Guindon & Gascuel, 2003). The robustness of the ML topologies was inferred by non-parametric bootstrap tests based on 100 data

Table 1. Strains used in this study and relevant information

Strain	Host plant	Geographical origin	Reference
B. lablabi sp. nov.			
CCBAU 23086^T	*L. purpureus*	Anhui, China	This study
CCBAU 23160	*A. hypogaea*	Anhui, China	This study
CCBAU 61434	*L. purpureus*	Sichuan, China	This study
CCBAU 61428	*L. purpureus*	Sichuan, China	This study
CCBAU 61430	*L. purpureus*	Sichuan, China	This study
B. elkanii USDA 76^T	*Glycine max*	USA	Kuykendall *et al.*, 1992
B. pachyrhizi PAC48^T	*Pachyrhizus erosus*	Costa Rica	Ramírez-Bahena *et al.*, 2009
B. jicamae PAC68^T	*Pachyrhizus erosus*	Honduras	Ramírez-Bahena *et al.*, 2009

pseudoreplicates (Felsenstein, 1985). The nucleotide substitution model was selected by Akaike Information Criterion (AIC), as implemented in Modeltest 3.7 (Posada & Crandall, 1998).

In the phylogenetic tree based on 16S rRNA gene sequences (Fig. 1), the three strains tested had identical gene sequences and were closely related to *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T, with 99.48 %, 99.48 % and 99.22 % similarities, respectively. This result was consistent with the results of 16S rRNA gene RFLP analysis. The topology of the ML tree (Supplementary Fig. S4) was very similar to that of the NJ tree.

In the IGS region sequence analysis, the three representative strains showed identical sequences. The topology of the NJ (Supplementary Fig. S5) and the ML phylogenetic (not shown) trees was the same. The IGS region sequence of strain CCBAU 23086^T had 95.25 %, 86.3 % and 86.6 % similarities with that of *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T, respectively. Since Willems *et al.* (2003) have hypothesized that two bradyrhizobial strains with IGS region sequence similarity <95.5 % usually belong to separate genospecies, our data suggested that the novel group represented a novel genospecies of the genus *Bradyrhizobium*.

The results of the phylogenetic analyses of the three housekeeping genes with the NJ and ML methods were similar. The NJ tree constructed using the combined sequences of the three genes is shown in Fig. 2 and the ML tree is available as Supplementary Fig. S6. The strains of the novel group were closely related to *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T, which confirmed the relationships obtained in analyses of the 16S rRNA gene. The sequence similarities of the *atpD*, *recA* and *glnII* genes were 98.4–100 % among the three representative strains of the novel group and were <96.5 % between the novel group and reference strains of related species (see Supplementary Table S1). The small differences between strains CCBAU 23086^T and CCBAU 23160 in the *atpD* gene sequence (98.98 % similarity) demonstrated that the strains were not a clone, although both shared the same BOX patterns.

Symbiotic (*nif* and *nod*) genes do not offer taxonomic information because they are located in easily interchangeable elements (plasmids or in symbiotic islands), but they are required for the successful establishment of the highly specific symbiosis between rhizobia and legumes. Therefore, comparisons of these symbiotic genes may reveal the host ranges of rhizobia. For this reason, the *nifH* and *nodC* genes are commonly analysed for the description of novel rhizobial species. The results of the phylogenetic analysis of these genes in the present study showed that the rhizobia isolated from *L. purpureus* and *A. hypogaea* had identical *nifH* and *nodC* gene sequences, implying that they could have the same host spectrum.

In the *nifH* gene phylogenetic tree (Supplementary Fig. S7), the closest relatives were strain CCBAU 83335 nodulating *Sophora alopecuriodes*, RST89 nodulating *Retama sphaerocarpa* and CCBAU 05065 nodulating *Vicia pseudorobus*, with 96.5 %, 96.5 % and 96.3 % sequence similarities, respectively. The *nifH* sequence similarities between the novel group and *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T were 92.2 %, 88.0 % and 88.5 %, respectively. When the *nodC* genes were considered, the similarities between the novel group and recognized species were <86 %, indicating an independent evolutionary history.

