INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Ouyabe *et al.*, *Int. J. Syst. Evol. Microbiol.* 2020;70:5054–5062

DOI 10.1099/ijsem.0.004381

MICROBIOLOGY SOCIETY

OPEN MICROBIOLOGY

Rhizobium dioscoreae sp. nov., a plant growth-promoting bacterium isolated from yam (*Dioscorea* species)

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Abstract

This study investigated endophytic nitrogen-fixing bacteria isolated from two species of yam (water yam, *Dioscorea alata* L.; lesser yam, *Dioscorea esculenta* L.) grown in nutrient-poor alkaline soil conditions on Miyako Island, Okinawa, Japan. Two bacterial strains of the genus *Rhizobium*, S-93^T and S-62, were isolated. The phylogenetic tree, based on the almost-complete 16S rRNA gene sequences (1476 bp for each strain), placed them in a distinct clade, with *Rhizobium miluonense* CCBAU 41251^T, *Rhizobium hainanense* I66^T, *Rhizobium multihospitium* HAMBI 2975^T, *Rhizobium freirei* PRF 81^T and *Rhizobium tropici* CIAT 899^T being their closest species. Their bacterial fatty acid profile, with major components of $C_{19:0}$ cyclo $\omega 8c$ and summed feature 8, as well as other phenotypic characteristics and DNA G+C content (59.65 mol%) indicated that the novel strains belong to the genus *Rhizobium*. Pairwise average nucleotide identity analyses separated the novel strains from their most closely related species with similarity values of 90.5, 88.9, 88.5, 84.5 and 84.4% for *R. multihospitium* HAMBI 2975^T, *R. tropici* CIAT 899^T, *R. hainanense* CCBAU 57015^T, *R. miluonense* HAMBI 2971^T and *R. freirei* PRF 81^T, respectively; digital DNA–DNA hybridization values were in the range of 26–42%. Considering the phenotypic characteristics as well as the genomic data, it is suggested that strains S-93^T and S-62 represent a new species, for which the name *Rhizobium dioscoreae* is proposed. The type strain is S-93^T (=NRIC 0988^T=NBRC 114257^T=DSM 110498^T).

Bacteria of the genus *Rhizobium* have been isolated from a variety of sources and are of great environmental and agricultural importance. Rhizobia, which are used extensively for their nitrogen-fixing ability, are generally isolated from the nodules of leguminous plants. However, in recent studies, novel species of *Rhizobium* have been isolated from environmental samples [1, 2] as well as non-leguminous plants, including rice [3–5], maize [6] and grasses [7]. In these non-leguminous plants, some species of *Rhizobium*, such as *Rhizobium leguminosarum*, have been reported to promote plant growth [8].

Yam (*Dioscorea* species) is a tropical tuber crop. At present, 644 species are classified in the family Dioscoreaceae [9], among which the six most cultivated as staple foods are *Dioscorea rotundata* Poir (white guinea yam), *Dioscorea cayenensis* Lam (yellow yam), *Dioscorea alata* L. (water yam), *Dioscorea esculenta* L. (lesser yam), *Dioscorea dumetorum* (Kunth) and *Dioscorea bulbifera* L. (aerial yam). Yams are important in the tropics and subtropics, including in the yam belt of West Africa and in other countries such as Sri Lanka and Japan. More than 60 million people in West Africa depend on yams for food [10]. However, yam cultivation requires adequate soil fertility with high organic matter content. In West Africa, yam is cultivated as the first crop after clearing forests or after long-term fallow [11], a practice that has led to serious deforestation. Despite this, yam tuber yields remain low because of the rapid decline in soil fertility [11], which in turn has led to an increase in cultivation area to meet demand. Research on increasing yam tuber yield through application of chemical fertilizers has failed to improve productivity [11, 12]. Although limited in non-leguminous tropical crops, the use of plant growth-promoting bacteria is a promising alternative

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Keywords: *D. alata*; ANIb; *D. esculenta*; pan-genome; yam.

Abbreviations: ANI, average nucleotide identity; COG, clusters of orthologous groups; dDDH, digital DNA–DNA hybridization; IAA, indole 3-acetic acid; MLSA, multilocus sequence analysis; MR, modified Rennie; TSA, tryptic soy agar.

The DDBJ/ENA/GenBank accession numbers are LC498520 (S-93^T) and LC483383 (S-62).

Three supplementary figures and three supplementary tables are available with the online version of this article.

