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Report of five unrecorded bacterial species in Korea belonging to the genus *Bradyrhizobium*

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Symbiotic bacteria belonging to the genus *Bradyrhizobium* were generally called 'rhizobia' which can fix nitrogen by nodulating legumes. Between 2000 and 2001, numerous *Bradyrhizobium* strains were isolated from root nodules. However, due to challenges in identification, many of these isolates remained unreported. Recent advances in phylogenetic analyses based on whole genome sequencing have resolved these difficulties in species identification. Consequently, five of these strains have now been re-identified and are described as previously unrecorded species in the Republic of Korea. As a result, we report *Bradyrhizobium elkanii* Glm-3 (=KACC 10989), *Bradyrhizobium australafricanum* Glm-4 (=KACC 10990), *Bradyrhizobium huanghuaihaiense* Glm-7 (=KACC 10993), these were isolated from root nodules of *Glycine max*, *Bradyrhizobium frederickii* Kus-5 (=KACC 11016) isolated from root nodules of *Kummerowia striata* and *Bradyrhizobium diazoefficiens* Leb-14 (=KACC 11026) isolated from root nodules of *Lespedeza bicolor*.

Keywords: *Bradyrhizobium*, Rhizobia, unrecorded species

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Introduction

The genus *Bradyrhizobium* was established by Jordan DC (1982), belongs to the family *Nitrobacteracea*. This genus comprises 72 validly published species, with *Bradyrhizobium japonicum* as the type species (https://lpsn.dsmz.de/genus/microbacterium; accessed 24 June 2024). They are a Gram-negative, non-spore-forming, rod-shaped and either motile or non-motile. The genus *Bradyrhizobium* is known as a predominant symbiont of legumes by nodulating (Parker, 2015) and most of them have ability to biological nitrogen fixation (BNF) (Vanlnsberghe *et al*., 2015). They are found mainly in soil, rhizosphere and plant root nodules, and are classified by their or and characteristics (Parker, 2015; Tao *et al*., 2021). The significance of *Bradyrhizobium* has led to studies on its diversity in Korea, many of strains were isolated from legumes or agricultural soils (Kwon *et al*., 2005; Kim *et al*., 2022). However, only six species are currently reported in Korea according to the National List of Species of Korea (https://species.nibr.go.kr; accessed 24 June 2024). Because *Bradyrhizobium* cannot be identified based on 16S rRNA region sequences alone, most of them are not identified with the correct scientific name (Vinuesa *et al*., 2005; Delamuta *et al*., 2013). In this study, we focused on re-identification of *Bradyrhizobium* strains isolated from plant in Korea during 2000−2001. These strains were preserved in Korea Agricultural Culture Collection (KACC) and describe the taxonomic properties of five unrecorded species of the genus *Bradyrhizobium*.

Materials and Methods

All strains have been deposited at the Korean Agricultural Culture Collection (KACC) which were isolated from nodules of *Glycine max*, *Lespedeza bicolor* or *Kummerowia striata* in 2001−2002 reported by Qian *et al*. (2003). The designated strain IDs, isolation sources and identification results are summarized in Table 1. All cultured strains were maintained in glycerol suspension (15%, v/v) at −80°C.

The 16S rRNA and *recA* gene sequencing of the strains pure-cultured by the above procedure was conducted by Macrogen. For getting the sequence of *recA* gene region, all strains were amplified with the TSrecAf and TSrecAr primer sets described by Stępkowski *et al*. (2005).

These gene sequences of the type strains of the *Bradyrhizobium* species were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic

Table 1. The taxonomic affiliations of isolated strains belonging to the genus *Bradyrhizobium*.

