Ensifer sojae sp. nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils

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Thirteen bacterial isolates from root nodules of soybean grown in saline-alkaline soils in the Chinese province of Hebei were identified as a unique group in the genus *Ensifer* based upon BOX-PCR patterns, sequencing analyses of 16S rRNA and housekeeping genes and DNA–DNA hybridization. Phenotypically, positive tests for acid production and negative results for reduction in litmus milk and sensitivity to 50 μg ampicillin ml^{-1}, as well as some other features, could differentiate the novel group from defined species of the *Ensifer–Sinorhizobium* group. The novel group had symbiotic gene sequences (*nodC* and *nifH*) that were identical or very similar to those of *Ensifer* (*Sinorhizobium*) *fredii*, and formed effective nodules with *Glycine max* (soybean), *Vigna unguiculata* and *Glycine soja*. Based upon the consensus of these analyses, a novel species, *Ensifer sojae* sp. nov., is proposed, with CCBAU 05684^T (=LMG 25493^T =HAMBI 3098^T) as the type strain. The DNA G+C content of strain CCBAU 05684^T was 60.9 mol% (T_m).

Soybean [*Glycine max* (L.) Merr.] is one of the world's most important legume crops and has been cultivated in China, the diversification centre of the plant, for more than 5000 years. Soybean forms root nodules with various symbiotic bacteria (Chen *et al.*, 2005) including the slow-growing species *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense* (Vinuesa *et al.*, 2008) and the fast-growing species *Ensifer* (*Sinorhizobium*) *fredii*, *Rhizobium tropici*, *Rhizobium* sp. (Hungria *et al.*, 2006), *Rhizobium oryzae* (Peng *et al.*, 2008) and *Mesorhizobium tianshanense* (Chen *et al.*, 1995). Most of these bacteria, except *Rhizobium tropici* and *Rhizobium* sp. (Hungria *et al.*, 2006), have been found in nodules of soybean grown in China. In addition, clear biogeography of soybean rhizobia has been shown in China (Han *et al.*, 2009; Man *et al.*, 2008; Wang *et al.*, 2009).

Abbreviation: IGS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and housekeeping gene sequences of isolates CCBAU 05606, CCBAU 05638, CCBAU 05640, CCBAU 05684^T, CCBAU 05646 and CCBAU 05699 are respectively GU593058–GU593062 and HM997087 (16S rRNA gene), GU994039–GU994043 and HM997088 (*atpD*), GU994047–GU994051 and HM997101 (*rpoB*), GU994052–GU994056 and HM997098 (*recA*), GU994057–GU994061 and HM997089 (*glnII*), GU994066–GU994070 and HM997097 (*nodC*) and GU994074–GU994078 and HM997096 (*nifH*).

Five supplementary figures are available with the online version of this paper.

Sinorhizobium was initially separated from *Rhizobium* by Chen *et al.* (1988), and it has been treated as a later synonym of *Ensifer* (Young, 2003). However, arguments continue over whether the name *Sinorhizobium* should be used in preference to *Ensifer* (Lindström & Young, 2009). At the time of writing, 11 *Sinorhizobium* species have been described, nine of which have been reclassified in *Ensifer* [the exceptions are *Sinorhizobium morelense*, described as a later heterotypic synonym of *Ensifer adhaerens* by Young (2003), and *Sinorhizobium americanum*]; a further four *Ensifer* species have been described, *E. adhaerens*, *Ensifer garamanticus*, *Ensifer mexicanus* and *Ensifer numidicus* (http://www.uniprot.org/taxonomy/227292; http://www.bacterio.cict.fr/e/ensifer.html). Among these species, *E.* (*S.*) *fredii* has been found mainly in temperate and subtropical regions of China in association with soybean. Previously, two species, *E.* (*S.*) *fredii* and *Ensifer* (*Sinorhizobium*) *xinjiangensis*, were proposed for the fast-growing rhizobia in root nodules of soybean based upon phenotypic characterization and DNA–DNA hybridization (Chen *et al.*, 1988; Peng *et al.*, 2002). However, *E.* (*S.*) *xinjiangensis* has subsequently been incorporated into *E.* (*S.*) *fredii* based upon multilocus sequence analysis (Martens *et al.*, 2008).