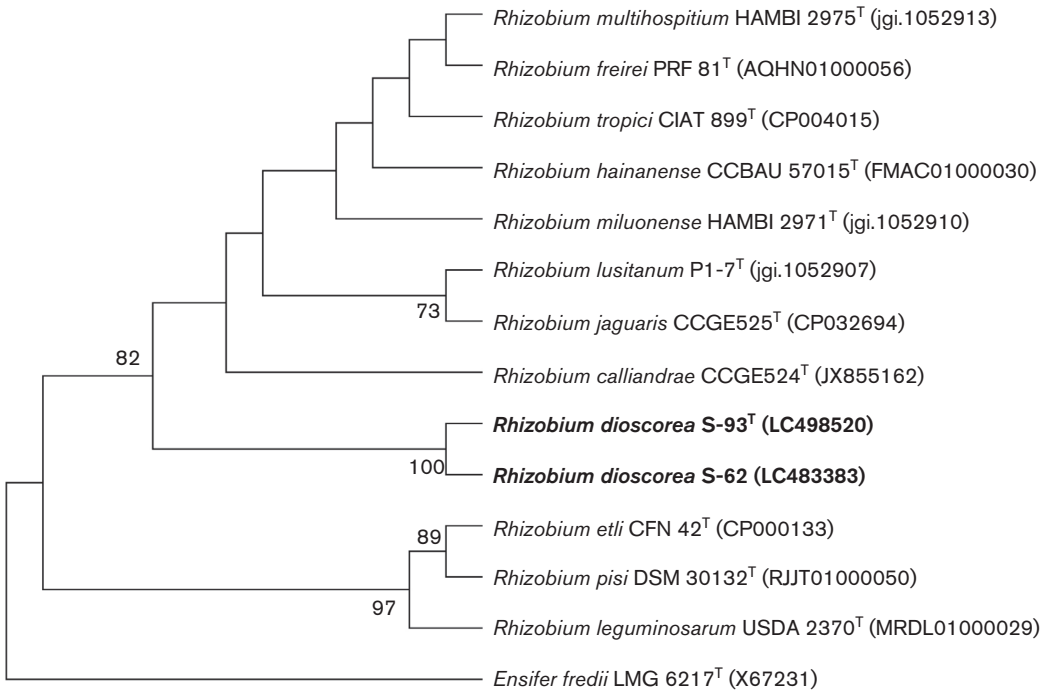

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of S-62 and S-93^T and their close species by Maximum Likelihood method. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Bootstrap values based on 1000 replications are given as percentages at the branching points. Numbers indicate percentages greater than 70%.

to chemical fertilizers. Recently, nitrogen-fixing bacteria have been isolated from yam species [13, 14].

This paper describes two Rhizobia strains, S-93^T and S-62, isolated from *Dioscorea* species as endophytic plant growth-promoting bacteria [15, 16]. A polyphasic taxonomic approach considering the genomic features of these strains suggest that they represent a new species of the genus *Rhizobium*.

ISOLATION AND ECOLOGY

Endophytic nitrogen-fixing bacteria were isolated from the roots, stems, leaves and tubers of water yam and lesser yam, using a culture-dependent method [17]. The plants were grown in pots containing a Holocene limestone soil with the typical pH range of pH 6.5–8.0. The soil is considered nutrient-poor and is locally referred to as *shimajiri mahaji* [18] on Miyako Island, Okinawa, Japan. The experimental conditions were as described in Ouyabe *et al.* [15, 16]. Briefly, samples were washed with sterile distilled water to remove dust and then surface-sterilized in a solution of 70% ethanol for 30 s and in 2.5% sodium hypochlorite (NaClO) for 60 s before four rinses in sterile distilled water. The samples were then macerated using a sterilized mortar and pestle containing 10 ml autoclaved normal saline (0.85% NaCl). One millilitre of plant macerate was streaked on plates containing solid nitrogen-free modified Rennie (MR; pH 6.8) medium [19] under laminar flow. Individual colonies were cultured on fresh MR medium and pure bacterial colonies were obtained. Strains S-93^T and S-62 were among those selected for further analysis. S-93^T was isolated from the root of *Dioscorea esculenta* L., accession E-1, and S-62 was isolated from the roots of *Dioscorea alata* L., accession A-62.

The plant growth-promotion ability of the two strains was evaluated by assessing their nitrogenase activity, hormone and siderophore production, and inorganic phosphate solubilization. An acetylene reduction assay was used to qualitatively determine nitrogen fixation ability [20], using a semi-solid nitrogen-free MR medium incubated at 30 °C for 5 days. Acetylene gas was injected into tubes to create an atmosphere with a final concentration of 10% (v/v). The amount of acetylene converted to ethylene was measured by injecting 1 ml atmosphere into a gas chromatograph equipped with a flame ionization detector and a Porapak N column (GC 2014; Shimadzu Corp.). Pakovskaya's agar [21] plates were prepared for qualitative and quantitative solubilization analysis of calcium phosphate. Bacteria were spotted on the plate in triplicate and incubated at 30 °C for 7 days. Strains forming visible clear halos around the colonies were considered calcium phosphate solubilizers. Quantitative analysis was performed by determining the solubilization index. Indole 3-acetic acid (IAA) production was also determined [22] in triplicate. Bacterial strains were grown in 300 ml flasks containing 50 ml Luria broth supplemented with L-tryptophan (0.5 mg ml^{-1}) for 48 h

Table 1. ANI and dDDH values of the novel strains and the most closely related *Rhizobium* species

Strains: 1, S-93^T; 2, S-62; 3, *Rhizobium multihospitium* HAMBI 2975^T; 4, *Rhizobium tropici* CIAT 899^T; 5, *Rhizobium hainanense* CCBAU 57015^T; 6, *Rhizobium miluonense* HAMBI 2971^T; 7, *Rhizobium freirei* PRF 81^T; 8, *Rhizobium lusitanum* P1-7^T. Values above and below the diagonal line of asterisks (*) represent dDDH and ANIb values, respectively.