Genus	Strain code	KACC[a] ID	Identification	16S rRNA similarity (%)	*recA* similarity (%)	Host	Location	Culture conditions
Bradyrhizobium	Glm-3	10989	*Bradyrhizobium elkanii*	100	100	*Glycine max*	Suwon-si, Gyeonggi-do	R2A[b] medium, 28°C, 5 days
	Glm-4	10990	*Bradyrhizobium ferriligni*	99.6	99.6	*Glycine max*	Suwon-si, Gyeonggi-do	
	Glm-7	10993	*Bradyrhizobium huanghuaihaiense*	100	100	*Glycine max*	Suwon-si, Gyeonggi-do	
	Kus-5	11016	*Bradyrhizobium frederickii*	100	97.7	*Kummerowia striata*	Suwon-si, Gyeonggi-do	
	Leb-14	11026	*Bradyrhizobium diazoefficiens*	100	98.2	*Lespedeza bicolor*	Suwon-si, Gyeonggi-do	

[a]Korean Agricultural Culture Collection (KACC).
[b]R2A agar medium containing yeast extract 0.05% (w/v), peptone 0.05% (w/v), casamino acid 0.05% (w/v), dextrose 0.05% (w/v), soluble starch 0.05% (w/v), dipotassium phosphate 0.03% (w/v), magnesium sulfate 0.005% (w/v), sodium pyruvate 0.03% (w/v) and agar 1.5% (w/v).

trees were reconstructed with three different algorithms, neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-likelihood (ML) (Felsenstein, 1981) and maximum-parsimony (MP) (Fitch, 1971) algorithm, in MEGA X (Tamura *et al*., 2021). Evolutionary distance matrices for the neighbor-joining and maximum-likelihood analyses were evaluated using Kimura 2 parameter model. Bootstrap analyses with 1,000 times were carried out for stability evaluation of tree topology (Felsenstein, 1985). Cell morphology was observed by a phase-contrast microscope (AX10; Carl Zeiss) and a transmission electron microscope (TEM; LEO 912AB; LEO Electron) after being grown on Reasoner's 2A agar medium at 28°C for 5 days. Gram staining was tested using a Gram staining kit (Sigma Aldrich, USA) according to the manufacturer's instructions. Catalase activity was tested by adding 3% (v/v) hydrogen peroxide solution (bioMérieux, France) and observing for bubbling, while oxidase activity was assessed by applying 1% (w/v) tetramethyl-p-phenylenediamine and noting any color change. Additional biochemical properties was determined using API 20NE kits (bioMérieux, France) according to the manufacturer's recommendations. In addition, media growth studies were performed. The media used were yeast mannitol agar (YMA), Reasoner's 2A (R2A) agar, nutrient agar (NA), potato dextrose agar (PDA), trypticase soy agar (TSA), yeast-peptone-dextrose (YPD) agar, Luria-Bertani (LB) agar, and marine agar solid media. The YMA medium consisted of 1 g/L yeast extract (BD Difco, USA), 2 g/L casamino acids (BD Difco, USA), 20 g/L Bacto Agar (BD Difco, USA), 1 g/L beef extract (Sigma Aldrich, USA), and 10 g/L maltose (Sigma Aldrich, USA). Other media ingredients were sourced from BD Difco in the USA and were prepared according to their respective instructions. All strains were inoculated on each solid media, and monitored at 3, 5, 7, and 10 days at 28°C.

Results and Discussion

The results of 16S rRNA and *recA* gene sequences analysis show that five strains were confirmed as unrecorded *Bradyrhizobium* species in Korea (Table 1). The phylogenetic trees between the isolated strains and closely related *Bradyrhizobium* type strains were presented in Figs. 1 and 2. The isolation of *Bradyrhizobium* strains obtained from *Lespedeza bicolor* and *Kummerowia striata* is taxonomically important report. On the other hand, the isolates isolated from *Glycine max* could be useful for agriculture.

Cellular morphology and the presence of flagella, which were examined by transmission electron microscope, were presented in Fig. 3. Flagella of *Bradyrhizobium elkanii* Glm-3 were only observed on this bacterium in dense clusters presented in Fig. 3. All of strains confirmed flagel-