As a standard method for species delineation (Graham *et al.*, 1991; Wayne *et al.*, 1987), DNA–DNA hybridization was performed between a representative strain, CCBAU 23086^T, and reference strains for the novel group (CCBAU 23160 and CCBAU 61434) and for related species (*B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T) using Marmur's method for total DNA isolation (Marmur, 1961) and the renaturation-rate technology described previously by De Ley *et al.* (1970).

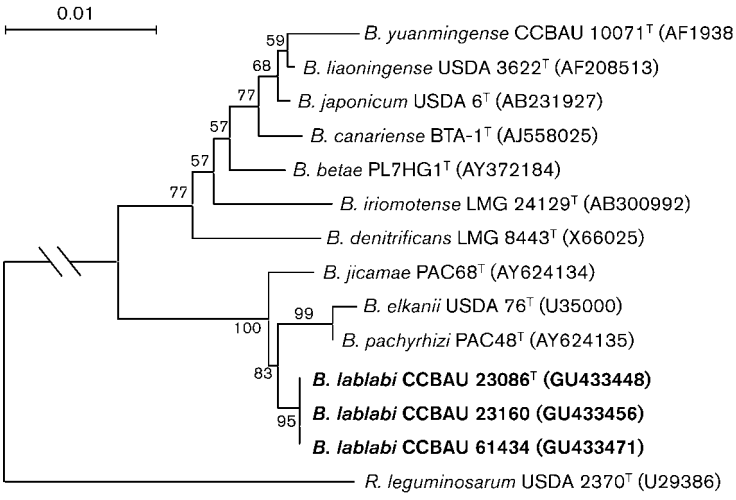

Fig. 1. Neighbour-joining tree reconstructed from 16S rRNA gene sequences showing the phylogenetic relationships of strain CCBAU 23086^T. Bootstrap values >50 % are indicated at nodes. The sequence of *Rhizobium leguminosarum* USDA 2370^T was used as an outgroup. Bar, 1 % nucleotide substitutions.

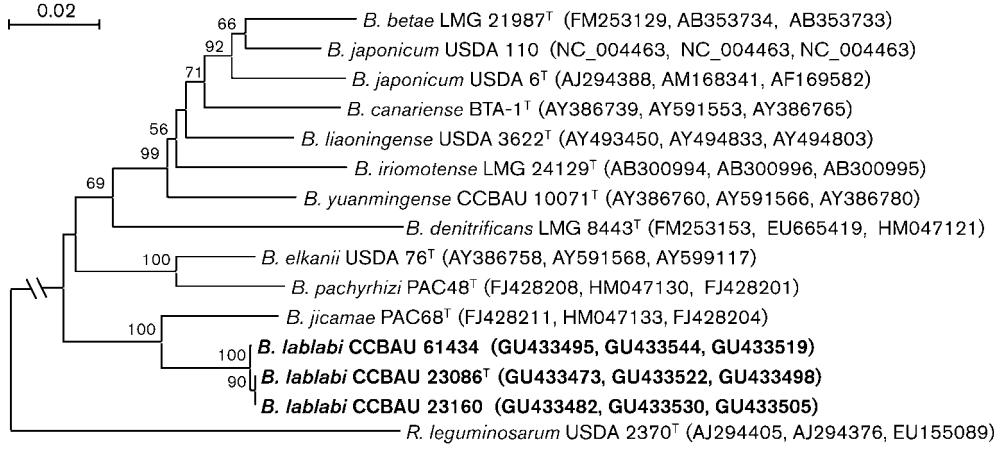

Fig. 2. Neighbour-joining phylogenetic tree based on *atpD*, *recA* and *glnII* gene sequences of *Bradyrhizobium lablabi* sp. nov. and all ten type strains of the genus *Bradyrhizobium*. Bootstrap values >50 % based on 1000 replicates are shown at each node. The GenBank accession numbers for the *atpD*, *recA* and *glnII* genes are shown in parentheses. Bar, 2 % nucleotide substitutions.

All experiments were performed three times and the mean DNA–DNA relatedness is presented in Supplementary Table S1. The DNA–DNA relatedness of strain CCBAU 23086^T with strains CCBAU 23160 and CCBAU 61434 was 100 % and 92.74 % (Supplementary Table S1), respectively. The results of DNA–DNA hybridization between strain CCBAU 23086^T and the related type strains *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T (Supplementary Table S1) indicated that strain CCBAU 23086^T showed significantly low relatedness values, in the range of 20.09–31.74 %. These values were lower than the threshold value of 70 % DNA–DNA relatedness that is generally accepted as the threshold for the definition of a species (Wayne *et al.*, 1987).