ANIb/dDDH	1	2	3	4	5	6	7	8
1	*	86.5	42.6	38.3	37.3	29.6	29.8	27.5
2	98.3	*	42.7	38.1	37.2	29.6	29.8	27.6
3	90.4	90.5	*	36.8	39.7	30.3	30.7	28.2
4	88.9	88.9	88.3	*	35.9	29.6	33.4	31.4
5	88.5	88.6	89.5	87.8	*	30.0	30.3	27.9
6	84.4	84.3	85.0	84.2	84.7	*	43.7	29.7
7	84.4	84.3	84.9	86.5	84.7	90.7	*	33.1
8	82.8	82.9	83.4	85.4	83.3	84.3	86.1	*

on a rotary shaker. The broth was centrifuged at 10000 ***g*** for 15 min. The cell-free supernatant was collected and the IAA concentration was measured at an optical density of 530 nm with a spectrophotometer. Siderophore production was evaluated on CAS plates following a previously described protocol [23]. Bacterial colonies were spotted on the CAS plates in triplicate and incubated at 30 °C for 7 days. The presence of visible clear halos around the colonies was indicative of siderophore production, the index of which was determined as the ratio of total halo diameter to colony diameter.

The acetylene reduction assay revealed the ability of S-93^T and S-62 to fix nitrogen. These strains were also found to produce IAA and to solubilize inorganic calcium-phosphates with similar indexes. However, siderophore production was detected only in S-93^T.

16S rRNA GENE PHYLOGENY

Nearly complete bacterial 16S rRNA gene sequences of strains S-93^T and S-62 were amplified and sequenced. A cell suspension (20 µl) was treated with Proteinase K (5 µl) at 60 °C for 20 min and 95 °C for 5 min to disrupt the cells. Amplification was done with the primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) using the Thermal Cycler PTC-200 (Marshall Scientific). PCR was performed as described in Arief *et al.* [24]. The ABI PRISM 310 Genetic Analyzer (Thermofisher Scientific) was used for sequencing. Obtained sequences of 1477 bp (S-93^T) and 1476 bp (S-62) long were used to perform a similarity search of the bacterial 16S database of the EzBioCloud [25].

The 16S rRNA gene-based phylogenetic tree was reconstructed to determine the taxonomic position of the two novel strains within the genus *Rhizobium* using MEGA X software [26] with *Ensifer fredii* LMG 6217^T as an outgroup.

A total of 31 strains, including S-93^T and S-62, were found to be closely related to a diverse variety of known *Rhizobium* species [15, 16]. The nearly complete 16S rRNA gene sequences of S-93^T and S-62 were used to determine their taxonomic position within the genus *Rhizobium*. *R. miluonense* CCBAU 41251^T (98.93% similarity to strain S-93^T), *R. multihospitium* HAMBI 2975^T (98.93%), *R. freirei* PRF 81^T (98.93%) *R. hainanense* I66^T (98.86%), and *R. tropici* CIAT 899^T (98.86%) were the closest species. Similarities with other species were less than 98.7%. The similarity between strains S-93^T and S-62 was 99.93%, place them in a separate clade with their most closely related species, as shown on the phylogenetic tree (Fig. 1), indicating that the two strains are possibly novel species. The phylogenetic tree was based on the 16S rRNA gene sequences of S-62 and S-93^T, and their close species by maximum-likelihood method. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. Bootstrap values based on 1000 replications are given as percentages at the branching points. Numbers indicate percentages greater than 70%. The novel strains and their closest related species share some common ecological features, such as having been isolated from tropical and subtropical soil conditions.

GENOMIC FEATURES

DNA–DNA hybridization of draft genomes

In addition to the nearly complete sequence of the 16S rRNA gene, the draft genomes of the two strains were sequenced as recommended by Chun *et al.* [27], who suggested that 16S rRNA gene similarity and DNA–DNA hybridization approaches can be used in combination for species classification. The two strains, S-93^T and S-62, were cultured on nutrient agar plates at 30 °C for 48 h. Sequencing of the whole genome was carried out using the DNeasy Blood and Tissue Kit (Qiagen.com) according to the manufacturer's instructions. The sequences were used to reconstruct the bacterial genome using the Unicycler version 0.4.7 program. The average nucleotide identity (ANI) of strains S-93^T and S-62 with their closely related type strains (i.e., *R. miluonense*

Table 2. Features of carbon assimilation and acid production of *Rhizobium dioscoreae* sp. nov. strains as determined by API tests and phylogenetically related species

Strains: 1, S-93^T; 2, S-62; 3, *Rhizobium miluonense* CCBAU 41251^T; 4, *Rhizobium hainanense* I66^T; 5, *Rhizobium multihospitium* CCBAU 83401^T; 6, *Rhizobium freirei* PRF 81^T; 7, *Rhizobium tropici* CIAT 899^T. +, Positive; –, negative; W, weakly positive; ND, no data.