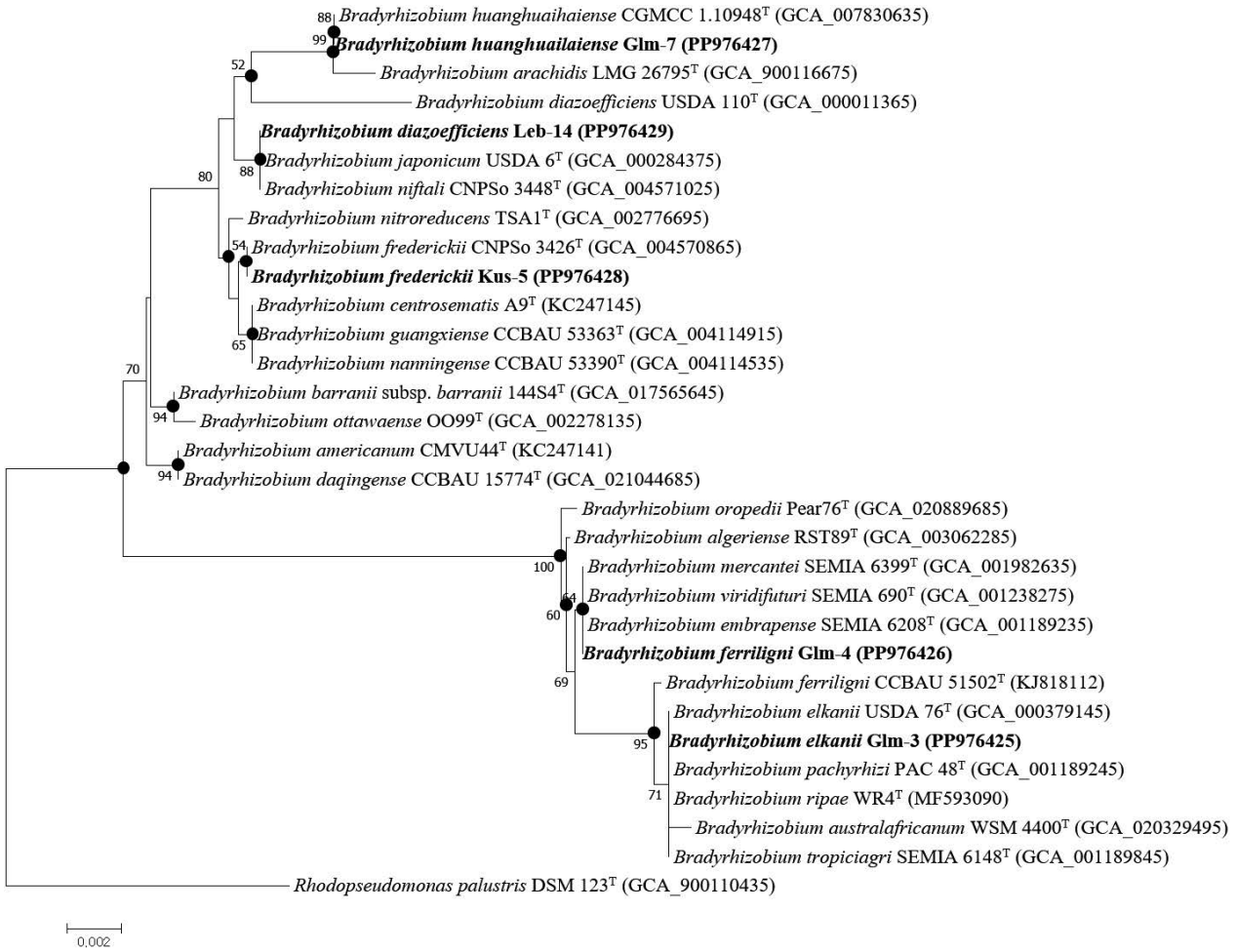

Fig. 1. Neighbor-joining phylogenetic tree showing the phylogenetic relationships of strains reported in this study and related species of *Bradyrhizobium*, based on 16S rRNA gene sequences. Numbers on nodes correspond to bootstrap values for branches (1,000 replicates); only values over 50% are shown. Filled circles indicate the corresponding nodes that were also recovered in trees constructed using the maximum-likelihood and maximum-parsimony algorithms. Scale bar, 0.002 substitutions per nucleotide.

lum depending on species. The phenotypic characteristics among five strains are listed in Table 2. The detailed description of each of *Bradyrhizobium* strains were described below.