In a survey of soybean rhizobia in Hebei Province, which surrounds Beijing and is the intermediate zone between the north-eastern, north-western and middle (subtropical) regions of China, we identified 13 rhizobial isolates from

root nodules of soybean grown in fields as representing a putative species in the *Ensifer–Sinorhizobium* group distinct from all defined species, based upon amplified 16S rRNA gene RFLP analysis. In order to clarify their taxonomic position, we performed a systematic study to compare these isolates with reference strains of related species.

The following reference strains were used in comparison with the novel isolates: *E.* (*S.*) *fredii* USDA 205^T and CCBAU 110, *Ensifer* (*Sinorhizobium*) *saheli* LMG 7837^T, *Ensifer* (*Sinorhizobium*) *kostiensis* HAMBI 1489^T, *Ensifer* (*Sinorhizobium*) *kummerowiae* CCBAU 71714^T, *Ensifer* (*Sinorhizobium*) *meliloti* USDA 1002^T, *Ensifer mexicanus* HAMBI 2910^T, *S. morelense* Lc04^T, *E. garamanticus* ORS 1400^T and *E. numidicus* ORS 1407^T. The novel isolates used in this study were: CCBAU 05684^T, CCBAU 05686 and CCBAU 05699 isolated from Huanghua county, CCBAU 05638, CCBAU 05639, CCBAU 05640, CCBAU 05642, CCBAU 05644, CCBAU 05646 and CCBAU 05647 from Hejian county and CCBAU 05603 and CCBAU 05606 from Cangxian county. All three counties are located in the southern region of Hebei Province and had saline-alkaline soils. Soil pH and electrical conductivity (mS m^{-1}) were respectively 8.33–8.71 and 11.6–11.9 in Cangxian county, 8.67 and 8.83 in Hejian county and 8.44 and 12.7 in Huanghua county. Standard procedures and YMA medium (Vincent, 1970) were used for isolation and purification. The isolates were incubated at 28 °C, except where indicated. Purified isolates were maintained in YMA at 4 °C for temporary storage and in 20 % (w/v) glycerol at −80 °C for long-term storage.

For ARDRA (amplified 16S rDNA restriction analysis), DNA samples were prepared as described by Terefework *et al.* (2001). PCR was carried out using primers P1 and P6 and the protocol described previously (Tan *et al.*, 1997). PCR products were digested separately with *Msp*I, *Hae*III, *Hin*fI and *Alu*I at 37 °C for 4 h and the digested fragments were separated by electrophoresis at 100 V (5 V cm^{-1}) for 4 h in 3 % (w/v) agarose gels. Restriction patterns were normalized, combined and clustered using GELCOMPAR II version 3.0. A UPGMA dendrogram (Sneath & Sokal, 1973) of ARDRA profiles was constructed based on the Dice correlation similarity coefficient. The 13 novel isolates had the same ARDRA patterns and clustered with defined species at a similarity of 97 % (Supplementary Fig. S1, available in IJSEM Online), demonstrating that the 13 isolates were members of the *Ensifer–Sinorhizobium* group.

RFLP analysis of the 16S–23S rRNA gene intergenic spacer (IGS) has been used to differentiate strains at the species or subspecies level (Gürtler & Stanisich, 1996). In the present study, primers FGPS6 and 23S-38 were used to amplify fragments as described by Rasolomampianina *et al.* (2005). Digestion of the amplified fragments by endonucleases *Msp*I, *Hae*III and *Hha*I and analysis of the digested fragments were conducted as described for ARDRA. Two RFLP patterns were obtained among the 13 novel isolates, which shared 95 % similarity: CCBAU 05646 had a unique pattern, while the remaining 12 isolates gave an identical, different pattern. Similarities varied from 68.5 to 79 % between the novel isolates and related species in the IGS-RFLP UPGMA dendrogram (Supplementary Fig. S2).

BOX-PCR and SDS-PAGE have been used as sensitive methods to distinguish closely related strains in the same species (Cho & Tiedje, 2000; Diouf *et al.*, 2000; Healy *et al.*, 2005; Nick & Lindström, 1994; Wang *et al.*, 2007). In the present study, BOX-PCR was carried out to analyse the diversity within the novel isolates and to compare them with related species. The primer BOXAIR (Versalovic *et al.*, 1994) and methods described by Nick & Lindström (1994) were used to amplify DNA fragments and to separate the PCR products by electrophoresis in 1.5 % (w/v) agarose gels. Again, two patterns in the BOX-PCR were found among the novel isolates, corresponding to CCBAU 05699 and the remaining isolates. They shared 92 % similarity with each other and clustered with reference strains of defined species at a similarity of 70 % (Supplementary Fig. S3). Preparation and electrophoresis of soluble proteins were performed according to Tan *et al.* (1997). In accordance with the results of BOX-PCR, two types in the SDS-PAGE were found, corresponding to CCBAU 05699 and the remaining 12 isolates (Supplementary Fig. S4).