Characteristics	1	2	3*	4*	5*	6*	7*
Acid production (API 50 CH):							
Glycerol	–	–	+	+	+	+	+
Erythritol	+	+	+	+	+	+	+
D-Ribose	–	–	ND	ND	ND	ND	ND
D-Xylose	+	+	+	+	+	W	+
L-Xylose	–	–	+	+	+	+	–
D-Adonitol	–	–	W	+	+	+	+
Methyl β-D-xylopyranoside	–	–	+	+	+	W	+
D-Galactose	+	+	ND	ND	ND	ND	ND
D-Fructose	W	W	+	+	+	W	+
L-Sorbose	–	–	+	W	W	W	–
L-Rhamnose	W	W	+	+	+	W	+
Dulcitol	–	–	W	W	W	–	–
Inositol	W	W	+	W	+	W	+
D-Sorbitol	–	–	+	+	W	W	–
Methyl α-D-mannopyranoside	–	–	–	–	W	–	–
Methyl α-D-glucopyranoside	–	–	+	+	+	W	+
Amygdalin	–	–	+	+	W	–	–
Arbutin	–	–	+	+	+	+	W
Salicin	–	–	+	W	+	+	W
Cellobiose	–	–	+	+	+	W	+
Lactose	–	–	+	W	+	W	W
Melibiose	–	–	+	W	+	W	W
Sucrose	–	–	+	W	+	W	+
Trehalose	–	–	+	+	+	W	+
Inuline	–	–	ND	ND	ND	ND	ND
Melezitose	–	–	–	W	–	–	–
Raffinose	–	–	+	W	+	W	W
Starch	–	–	ND	ND	ND	ND	ND
Glycogen	–	–	+	+	+	+	–
Xylitol	–	–	+	+	W	W	–
Gentiobiose	–	–	+	+	+	W	W
D-Turanose	–	–	+	+	+	W	W
D-Lyxose	W	–	+	+	+	W	+
D-Tagatose	–	–	+	W	W	W	–

Continued

Table 2. Continued

Characteristics	1	2	3*	4*	5*	6*	7*
D-Fucose	+	+	ND	ND	ND	ND	ND
L-Fucose	+	+	ND	ND	ND	ND	ND
D-Arabitol	–	–	+	+	+	W	+
L-Arabitol	–	–	W	+	W	+	W
Potassium gluconate	–	–	ND	ND	ND	ND	ND
Potassium 2-cetogluconate	–	–	ND	ND	ND	ND	ND
Potassium 5-cetogluconate	–	–	ND	ND	ND	ND	ND
L-Arabinose	+	+	ND	ND	ND	ND	ND
Assimilation and hydrolysis (API 20NE):							
D-Arabinose	W	–	+	+	+	W	+
D-Glucose	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	W	+
N-Acetylglucosamine	+	+	+	W	+	+	–
Aesculin (hydrolysis)	+	+	ND	ND	ND	ND	ND
Maltose	+	+	+	+	+	W	+
Aesculin ferric citrate	+	+	ND	ND	ND	ND	ND
Potassium gluconate	–	–	ND	ND	ND	ND	ND
Capric acid	–	–	ND	ND	ND	ND	ND
Adipic acid	–	–	ND	ND	ND	ND	ND
Malic acid	+	+	ND	ND	ND	ND	ND
Citric acid (as trisodium citrate)	+	+	ND	ND	ND	ND	ND
Phenylacetic acid	–	–	ND	ND	ND	ND	ND
Potassium nitrate reduction	+	+	ND	ND	ND	ND	ND
Hydrolysis:							
L-Arginine	–	–	ND	ND	ND	ND	ND
Urease	–	–	ND	ND	ND	ND	ND
Gelatin (bovine origin)	–	–	ND	ND	ND	ND	ND

*Data of the closely related species were obtained from [47].

CCBAU 41251^T, *R. hainanense* I66^T, *R. multihospitium* HAMBI 2975^T, *R. freirei* PRF 81^T and *R. tropici* CIAT 899^T), selected based on the results of the 16S rRNA gene data, was calculated using the averagenucleotide identity-BLAST (ANIb) algorithm [28]. Digital DNA–DNA hybridization (dDDH) was also calculated using the BLAST+ method of the Genome-to-Genome Distance Calculator version 2.1 [29]. The results were based on formula 2 [30].

Results of the genome analysis revealed that the G+C contents of these strains were 59.65 and 59.55 mol% for S-93^T and S-62, respectively, which are in the range of the genus *Rhizobium* [31]. dDDH has been used as a standard technique in the classification of prokaryotes [29]. The results of the dDDH analyses in this study indicated a DNA–DNA relatedness of 86.5% between S-93^T and S-62. DNA–DNA relatedness was in the range 27.5–42.6% between S-93^T and closely related *Rhizobium* species (Table 1). As the DNA–DNA relatedness value for the definition of bacteria species should be approximately or greater than 70% [32, 33], the values obtained in this study indicate that S-93^T and S-62 belong to the same species, but are distinct from the validly published species of the genus *Rhizobium*.