Description of *Bradyrhizobium elkanii* Glm-3 (=KACC 10989)

Cells are Gram-negative, flagellated or non-flagellated, non-spore-forming rods (0.8 μm × 1.5 μm). Colonies are circular and white colored with less than 1 mm in diameter within 5 days at 28°C on R2A medium. Catalase-negative and oxidase-positive. Positive for nitrate reduction and urease activity; but negative for indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatin hydrolysis and β-galactosidase activity. According to API 20 NE test results, all assimilations were negative; D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. It grow on YMA, R2A, NA, PDA media within 3 days at 28°C; but weakly grow on YPD, TSA, LB, Marine agar media within 5 days or more at 28°C in the aerobic condition. Strain Glm-3 (=KACC 10989) was isolated from *Glycine max* sampled from Suwon-si, Gyeonggi-do. The GenBank accession number for the 16S rRNA gene and *recA* gene sequence of strain Glm-3 is PP986425 and PP990516, respectively.

Description of *Bradyrhizobium ferriligni* Glm-4 (=KACC 10990)

Cells are Gram-negative, flagellate, non-spore-forming rods (0.7 μm × 1.7 μm). Colonies are circular and white colored with less than 1 mm in diameter within 5 days at 28°C on R2A medium. Catalase-negative and oxidase-