In order to clarify the phylogenetic position of the 13 new isolates, CCBAU 05684^T, CCBAU 05638, CCBAU 05640, CCBAU 05646, CCBAU 05606 and CCBAU 05699 were analysed, representing the three isolation sites. The 16S rRNA gene was amplified as described for ARDRA and the PCR products were sequenced directly according to Hurek *et al.* (1997). Sequences acquired in this work and those of related strains obtained from the GenBank database were aligned using the CLUSTAL W program in the MEGA 4 software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Swofford, 1993) methods based upon Jukes–Cantor distances, using the same software. Bootstrap analysis was based on 1000 replications. Since no significant topological differences were found among the phylogenetic trees reconstructed using the different methods, only the neighbour-joining tree is shown (Fig. 1). The six representative isolates shared 99.9–100 % sequence similarity with each other in the 16S rRNA gene. Their closest relative was *E.* (*S.*) *saheli* LMG 7837^T (99.3 % similarity), followed by *E. mexicanus* HAMBI 2910^T (99.1 %), *E.* (*S.*) *fredii* USDA 205^T and *Sinorhizobium americanum* CFNEI 156^T (99 %) (Table 1).

Recently, analysis of multiple protein-encoding housekeeping genes has been widely applied to the investigation of taxonomic relationships (Martens *et al.*, 2008; Turner & Young, 2000; Vinuesa *et al.*, 2005a, b). PCR amplification and sequencing were carried out according to the following references: Martens *et al.* (2008) for the partial *rpoB* gene, Vinuesa *et al.* (2005a, b) for the partial *atpD* and *recA* genes and Turner & Young (2000) for the partial *glnII* gene. Phylogenetic trees were reconstructed using the neighbour-joining method and Kimura's two-parameter model. The

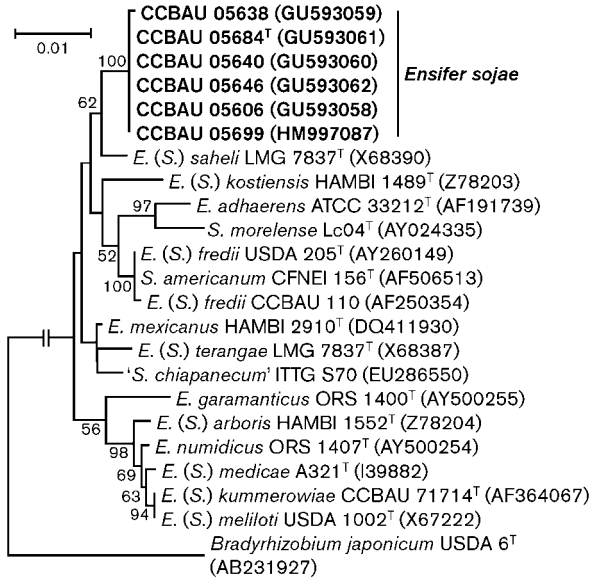

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of isolates of *Ensifer sojae* sp. nov. and related species of the *Ensifer–Sinorhizobium* group. GenBank accession numbers are shown in parentheses. Bootstrap probability values ⩾50 % are indicated at branch points. Bar, 1 % nucleotide substitutions.

six representative isolates showed 99.7–100 % similarity with each other in *rpoB*, *atpD*, *glnII* and *recA* gene sequences. They were more closely related to *E.* (*S.*) *saheli* and *E.* (*S.*) *kostiensis* than to other species. The similarities between the novel isolates and the type strains of *E.* (*S.*) *saheli* and *E.* (*S.*) *kostiensis* were 91.3 and 90.8 % for *rpoB*, 94.7 and 92.4 % for *atpD*, 91.1 and 91 % for *recA* and 93.4 and 90.9 % for *glnII* (Table 1). In the phylogenetic tree reconstructed from concatenated housekeeping gene sequences (*rpoB*, *atpD*, *recA* and *glnII*), the novel isolates showed similar relationships to those revealed by the 16S rRNA gene tree, i.e. they were closely related to *E.* (*S.*) *saheli* (Fig. 2; trees for the individual gene sequences are shown in Supplementary Fig. S5). These sequence similarities suggested that the novel isolates may represent a species distinct from defined species.