Table 3. Cellular fatty acid profiles of strains S-93^T and S-63 and related type strains

Strains: 1, S-93^T; 2, S-62; 3, *Rhizobium miluonense* CCBAU 41251^T; 4, *Rhizobium hainanense* I66^T; 5, *Rhizobium multihospitium* CCBAU 83401^T; 6, *Rhizobium tropici* CIAT 899^T; 7, *Rhizobium leguminosarum* USDA 2370^T. –, Not detected or detected at <0.1%.

Fatty acids	1	2	3†	4†	5†	6†	7†
$C_{16:0}$	5.1	5.3	11.7	6.3	2.9	10.0	6.3
$C_{15:0}$ iso 3-OH	3.3	1.1	5.7	2.9	7.8	14.0	–
$C_{17:0}$ iso	3.2	4.0	–	1.3	2.7	1.0	1.2
$C_{17:0}$	2.2	2.7	–	0.3	1.5	–	–
$C_{16:0}$ 3-OH	3.2	1.4	6.5	3.5	4.9	8.0	1.7
$C_{18:0}$	3.5	3.6	2.8	3.9	1.2	–	3.1
$C_{18:1}$ ω7*c* 11-methyl	1.2	–	–	0.8	1.4	5.0	2.0
$C_{19:0}$ cyclo *ω8c*	39.5	44.3	21.0	38.8	–	10.0	1.7
$C_{18:1}$ 2-OH	2.9	–	–	1.9	3.8	–	–
$C_{18:0}$ 3-OH	4.6	2.1	2.2	–	3.0	2.0	1.8
Summed feature 2*	1.0	–	4.4	–	2.9	–	12.8
Summed feature 8*	26.2	30.6	44.2	30.1	43.1	–	57.9

*Summed features denote two or more fatty acids that could not be separated into known individual fatty acids. Summed feature 2 comprised $C_{14:0}$ 3-OH and/or $C_{16:1\ iso}$ I and an unknown equivalent with a chain length of 10.928, and summed feature 8 comprised $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*. Summed feature 8 corresponds to the summed feature 7 of Tighe *et al.* [46].
†Data were obtained from Zhang *et al.* [48], Tighe *et al.* [46], Han *et al.* [49] and Gil-Serrano *et al.* [50].

To confirm the genetic relationship between the novel strains and their most closely related species, the ANIb test was performed. The genome sequences of six *Rhizobium* species (*R. multihospitium* HAMBI 2975^T, *R. tropici* CIAT 899^T, *R. hainanense* CCBAU 57015^T, *R. miluonense* HAMBI 2971^T, *R. freirei* PRF 81^T and *R. lusitanum* P1-7^T) were downloaded from the GenBank database and compared with each other and with S-93^T and S-62. The ANIb value between S-93^T and S-62 was 98.3%. The ANIb values of the isolates to their most closely related strains ranged from 82.88 to 90.70% (Table 1). Based on the recommended ANI threshold value of 95–96% for bacterial species identification [32], the ANIb results of this study confirmed that S-93^T and S-62 are novel species. The genomic features of the two strains are provided as Table S1 (available in the online version of this article).

Multilocus sequence analysis

Multilocus sequence analysis (MLSA) has been proposed as either a complementary or alternative high-resolution method for elucidating the phylogenetic relationship between species within bacterial genera [34]. MLSA was used in this study as an additional method to confirm the results obtained from the dDDH. Genome-based phylogenetic analysis was carried out by using an original pipeline for all closely related species. Orthologous protein sequences were extracted based on the bidirectional best BLAST hit analysis for all genomic pairs using BLASTp version 2.5.0 (National Center for Biotechnology Information, NCBI) [35] with a minimum identity of 35% and a minimum sequence coverage of 60% in both directions. Amino acid sequences in each ortholog group were aligned using MAFFT 7.310 [36] in the L-INS-i mode. All alignments were trimmed using Gblocks 0.91b [37] with 'stringent' block parameters, followed by the concatenation of all trimmed alignments. Where necessary, multiple alignments of amino acid sequences were back-translated to their original nucleotide sequences codon-by-codon, and the resultant nucleotide sequence alignments were trimmed and concatenated as described above. The concatenated amino acid and/or nucleotide sequences were subjected to phylogenetic analysis by the maximum-likelihood method using RAxML 8.2.11 [38] with the rapid bootstrap option; the JTT amino acid substitution model and/or the GTR nucleotide substitution model were used. Phylogenetic analysis by the neighbour-joining method was performed using CLUSTAL_W 2.1 [39] with the default settings.

The phylogenetic tree reconstructed using MLSA is shown in Fig. S1. The results confirmed that S-93^T and S-62 are two strains of the same species. Moreover, among the closely related species identified using the 16S rRNA gene sequences analysis, the MLSA-based phylogenetic tree revealed *R. multihospitium* HAMBI 2975^T as the species most closely related to the two strains reported in this study.