The G+C content of the DNA was measured by the thermal denaturation method of De Ley *et al.* (1970) using *Escherichia coli* K-12 as a standard. The DNA G+C content of the three strains was 60.14–62.85 mol% (T_m), which was within the range expected for recognized members of the genus *Bradyrhizobium*.

To discern and describe the novel genomic species, the cellular fatty acid profile of strain CCBAU 23086^T was assayed together with those of *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T as reported in other studies (de Lajudie *et al.*, 1998; Quan *et al.*, 2005; Tighe *et al.*, 2000). The strains were grown on YMA medium for 7 days at 28 °C. Fatty acid methyl esters were prepared and separated using a previously described method (Sasser, 1990) and identified with the MIDI Sherlock Microbial Identification System (Sherlock license CD v.6.0), using the TSBA6 database.

A total of 16 fatty acids were detected in strain CCBAU 23086^T. According to this analysis, the presence of $C_{16:1}\omega 9c$, summed feature 3 and summed feature 7 was common for the novel group and for the type strains of *B. jicamae*, *B. pachyrhizi* and *B. elkanii*. The dominant fatty acid for strain CCBAU 23068^T was $C_{18:1}\omega 7c$. This was different to the dominant fatty acid found in the three related species, but was consistent with the major fatty acid found for other species of the genus *Bradyrhizobium*, such as *B. japonicum* and *Bradyrhizobium liaoningense* (Islam *et al.*, 2008). Several fatty acids ($C_{12:0}$ 2-OH, $C_{14:0}$ iso 3-OH, $C_{15:1}$ iso F and $C_{17:0}$ 2-OH) were found in small amounts only in strain CCBAU 23086^T of the novel group. Detailed results are presented in Supplementary Table S2.

The phenotypic features of the three new isolates and the related type strains of species of the genus *Bradyrhizobium* were determined according to the method described by Gao *et al.* (1994). Tested features included the utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of NaCl, growth at varying pH values and the temperature range for growth (Gao *et al.*, 1994). Biochemical tests including the activities of catalase, urease, oxidase and nitrate reductase and reduction of litmus milk, Nile blue and methylene blue were performed according to Smibert & Krieg (1994). The three strains of the novel species tested in this study had the same characteristics in most cases except that strain CCBAU 23086^T could not utilize sodium acetate as a sole carbon source and was resistant to tetracycline hydrochloride (150 μg ml^{-1}). Strains CCBAU 23086^T, CCBAU 23160 and CCBAU 61434 were further compared with the type strains of their closest relatives, *B. jicamae* PAC68^T, *B. pachyrhizi* PAC48^T (Ramírez-Bahena *et al.*, 2009) and *B. elkanii* USDA 76^T. Distinctive features of the novel group of strains are shown in Table 2 and more features are presented in the description of the novel species.

Nodulation and nitrogen-fixation abilities are important characteristics of the genus *Bradyrhizobium* and the

Table 2. Distinctive features of *B. lablabi* sp. nov. and its closest relatives

Strains: 1, *B. lablabi* sp. nov. CCBAU 23086^T; 2, *B. lablabi* sp. nov. CCBAU 23160; 3, *B. lablabi* sp. nov. CCBAU 61434; 4, *B. jicamae* PAC68^T; 5, *B. elkani* USDA 76^T; 6, *B. pachyrhizi* PAC48^T. +, Growth or resistant; −, no growth or sensitive; W, weak; ND, not determined. Data were obtained in this study unless indicated.