DNA–DNA relatedness is considered to be an essential criterion for species definition (Graham *et al.*, 1991; Wayne *et al.*, 1987). In the present study, total DNA was extracted from each isolate according to Marmur (1961) and DNA–DNA relatedness was estimated with the spectrophotometric method as described by De Ley *et al.* (1970). DNA–DNA hybridization between strain CCBAU 05684^T and four of the novel isolates (CCBAU 05638, CCBAU 05640, CCBAU 05646 and CCBAU 05699) was 93.8–97.4 %, indicating that the five isolates represent the same genomic species. CCBAU 05684^T showed the highest relatedness (46.9 %) to *E.* (*S.*) *saheli* LMG 7837^T, followed by *E. mexicanus* HAMBI 2910^T and *E.* (*S.*) *kostiensis* HAMBI 1489^T (41.9 and 40.3 %, respectively) (Table 1). These results indicate that the novel isolates represent a distinct species when the species definition threshold value of 70 % DNA–DNA relatedness is considered (Wayne *et al.*, 1987).

Using the thermal denaturation method (Marmur & Doty, 1962) and *Escherichia coli* K-12 DNA as a standard, the DNA G+C content of isolates CCBAU 05684^T, CCBAU 05638, CCBAU 05640 and CCBAU 05646 was determined to be 59.3–62.8 mol%, within the range reported for the *Ensifer–Sinorhizobium* group (Chen *et al.*, 1988).

Symbiosis and the formation of effective nitrogen-fixing nodules on legumes are important features of rhizobia. It has been documented that phylogenies of symbiotic genes

Table 1. Sequence similarities for 16S rRNA (*rrs*), *rpoB*, *atpD*, *recA* and *glnII* genes and DNA–DNA relatedness among the novel isolates and related type strains

Strain	Sequence similarity with CCBAU 05684^T (%)					DNA–DNA relatedness with CCBAU 05684^T (%)
	rrs	*rpoB*	*atpD*	*recA*	*glnII*	
Ensifer sojae sp. nov.						
CCBAU 05638	99.9	100	100	100	100	93.8
CCBAU 05640	100	100	100	100	100	97.4
CCBAU 05646	100	100	100	100	100	96.1
CCBAU 05606	100	100	100	100	100	ND
CCBAU 05699	100	99.7	100	99.7	100	94.5
E. (*S.*) *saheli* LMG 7837^T	99.3	91.3	94.7	91.1	93.4	46.9
E. mexicanus HAMBI 2910^T	99.1	90.3	92.0	88.9	91.1	41.9
E. (*S.*) *kostiensis* HAMBI 1489^T	98.6	90.8	92.4	91.0	90.9	40.3
E. (*S.*) *fredii* USDA 205^T	99.0	90.3	89.7	88.3	92.0	26.3
E. (*S.*) *kummerowiae* CCBAU 71714^T	98.2	90.6	90.3	91.0	90.5	18.6
E. (*S.*) *terangae* LMG 7834^T	98.6	89.5	91.2	88.0	92.2	16.4

ND, Not done.