Pan-genome analysis

The Pan-Genome Analysis Pipeline (PGAP) tool [40] was used to identify core, accessory, and unique protein families in S-93^T and S-62 and their closest species

(*R. multihospitium* HAMBI 2975^T). Families are based on the clusters of orthologous groups (COG) of proteins database. Genomic protein sequence data were obtained from the NCBI database. Pan-genome analysis is increasingly being used as a tool for clarifying and improving conventional and other genomic-based methods of bacterial classification [41]. The genomes of strains S-93^T and S-62 were compared with that of *R. multihospitium* HAMBI 2975^T, which was shown to be their closest relative by the genome-based phylogenetic analysis. For pan-genome analysis, the protein sequence data for S-93^T, S-62 and *R. multihospitium* HAMBI 2975^T were obtained with reference to Refseq assembly accessions GCF_009176305, GCF_009176325 and GCF_900094585, respectively.

The genomes of the three strains comprise 7576 protein-encoding gene families, 4965 of which belong to the core genome (Fig. S2). In strains S-93^T and S-62, 280 (5%) and 431 (7%) gene families, respectively, were included in the unique genomes, whereas 1148 (17%) gene families were assigned to *R. multihospitium* HAMBI 2975^T. The distribution of COG categories in the pan-genome is shown in Fig. S3. In the core genomes, major categories were related to transport, transcription and primary metabolic processes, including amino acid transport and metabolism (E), carbohydrate transport (G), cell wall/membrane/envelope biogenesis and metabolism (M), and transcription (K). Moreover, a large number of gene families related to transcription were included in the accessory genomes of strains S-93^T and S-62 compared with other categories, and these gene families accounted for a large proportion of the unique genome of *R. multihospitium* HAMBI 2975^T. These results indicate that strains S-93^T and S-62 are relatively close to each other and likely diverged from *R. multihospitium*. Interestingly, genes related to the bacterial type IV secretion system (T4SS), also known as the legume–rhizobia symbiosis system [42], were found in the accessory genome of strains S-93^T and S-62 (Table S2), as well as the type VI secretion system (T6SS) (Table S3) involved in the host plant infection [43]. Protein sequences encoded by homologous genes were identified in some of the *Rhizobium* genomes. These homologous proteins showed 70–80% similarity values to those of strains S-93^T and S-62 (details not shown). Thus, the T4SS and T6SS of strains S-93^T and S-62 could potentially be adapted to the yam environment for infection and symbiosis as endophytic beneficial bacteria.

PHYSIOLOGY AND CHEMOTAXONOMY

The phenotypic characterization of S-93^T and S-62 was performed using the API 20NE and API 50 CHB/E tests (bioMérieux) according to manufacturer's instructions for carbohydrate assimilation and acid production. The API tests were incubated at 30 °C for 24–48 h. Biochemical reactions were recorded as positive or negative and eventually weak according to the change in colour observed and interpreted with reference to the manufacturer's manual.

The phenotypic characteristics of the strains S-93^T and S-62 were analysed in terms of sole carbon utilization and fatty acid profile. API 50CH and API 20NE tests were used to evaluate the utilization of carbon sources. The results are presented in Table 2.

The cellular fatty acid profiles of S-93^T and S-62 were determined and compared with those of close species, referenced from published papers. The strains were grown on trypticsoy agar medium for 48 h at 30 °C and lyophilized. The lyophilized cells were used for fatty acid analysis, as described in Mueller *et al.* [44]. About 20 mg dried cells were saponified and methylated. The protocol of the Sherlock Microbial Identification System (MIDI) [45] was followed and gas chromatography (6890 N, Agilent Technologies) was performed using MIDI Sherlock software version 6.2 and the TSBA6 database version 6.2 for the identification of the fatty acids.

Fatty acid profiling can be used as reliable method for differentiation of bacteria [46]. In this study, a total of 13 and 10 fatty acids were found in S-93^T and S-62, respectively. The results showed that the major fatty acids of S-93^T and S-62 are $C_{19:0}$ cyclo *ω8c* and those from summed feature 8 (Table 3). However, some differences were observed in fatty acids $C_{18:1}$ ω7*c* 11-methyl, $C_{18:1}$ 2-OH, and those from summed feature 2, which were found in S-93^T but were not detected or were detected in low amounts in S-62. The fatty acid profile containing $C_{19:0}$ cyclo ω8*c* and summed feature 8 ($C_{18:1}$ ω7*c* and/or $C1_{8:1}$ ω6*c*) supports inclusion of the strains in the genus *Rhizobium* [46]

DESCRIPTION OF *RHIZOBIUM DIOSCOREAE* SP. NOV.

Rhizobium dioscoreae (di.os.co.re'ae. N.L. gen. n. *dioscorea* of the plant genus *Dioscorea* species).

Cells are Gram-strain negative, aerobic non-spore forming rods (0.4–0.5×2–2.2 µm). Colonies on MR medium (pH 6.8) are circular, opaque and white at 30 °C after 48 h incubation. Optimal cell growth occurs between pH 7 and 10. Acid production is positive for erythritol, D-xylose, D-galactose, D-fucose, L-fucose and aesculin, and a negative reaction is found for glycerol, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, methyl- α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, malic acid, trisodium citrate, reduction of potassium nitrate, fermentation of D-glucose, and hydrolysis of esculin ferric citrate are positive, and assimilation of potassium gluconate, capric acid, adipic acid and phenylacetic acid are negative. In addition, reactions are negative for arginine dihydrolase, gelatin hydrolysis and urease in the API 20NE test. Major fatty acids are $C_{19:0}$ cyclo *ω8c*, $C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*. The DNA G+C content of the type strain is 59.65 mol%.