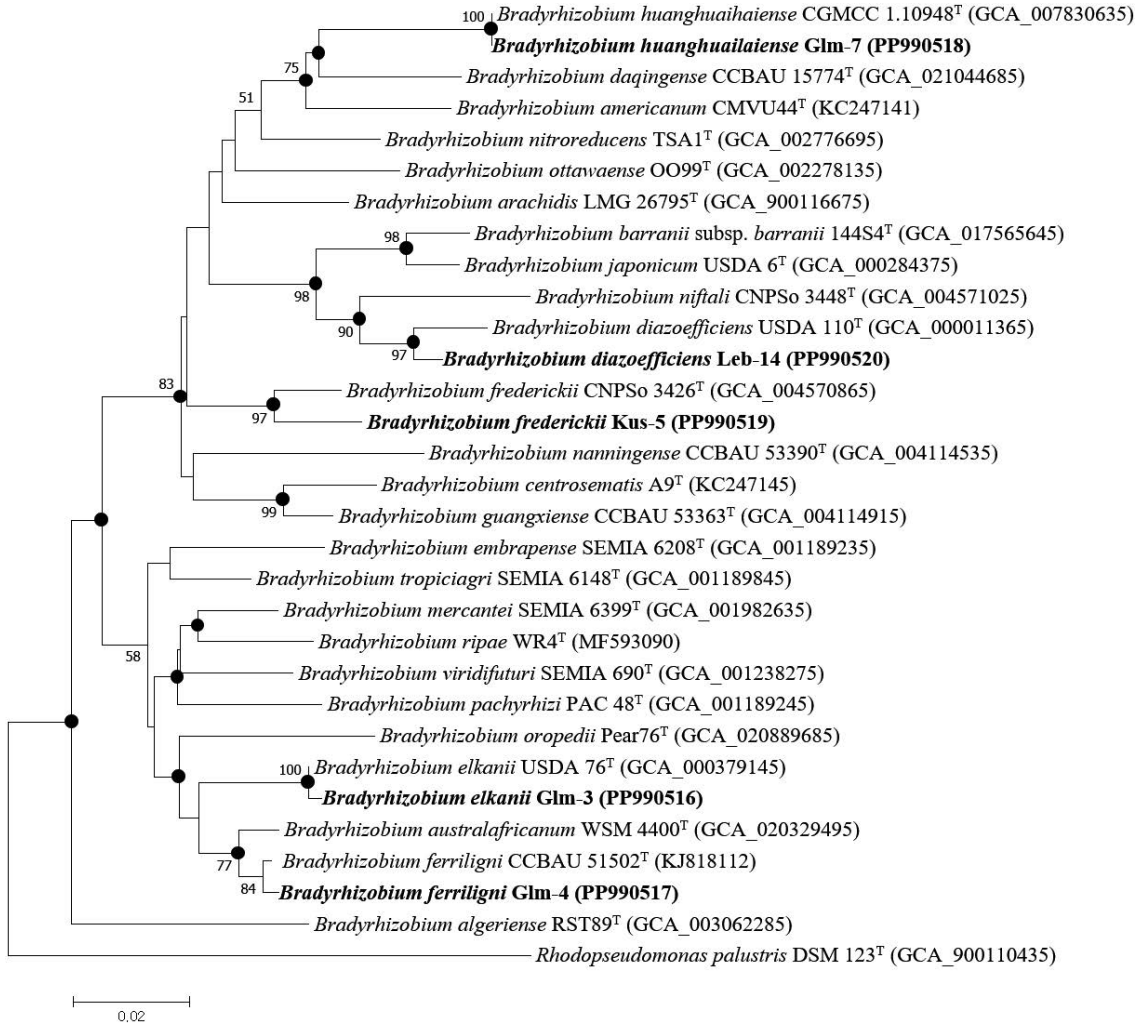

Fig. 2. Neighbor-joining phylogenetic tree showing the phylogenetic relationships of strains reported in this study and related species of *Bradyrhizobium*, based on *recA* gene sequences. Numbers on nodes correspond to bootstrap values for branches (1,000 replicates); only values over 50% are shown. Filled circles indicate the corresponding nodes that were also recovered in trees constructed using the maximum-likelihood and maximum-parsimony algorithms. Scale bar, 0.02 substitutions per nucleotide.

positive. Positive for urease activity; but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatin hydrolysis and β-galactosidase activity. According to API 20 NE test results, it assimilates D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine and D-maltose; but does not assimilate potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. It grow on YMA, R2A, NA and PDA media within 3 days at 28°C; but weakly grow on YPD, TSA, LB and Marine agar media within 5 days or more at 28°C in the aerobic condition. Strain Glm-4 (=KACC 10990) was isolated from *Glycine max* sampled from Suwon-si, Gyeonggi-do. The GenBank accession number for the 16S rRNA gene and *recA* gene sequence of strain Glm-4 is PP986426 and PP990517, respectively.

Description of *Bradyrhizobium huanghuaihaiense* Glm-7 (=KACC 10993)

Cells are Gram-negative, flagellated, non-spore-forming rods (0.8 μm × 1.8 μm). Colonies are circular and white colored with 1 mm in diameter within 5 days at 28°C on R2A medium. Catalase-positive and oxidase-positive. Positive for urease activity; but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatin hydrolysis and β-galactosidase activity. According to API 20 NE test results, all assimilations were negative; D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. It grow on YMA, R2A, NA and PDA media within 3 days at 28°C; but weakly grow on YPD, LB and Marine agar media within