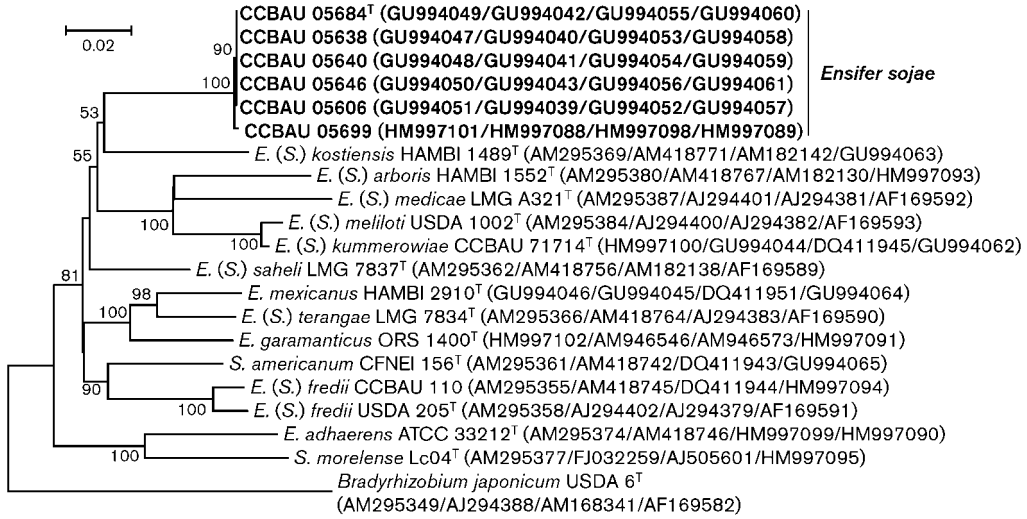

Fig. 2. Neighbour-joining tree constructed with combined sequences of housekeeping genes *rpoB*, *atpD*, *recA* and *glnII* showing phylogenetic relationships of isolates of *E. sojae* sp. nov. and related species of the *Ensifer–Sinorhizobium* group. GenBank accession numbers are shown in parentheses in the order *rpoB*/*atpD*/*recA*/*glnII*. Bootstrap probability values $\geqslant$50 % are indicated at branch points. Bar, 2 % nucleotide substitutions.

(*nod* and *nif*) are generally not congruent with those based on the 16S rRNA gene sequence, but show correlation with host range (Haukka *et al.*, 1998; Laguerre *et al.*, 2001). In this work, partial *nodC* and *nifH* genes of representative isolates were amplified and sequenced as described previously (Laguerre *et al.*, 2001). Interestingly, the *nodC* sequences of all representative isolates were identical and were 100 % identical to those of *E.* (*S.*) *fredii* and *E.* (*S.*) *saheli* strains. The novel isolates also had identical *nifH* sequences to *E.* (*S.*) *fredii* strains and showed 91.9 % *nifH* gene sequence similarity with the type strain of *E.* (*S.*) *saheli* (Fig. 3). These results are explained by the fact that the novel isolates and *E.* (*S.*) *fredii* have the same host. The results of cross-nodulation tests supported the symbiotic gene phylogenies. Strain CCBAU 05684^{T} formed pink nodules on *Glycine max*, *Vigna unguiculata* and *Glycine soja*. Ineffective symbioses were formed with *Leucaena leucocephala* and *Phaseolus vulgaris* and no nodules were formed with *Medicago sativa*, *Trifolium repens*, *Melilotus* sp., *Lotus corniculatus*, *Sesbania cannabina*, *Acacia farnesiana* or *Pisum sativum*. In short, symbiotic gene sequence analysis and nodulation tests demonstrated that the novel isolates have symbiotic characteristics similar to those of *E.* (*S.*) *fredii* (Scholla & Elkan, 1984).

Phenotypic features of isolates CCBAU 05684^{T}, CCBAU 05606, CCBAU 05638, CCBAU 05640, CCBAU 05646 and CCBAU 05699 were determined in comparison with type strains of related species according to the method described by Gao *et al.* (1994). One hundred and thirty phenotypic features were measured, including utilization of sole carbon and nitrogen sources, resistance to antibiotics (at 5, 50, 100 and 300 μg ml^{-1}), tolerance of NaCl (1–5 %, w/v), pH and temperature ranges for growth, production of catalase, urease, phenylalaninase and oxidase, methyl blue, Nile blue and nitrate reduction, methyl red and Voges–Proskauer reactions, hydrolysis of casein, gelatin, starch and Tween 80, production of indole and 3-ketolactose, growth in Luria–Bertani broth and litmus milk experiments. The results showed that the six representative isolates had similar features and could be differentiated from the type strains of related species by several characteristics, such as positive tests for acid production and a negative reaction for reduction in litmus milk and sensitivity to 50 μg ampicillin ml^{-1} (Table 2).

Based on the genotypic and phenotypic results obtained in this study, the 13 isolates formed a unique group and were divided into two clones: CCBAU 05699 and the remaining 12 isolates. Considering that they were isolated from three sites with similar soil characteristics (saline-alkaline) in the same region, it could be suggested that this symbiotic bacterium might have evolved recently and has not been widely distributed. We believe that these soybean rhizobial isolates represent a novel species. In light of the nomenclatural argument mentioned above, if the genera *Ensifer* and *Sinorhizobium* are synonyms, the correct name for the single taxon is *Ensifer*; we therefore propose the name *Ensifer sojae* sp. nov. to accommodate the novel isolates.