The type strain, S-93^T (=NRIC 0988^T=NBRC 114257^T=DSM 110498^T), was isolated from the roots of lesser yam (*Dioscorea esculenta* L.), grown in nutrient-poor alkaline soils on Miyako Island in Okinawa Prefecture, Japan, as endophytic nitrogen-fixing bacteria. Strain S-62, isolated from the root of water yam (*Dioscorea alata* L.), is also classified in this species.

16S rRNA gene sequences of the two novel strains were submitted to the DDJB/ENA/GenBank under the accession numbers LC498520 (S-93^T) and LC483383 (S-62). The sequences of the draft genome were deposited in the DDBJ under the accession numbers BLAJ01000000 (S-93^T) and BLAI01000000 (S-62).

Funding information
This study was financially supported by the scholarship fund of the Ministry of Education, Culture, Sport, Science and Technology, Japan, and by JSPS KAKENHI grant number 15K14680.

Acknowledgements
The authors would like to express their gratitude to Dr Tomohiko Tamura (National Institute of Technology and Evolution, Chiba, Japan) and Professor Ken-ichiro Suzuki (Laboratory of Fermentation Microbiology, Tokyo University of Agriculture, Tokyo, Japan) for their assistance with the analysis and identification of the bacterial fatty acids. The authors are also grateful to Professor Aharon Oren (The Hebrew University of Jerusalem, Jerusalem, Israel) for his help in reviewing the etymology of the proposed species name.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Sheu S-Y, Huang H-W, Young C-C, Chen W-M. *Rhizobium alvei* sp. nov., isolated from a freshwater river. *Int J Syst Evol Microbiol* 2015;65:472–478.
2. Zhang S, Yang S, Chen W, Chen Y, Zhang M *et al*. *Rhizobium arenae* sp. nov., isolated from the sand of Desert Mu Us, China. *Int J Syst Evol Microbiol* 2017;67:2098–2103.
3. Zhang X-X, Tang X, Sheirdil RA, Sun L, Ma X-T. *Rhizobium rhizoryzae* sp. nov., isolated from rice roots. *Int J Syst Evol Microbiol* 2014;64:1373–1377.
4. Zhang X-X, Gao J-S, Cao Y-H, Sheirdil RA, Wang X-C *et al*. *Rhizobium oryzicola* sp. nov., potential plant-growth-promoting endophytic bacteria isolated from rice roots. *Int J Syst Evol Microbiol* 2015;65:2931–2936.
5. Zhao J-J, Zhang J, Sun L, Zhang R-J, Zhang C-W *et al*. *Rhizobium oryziradicis* sp. nov., isolated from rice roots. *Int J Syst Evol Microbiol* 2017;67:963–968.
6. Celador-Lera L, Menéndez E, Peix A, Igual JM, Velázquez E *et al*. *Rhizobium zeae* sp. nov., isolated from maize (*Zea mays* L.) roots. *Int J Syst Evol Microbiol* 2017;67:2306–2311.
7. Zhang L, Shi X, Si M, Li C, Zhu L *et al*. *Rhizobium smilacinae* sp. nov., an endophytic bacterium isolated from the leaf of *Smilacina japonica*. *Antonie van Leeuwenhoek* 2014;106:715–723.
8. Mano H, Morisaki H. Endophytic bacteria in the rice plant. *Microbes Environ* 2008;23:109–117.
9. Govaerts R, Wilkin P, Saunders RMK. *World Checklist of Dioscoreales, Yams and Their Allies*. UK: Royal Botanic Gardens, Kew publishing; 2007.
10. Asiedu R, Sartie A. Crops that feed the World 1. Yams for income and food security. *Food Secur* 2010;2:305–315.
11. Diby LN, Hgaza VK, Tie B, Assa A, Carsky R *et al*. Productivity of yams (*Dioscorea* spp.) as affected by soil fertility. *J Anim Plant Sci* 2009;5:494–506.
12. Ettien DJB, Kouadio KH, N'goran KE, Yao-Kouamé A, Girardin O. Improving the performance of a traditional variety of yam produced under ferralsol poor in organic matter in the forest areas of Côte d'Ivoire. *IJAAR* 2014;4:76–84.
13. Rezaei AQ, Kikuno H, Babil P, Tanaka N, Park B *et al*. Nitrogen-fixing bacteria is involved with the lesser yam (Dioscorea esculenta L.) growth under low fertile soil condition. *Trop Agric Develop* 2017;61:40–47.
14. Takada K, Kikuno H, Babil P, Shiwachi H. Analysis of the source of nitrogen during water yam (*Dioscorea alata* L.) growth using δ15N observations. *Trop Agric Develop* 2018;62:124–131.
15. Ouyabe M, Kikuno H, Tanaka N, Babil P, Shiwachi H. Nitrogen-fixing endophytic bacteria of water yam (*Dioscorea alata* L.) in relation with fertilization practices. *Trop Agric Develop* 2019a;63:122–130.
16. Ouyabe M, Kikuno H, Tanaka N, Babil P, Shiwachi H. Contribution of biological nitrogen fixation in lesser yam (Dioscorea esculenta L.) associated with endophytic diazotrophic bacteria. *Trop Agric Develop* 2019;63:131–139.
17. Ghiasian M, Sepahy AA, Amoozegar MA, Saadat-mand S, Shavandi M. Bacterial diversity determination using culture-dependent and culture-independent methods. *GJESM* 2017;3:153–164.
18. Shiwachi H, Kikuno H, Ohata J, Kikuchi Y, Irie K. Growth of water yam (*Dioscorea alata* L.) under alkaline soil conditions. *Trop Agric Develop* 2015;59:76–82.
19. Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B *et al*. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl Environ Microbiol* 2001;67:5285–5293.
20. Magnani GS, Didonet CM, Cruz LM, Picheth CF, Pedrosa FO *et al*. Diversity of endophytic bacteria in Brazilian sugarcane. *Genet Mol Res* 2010;9:250–258.
21. Paul D, Sinha SN. Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India. *Ann Agrar Sci* 2017;15:130–136.
22. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. *Plant Physiol* 1951;26:192–195.
23. Louden BC, Haarmann D, Lynne AM. Use of blue agar CAS assay for siderophore detection. *J Microbiol Biol Educ* 2011;12:51–53.
24. Arief II JBSL, Astawan M, Fujiyama K, Witarto AB. Identification and probiotic characteristics of lactic acid bacteria isolated from Indonesian local beef. *Asian J Anim Sci* 2015;9:25–36.
25. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y *et al*. Introducing EzBioCloud: a taxonomically United database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
26. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018;35:1547–1549.
27. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR *et al*. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.
28. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P *et al*. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
29. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
30. Techo S, Shiwa Y, Tanaka N, Fujita N, Miyashita M *et al*. *Enterococcus florum* sp. nov., isolated from a cotton flower (*Gossypium hirsutum* L.). *Int J Syst Evol Microbiol* 2019;69:2506–2513.
31. Kuykendall LD, Young JM, Martínez-Romero E, Kerr A *et al*, ed. *Bergey's Manual of Systematics of Archaea and Bacteria*. Athens, USA: John Wiley & Sons; 2015. pp. 1–36.

32. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 2009;106:19126–19131.
33. Wayne LG, Moore WEC, Stackebrandt E, Kandler O, Colwell RR, Brenner DJ, Grimont PAD *et al*. Report of the AD hoc Committee on reconciliation of approaches to bacterial Systematics. *Int J Syst Evol Microbiol* 1987;37:463–464.
34. Glaeser SP, Kämpfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. *Syst Appl Microbiol* 2015;38:237–245.
35. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J *et al*. BLAST+: architecture and applications. *BMC Bioinformatics* 2009;10:421.
36. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30:772–780.
37. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol* 2007;56:564–577.
38. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;30:1312–1313.
39. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA *et al*. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23:2947–2948.
40. Zhao Y, Wu J, Yang J, Sun S, Xiao J *et al*. PGAP: pan-genomes analysis pipeline. *Bioinformatics* 2012;28:416–418.
41. Caputo A, Fournier P-E, Raoult D. Genome and pan-genome analysis to classify emerging bacteria. *Biol Direct* 2019;14:5.
42. Deakin WJ, Broughton WJ. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat Rev Microbiol* 2009;7:312–320.
43. Bernal P, Llamas MA, Filloux A. Type VI secretion systems in plant-associated bacteria. *Environ Microbiol* 2018;20:1–15.
44. Mueller HW, O'Flaherty JT, Wykle RL. Ether lipid content and fatty acid distribution in rabbit polymorphonuclear neutrophil phospholipids. *Lipids* 1982;17:72–77.
45. Sasser M. *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc; 1990.
46. Tighe SW, de Lajudie P, Dipietro K, Lindström K, Nick G *et al*. Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock microbial identification system. *Int J Syst Evol Microbiol* 2000;50 Pt 2:787–801.
47. Dall'Agnol RF, Ribeiro RA, Ormeño-Orrillo E, Rogel MA, Delamuta JRM *et al*. *Rhizobium freirei* sp. nov., a symbiont of *Phaseolus vulgaris* that is very effective at fixing nitrogen. *Int J Syst Evol Microbiol* 2013;63:4167–4173.
48. Zhang RJ, Hou BC, Wang ET, Li Y, Zhang XX *et al*. *Rhizobium tubonense* sp. nov., isolated from root nodules of *Oxytropis glabra*. *Int J Syst Evol Microbiol* 2011;61:512–517.
49. Han TX, Wang ET, Wu LJ, Chen WF, Gu JG *et al*. *Rhizobium multihospitium* sp. nov., isolated from multiple legume species native of Xinjiang, China. *Int J Syst Evol Microbiol* 2008;58:1693–1699.
50. Gil-Serrano AM, González-Jiménez I, Tejero-Mateo P, Megías M, Romero-Vazquez MJ. Analysis of the lipid moiety of lipopolysaccharide from *Rhizobium tropici* CIAT899: identification of 29-hydroxytriacontanoic acid. *J Bacteriol* 1994;176:2454–2457.