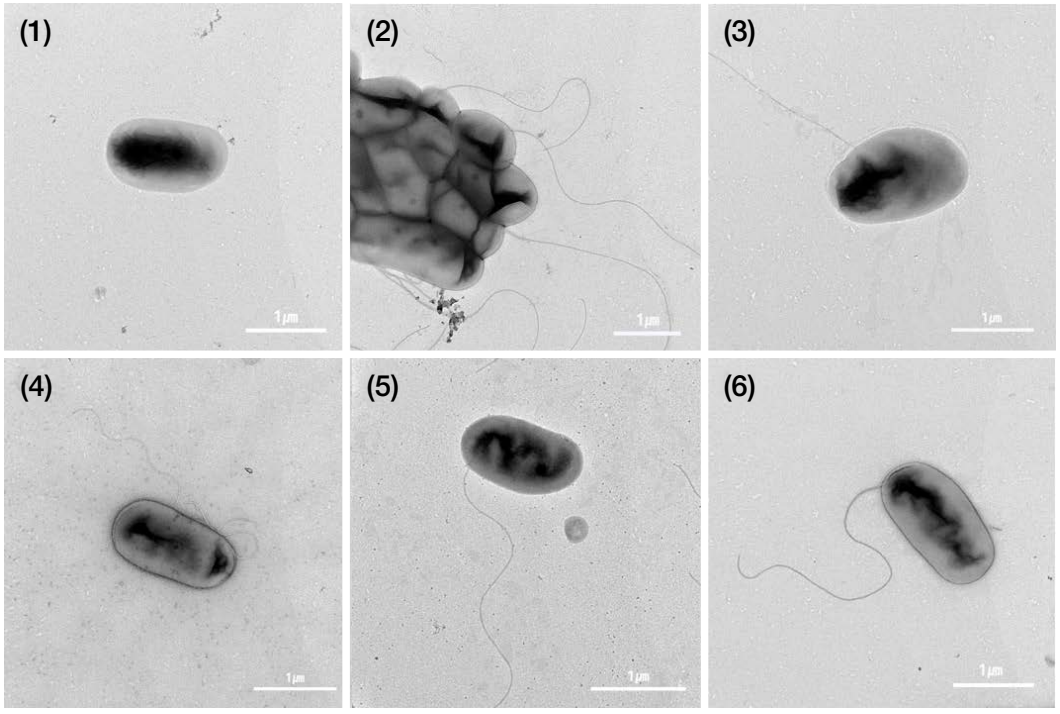

Fig. 3. Transmission electron micrographs of the *Bradyrhizobium* strains. Strain: 1 and 2, *Bradyrhizobium elkanii* Glm-3 (=KACC 10989); 3, *Bradyrhizobium australafricanum* Glm-4 (=KACC 10990); 4, *Bradyrhizobium huanghuaihaiense* Glm-7 (=KACC 10993); 5, *Bradyrhizobium frederickii* Kus-5 (=KACC 11016); 6, *Bradyrhizobium diazoefficiens* Leb-14 (=KACC 11026).

Table 2. Differential phenotypic characteristics among the isolated strains classified into the genus.

Characteristics	1	2	3	4	5
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell size (μm)	0.8−1.0×1.5	0.7−1.1×1.2−1.7	0.8−1×1.5−1.8	0.9×1.5	0.9×1.5
Flagellum	Present	Present	Present	Present	Present
Catalase/oxidase	−/+	−/+	+/+	+/+	−/+
Activity:					
arginine dihydrolase	−	−	−	−	+
nitrate reduction	+	−	−	+	−
urease activity	+	+	+	+	+
Assimilation:					
D-glucose	−	+	−	−	−
L-arabinose	−	+	−	−	−
D-mannose	−	+	−	−	−
D-mannitol	−	+	−	−	−
N-acetylglucosamine	−	+	−	−	−
D-maltose	−	+	−	−	−

Strain: 1, *Bradyrhizobium elkanii* Glm-3 (=KACC 10989); 2, *Bradyrhizobium ferriligni* Glm-4 (=KACC 10990); 3, *Bradyrhizobium huanghuaihaiense* Glm-7 (=KACC 10993); 4, *Bradyrhizobium frederickii* Kus-5 (=KACC 11016); 5, *Bradyrhizobium diazoefficiens* Leb-14 (=KACC 11026).
All strains are positive for oxidase and urease activity, but negative for Gram-staining, aesculin hydrolysis, gelatin hydrolysis glucose fermentation, indole production and β-galactosidase activity; +, positive; −, negative.

5 days or more at 28°C in the aerobic condition. The TSA media did not grow. Strain Glm-7 (=KACC 10993) was isolated from *Glycine max* sampled from Suwon-si, Gyeonggi-do. The GenBank accession number for the 16S rRNA gene and *recA* gene sequence of strain Glm-3 is PP986427 and PP990518, respectively.

Description of *Bradyrhizobium frederickii* Kus-5 (=KACC 11016)

Cells are Gram-negative, flagellated, non-spore-forming rods (0.9 μm × 1.5 μm). Colonies are circular and white colored with 1 mm in diameter within 5 days at 28°C on R2A medium. Catalase-positive and oxidase-positive. Positive for nitrate reduction and urease activity; but negative for indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatin hydrolysis and β-galactosidase activity. According to API 20 NE test results, all assimilations were negative; D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. It grow on YMA, R2A, NA and PDA media within 3 days at 28°C; but weakly grow on LB and Marine agar media within 5 days or more at 28°C in the aerobic condition. The YPD and TSA media did not grow. Strain Kus-5 (=KACC 11016) was isolated from *Kummerowia striata* sampled from Suwon-si, Gyeonggi-do. The GenBank accession number for the 16S rRNA gene and *recA* gene sequence of strain Kus-5 is PP986428 and PP990519, respectively.