Characteristic	1	2	3	4	5	6
Generation time (h) in YM broth	10–12	10–12	10–12	6–7*	7–8*	>6*
Utilization of sole carbon sources:						
Adipic acid	−	−	−	−†	−	+
D-Glucose	W	W	W	+	+	+
Inulin	+	+	+	+	−	−
Maltose	+	+	+	−	+	+
Melezitose	−	−	−	+	−	−
Salicin	W	W	W	W	−	−
Sodium pyruvate	+	+	+	−	+	+
L-Rhamnose	W	W	W	−	−	+
Sodium acetate	−	+	+	+	+	+
Sodium citrate	+	+	+	−	+	−
Sodium formate	−	−	−	−	+	+
L-Threonine	+	+	+	−	+	+
Utilization of sole nitrogen sources:						
D-Glutamic acid	+	+	+	−	+	+
L-Threonine	−	−	−	−	+	+
L-Cystine	−	−	−	−	+	+
Resistance to (μg ml^{-1})						
Ampicillin (50)	+	+	+	−	+	+
Streptomycin sulfate (5)	+	+	+	−	+	+
Tetracycline HCl (150)	+	−	−	−	+	+
Erythromycin (5)	+	+	+	−	+	+
Erythromycin (50)	−	−	−	−	+	+
Gentamicin sulfate (5)	+	+	+	+†	+	+
Chloramphenicol (50)	+	+	+	−	+	+
Growth in/at:						
1 % (w/v) NaCl	−	−	−	+	+	−
pH 5.0	W	W	W	−	+	+
pH 10.0	+	+	+	−	−	−
37 °C	+	+	+	−	+	+†

*Data from Ramírez-Bahena *et al.* (2009).

†The data from this study were not consistent with those reported previously.

host range is an important feature for the description of novel rhizobial species (Graham *et al.*, 1991). In the present study, cross nodulation tests performed in vermiculite moistened with N-free solution (Vincent, 1970) indicated that strains CCBAU 23086^T, CCBAU 23160 and CCBAU 61434 could nodulate *L. purpureus*, *A. hypogaea* and *Vigna unguiculata*, but not *Glycine max*, *Trifolium repens*, *Lotus corniculatus*, *Vigna radiata*, *Pisum sativum* or *Medicago sativa* under laboratory conditions.

According to all the results obtained in this study, it is concluded that the five new strains represent a novel species in the genus *Bradyrhizobium*. The name *Bradyrhizobium lablabi* sp. nov. is proposed for this taxon with strain CCBAU 23086^T designated as the type strain.

Description of *Bradyrhizobium lablabi* sp. nov.

Bradyrhizobium lablabi (lab′la.bi. N.L. gen. n. lablabi of *Lablab* referring to the fact that the bacterium was isolated from a root nodule of *Lablab purpureus*).

Cells are Gram-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, convex and translucent and have a diameter of 1 mm after incubation for 7–10 days at 28 °C. The generation time is 10–12 h in YM broth. The pH range for growth is 5–10, with optimum growth at pH 7.0. Growth occurs between 10 °C and 37 °C, with optimum growth at 28 °C. Does not grow in the presence of 1 % (w/v) NaCl. In addition to the carbon sources listed in Table 2, the type strain is also able to utilize D-galactose, sodium DL-malate, D-ribose, sodium D-gluconate, D-arabinose, hippuric acid, sodium succinate,

D-sorbitol, sorbose, soluble starch, tartaric acid, D-xylose, L-proline and calcium gluconate as sole carbon sources. Does not utilize adipic acid, D-amygdalin, lactose, D-mannose, L-methionine, melibiose, raffinose, sucrose, syringic acid, L-arginine, DL-asparagine, glycine, L-glycine, dextrin or dulcitol as a sole carbon source. Is able to grow on DL-alanine, L-arginine, hypoxanthine, L-isoleucine, L-phenylalanine, L-valine, L-aspartic acid and L-lysine as sole nitrogen sources. No growth with glycine, L-glutamic acid or L-methionine as sole nitrogen sources. The type strain is resistant to (μg ml^{-1}), chloramphenicol (5), kanamycin (5), neomycin sulfate (5), tetracycline hydrochloride (150) and gentamicin (5). Detailed distinctive features and the fatty acid content are shown in Table 2 and Supplementary Table S2. Strains can be distinguished by their housekeeping gene sequences and by DNA–DNA hybridization (Supplementary Table S1).

The type strain, CCBAU 23086^T (=LMG 25572^T=HAMBI 3052^T), was isolated from effective nodules of *L. purpureus* in Anhui province, China. The DNA G+C content of the type strain is 60.14 mol%. Additional strains are CCBAU 23160 and CCBAU 61434.

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