Description of *Ensifer sojae* sp. nov.

Ensifer sojae (so′ja.e. N.L. gen. n. *sojae* of soja, of soybean, referring to the source of the first isolates).

Cells are Gram-negative, aerobic, motile, non-spore-forming rods, 0.4–0.5 μm wide and 0.7–0.9 μm long. Colonies on YMA are circular, convex, white-opaque and

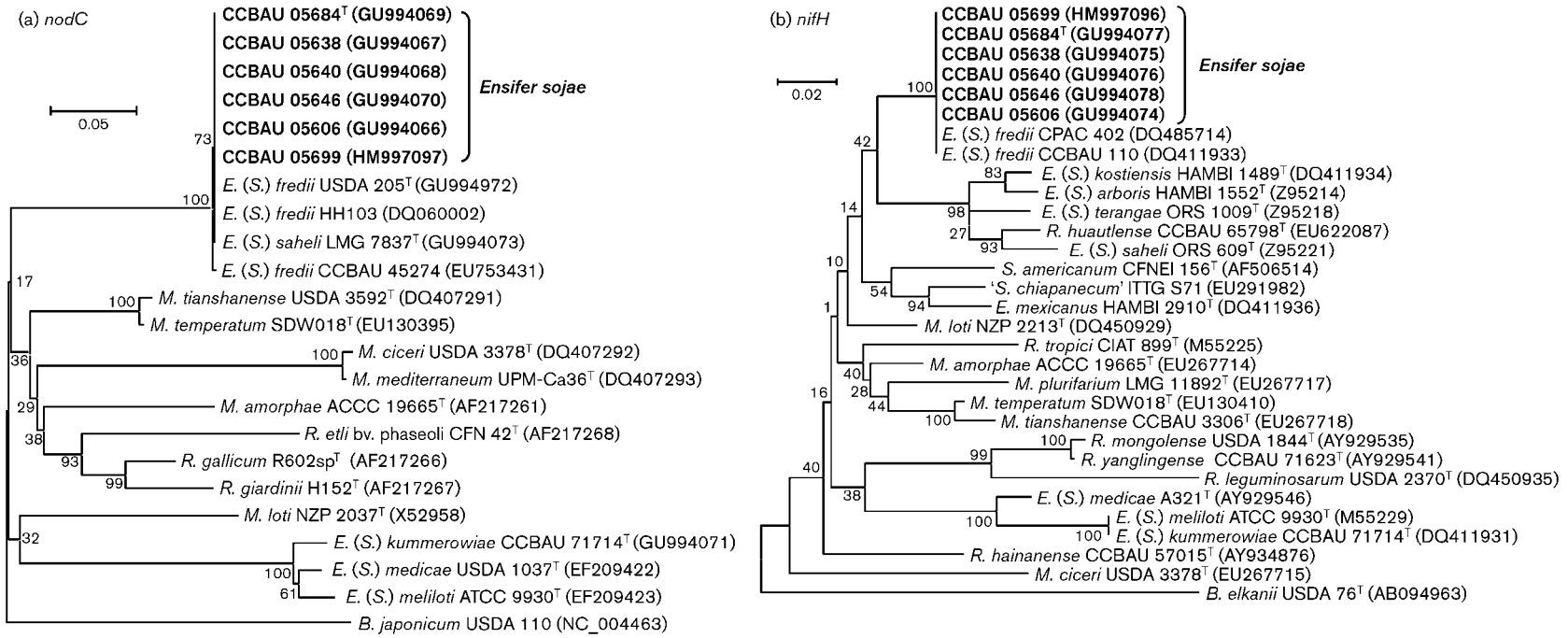

Fig. 3. Comparison of partial *nodC* (a) and *nifH* (b) gene phylogenies. The trees were reconstructed by using the neighbour-joining method from a Kimura 2-parameter distance matrix of the sequences. Bootstrap percentages based on 1000 replicates are shown at each node. Bars, 5 % (a) and 2 % (b) nucleotide substitutions. *B.*, *Bradyrhizobium*; *M.*, *Mesorhizobium*; *R.*, *Rhizobium*.