Description of *Bradyrhizobium diazoefficiens* Leb-14 (=KACC 11026)

Cells are Gram-negative, flagellated, non-spore-forming rods (0.9 μm × 1.5 μm). Colonies are circular and white colored with 1 mm in diameter within 5 days at 28°C on R2A medium. Catalase-negative and oxidase-positive. Positive for arginine dihydrolase and urease activity; but negative for nitrate reduction, indole production, glucose fermentation, aesculin hydrolysis, gelatin hydrolysis and β-galactosidase activity. According to API 20 NE test results, all assimilations were negative; D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. It grow on YMA, R2A, NA and PDA media within 3 days at 28°C; but weakly grow on LB and Marine agar media within 5 days or more at 28°C in the aerobic condition. The YPD and TSA media did not grow. Strain Leb-14 (=KACC 11026) was isolated from *Lespedeza bicolor* sampled from Suwon-si, Gyeonggi-do. The GenBank accession number for the 16S rRNA gene and *recA* gene sequence of strain Glm-3 is PP986429 and PP990520, respectively.

Conflicts of Interest

The author of this paper has no affiliation with any interests and is solely responsible for the paper.

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References

Delamuta, J.R.M., R.A. Ribeiro, E. Ormeno-Orrillo, I.S. Melo, E. Martínez-Romero and M. Hungria. 2013. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. International Journal Systematic and Evolutionary Microbiology 63:3342-3351.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. Journal of Molecular Evolution 17:368-376.

Felsenstein, J. 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution 39:783-791.

Fitch, W.M. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. Systematic Biology 20:406-416.

Jordan, D. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. International Journal Systematic and Evolutionary Microbiology 32:136-139.

Kim, Y.-e., H. Shin, Y. Yang and H.-G. Hur. 2022. Geographical distribution and genetic diversity of *Bradyrhizobium* spp. isolated from Korean soybean root nodules. Applied Biological Chemistry 65:39.

Kwon, S.W., J.Y. Park, J.S. Kim, J.W. Kang, Y.H. Cho, C.K. Lim and G.B. Lee. 2005. Phylogenetic analysis of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences. International Journal of Systematic and Evolutionary Microbiology 55:263-270.

Parker, M.A. 2015. The spread of *Bradyrhizobium* lineages across host legume clades: from Abarema to Zygia. Microbial Ecology 69:630-640.

Qian, J., S-.W. Kwon and M.A. Parker. 2003. rRNA and *nifD* phylogeny of *Bradyrhizobium* from sites across the Pacific Basin. FEMS Microbiology Letters 219:159-165.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Stępkowski, T., M. Lionel, K. Agnieszka, M. Alison, J.L. Ian and H. John. 2005. European Origin of *Bradyrhizobium* Populations Infecting Lupins and Serradella in Soils of Western Australia and South Africa. Applied and Environmental Microbiology 71:7041-7052.

Tamura, K., G. Stecher and S. Kumar. 2021. MEGA11: molecular evolutionary genetics analysis version 11. Molecular

Biology and Evolution 38:3022-3027.

Tao, J., S. Wang, T. Liao and H. Luo. 2021. Evolutionary origin and ecological implication of a unique *nif* island in free-living *Bradyrhizobium* lineages. The ISME Journal 15: 3195-3206.

VanInsberghe, D., K.R. Maas, E. Cardenas, C.R. Strachan, S.J. Hallam and W.W. Mohn. 2015. Non-symbiotic *Bradyrhizobium* ecotypes dominate North American forest soils. The ISME Journal 9:2435-2441.

Vinuesa, P., M. León-Barrios, C. Silva, A. Willems, A. Jarabo-Lorenzo, R. Pérez-Galdona, D. Werner and E. Martínez-Romero. 2005. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (*Papilionoideae*: *Genisteae*) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium genospecies* alpha and *Bradyrhizobium genospecies* beta. International Journal Systematic and Evolutionary Microbiology 55:569-575.

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