Table 2. Distinctive features of isolates of *Ensifer sojae* sp. nov. and their closest relatives

Strains: 1, *E. sojae* sp. nov. CCBAU 05684^T, CCBAU 05638, CCBAU 05640, CCBAU 05646, CCBAU 05606 and CCBAU 05699; 2, *E.* (*S.*) *saheli* LMG 7837^T; 3, *E.* (*S.*) *terangae* LMG 7834^T; 4, *E.* (*S.*) *fredii* USDA 205^T; 5, *E.* (*S.*) *kummerowiae* CCBAU 71714^T; 6, *E. mexicanus* HAMBI 2910^T; 7, *E.* (*S.*) *kostiensis* HAMBI 1489^T; 8, *E. garamanticus* ORS 1400^T; 9, *E. numidicus* ORS 1407^T. Data were obtained in this study. +, Positive; −, negative; ND, not done.

Characteristic	1	2	3	4	5	6	7	8	9
Utilization as sole carbon source of:									
D-Arabinose	−	−	−	+	−	+	−	+	+
Calcium malonate	−	+	−	+	−	−	−	−	−
Dulcitol	−	−	+	−	+	−	−	−	+
D-Amygdalin	−	−	−	+	−	−	−	+	−
D-Fructose	+	−	+	+	−	+	+	+	+
Lactose	−	+	+	+	−	−	+	+	+
Sodium DL-malate	+	−	−	+	−	+	+	−	+
(+)-D-Mannose	−	+	+	+	−	+	−	+	+
Sodium pyruvate	+	−	−	+	−	+	+	−	+
Salicin	−	+	−	+	+	−	−	+	+
Sodium citrate	+	+	+	−	−	+	−	+	+
Sodium succinate	+	+	−	+	−	+	+	−	+
Soluble starch	−	+	−	+	−	−	−	−	−
L-Arginine	−	−	−	+	−	+	+	+	−
L-Proline	−	−	+	+	−	+	+	+	+
Utilization as sole nitrogen source of:									
L-Aspartic acid	−	+	+	+	−	+	−	+	−
D-Glutamic acid	−	−	−	+	+	−	−	+	+
L-Valine	+	+	−	−	−	+	+	−	+
L-Methionine	+	+	−	+	−	+	+	+	+
Growth at pH 10	−	+	−	+	+	+	−	+	+
Resistance to (μg ml^{-1}):									
Ampicillin (50)	−	+	+	+	+	+	+	−	−
Kanamycin sulfate (5)	−	+	+	−	−	+	+	+	+
Neomycin sulfate (5)	−	−	−	+	+	+	−	+	+
Streptomycin sulfate (5)	−	+	−	+	−	−	−	+	+
Polymyxin B sulfate (5)	−	−	+	+	+	+	−	ND	ND
Erythromycin (5)	−	+	−	+	+	+	−	+	+
Chloramphenicol (5)	−	+	+	+	+	−	−	+	+

usually have a diameter of 1–3 mm within 3–4 days at 28 °C. Growth occurs at 12–37 °C and pH 6–9 on YMA. In addition to the features presented in Table 2, the type strain can use D-galactose, D-glucose, inositol, maltose, melibiose, raffinose, L-rhamnose, D-ribose, sodium acetate, D-sorbitol, sucrose and (+)-D-xylose as sole carbon sources and α-L-aminopropionic acid, L-arginine, L-cystine, hypoxanthine, L-isoleucine, L-phenylalanine, L-valine, L-threonine and L-methionine as sole nitrogen sources. The type strain does not use adipic acid, inulin, calcium gluconate, dextrin, *meso*-erythritol, melezitose, sodium formate, sodium D-gluconate, soluble starch, syringic acid, potassium sodium tartrate, vanillic acid, glycine, DL-asparagine, L-methionine or L-threonine as sole carbon sources. Tests for urease, oxidase and litmus milk peptonization are positive. Tests negative for nitrate reductase, indole production, growth in Luria–Bertani broth (Sigma), production of 3-ketolactose and hydrolysis of starch, gelatin and casein. Reduces Nile blue. Methyl red and Voges–Proskauer reactions are negative.

The type strain, CCBAU 05684^T (=LMG 25493^T =HAMBI 3098^T), was isolated from effective nodules of *Glycine max* in Hebei Province, China. The DNA G+C content of the type strain is 60.9 mol% (T_m).

Acknowledgements

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