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Genome Insight and Description of Previously Uncultured N_2-Fixing Bacterium *Rhizobium terricola* sp. nov., Isolated from Forest Rhizospheric Soil by Using Modified Culture Method

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Abstract: A bacterial strain S-51^T was isolated from rhizospheric forest soil at Kyonggi University during the study of previously uncultured bacterium. The phylogenetic analysis was based on 16S rRNA gene sequences that indicated that strain S-51^T belonged to the genus *Rhizobium* within the family *Rhizobiaceae*. The closest members of strain S-51^T were *Rhizobium naphthalenivorans* TSY03b^T (98.2% sequence similarity) and *Rhizobium selenitireducens* ATCC BAA-1503^T (98.1%). The sequence similarities of other members were <97.7%. The sole respiratory quinone was Q-10 and the major polar lipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylcholine, and unidentified glycolipid. The principal fatty acids were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), cyclo-$C_{19:0}\omega8c$, and $C_{18:0}$. The DNA G+C content was 63.1 mol%. The genome was 4930044 bp long and contained N_2-fixing genes, such as *fixF*, *ntrC*, and *ptsN*, in addition to respiratory nitrate reductase genes, such as *narC*, *narG*, *narH*, *narI*, and *narJ*. The average nucleotide identity (ANIu), average amino acid identity (AAI), and digital DNA-DNA hybridization (dDDH) relatedness between strain S-51^T and phylogenetically related species were ≤82.6, ≤83.6 and ≤25.3%, respectively, much lower than the species delineation thresholds. Based on the polyphasic taxonomic study, strain S-51^T represents a new species in the genus *Rhizobium*, for which the name *Rhizobium terricola* is proposed. The type strain is S-51^T (=KACC 19117^T = KEMB 9005-539^T = NBRC 112711^T).

Keywords: *Rhizobium terricola* sp. nov.; uncultured bacterium; forest soil; N_2-fixation; α-*Proteobacteria*

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1. Introduction

Advances in technology and metagenomic studies have revealed that most of the environmental microorganisms are 'unculturable', 'uncultured', or 'not-yet cultured' on synthetic media in the laboratory [1]. Uncultured microbes encompass most of the microbial diversity on this planet, as around 99% of microorganisms are still uncultivated [2]. There are various normal to complex reasons for failing to cultivate these microbes in the laboratory, such as lack of exact or correct nutrients, unsuitable pH, excessive use of rich-nutrient media, insufficient or improper incubation time, inappropriate temperature, specific growth signal, dependence on other microbes, and specially the lack of natural growth environment in the laboratory setup [1–3].

The genus *Rhizobium* has been described as root and/or stem-nodule bacteria by Frank [4]. To date, there are 117 species of the genus *Rhizobium* with validly published names (https://lpsn.dsmz.de/genus/rhizobium (accessed on 25 June 2022)). Representatives of the genus *Rhizobium* are characterized as motile, non-spore-forming, Gram-stain-negative, rod-shaped, and containing DNA G+C between 57 and 66 mol% and $C_{18:1}\omega7c$ as a principal fatty acid [5,6]. The members of the genus *Rhizobium* are well distributed in soil and are significant for their use in agriculture, as most of the species are able to fix

nitrogen in legume plants [5]. Most of the *Rhizobium* species have been isolated from the root nodule of leguminous plants [5,7]. However, in recent years, novel members of the genus *Rhizobium* have also been isolated from non-legume niches, such as activated sludge, bioreactor, sand dunes, pesticide-contaminated sites, effluent treatment plant, sediment, sea water, fresh water, and groundwater [6,8–14]. This study describes the polyphasic and taxonomic characteristics of strain S-51^T isolated from forest rhizospheric soil during the study of uncultured soil bacteria.

Due to their nitrogen fixing capability, the members of the genus *Rhizobium* are agriculturally and environmentally valuable [15,16]. The species of *Rhizobium* primarily inhabit the root or stem nodules of plants and provide a nitrogen source to their host plants [16]. However, the free-living nitrogen fixing species such as S-51^T isolated in this study are equally important, as these species could contribute adequate amounts of elemental nitrogen to the soil.

2. Material and Methods

2.1. Isolation and Ecology

Strain S-51^T was isolated from the rhizospheric loamy sandy soil in Kyonggi University's forest, located at Suwon, Gyeonggi-do, Republic of Korea (37°18′5″ N and 127°1′56″ E). A modified culture method using a six-well transwell plate (Corning Inc., Corning, NY, US) was used for isolation. Debris-free sieved soil (~3 g) was kept on the bottom of the transwell plate and 3 mL of diluted (1/10) R2A (Reasoner's 2A; KisanBio, Seoul, Republic of Korea) broth was added to the insert. After that, 100 µL of soil suspension (1 g of soil in 9 mL of distilled water) was added to the insert. Then, the transwell plate was incubated in a shaker at 130 rpm at 28 °C for 6 weeks. After 6 weeks, the enriched culture was serially diluted, and then 100 µL of each dilution was spread on 1/10 R2A agar and incubated at 28 °C for 4 weeks [17]. The short-term maintenance and long-term preservation were performed as described previously [18].

2.2. 16S rRNA Phylogeny

Genomic DNA of strain S-51^T was extracted using the InstaGene Matrix kit (Bio-Rad; Hercules, CA, US) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed with PCR using the primers 27F and 1492R [19]. Sequencing was carried out using an Applied Biosystems 3770XL DNA analyzer with a BigDye Terminator cycle sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, US). Nearly complete sequences of 16S rRNA genes were assembled with the SeqMan software (DNASTAR Inc., Madison, WI, US). For type material sequences, the nearest phylogenetic neighbours were identified using the EzBioCloud database [20]. In addition, non-type material sequences were compared with the top hits of Megablast (GenBank). All the 16S rRNA gene sequences of the closest phylogenetic members were retrieved from the NCBI GenBank and aligned using SILVA alignment (https://www.arb-silva.de (accessed on 14 March 2018)). Phylogenetic trees were reconstructed using the three tree making methods: neighbor-joining [21], maximum-likelihood [22], and maximum-parsimony [23] algorithms with MEGA7 [24]. The evolutionary distances were calculated according to the Kimura 2-parameter model [25] and bootstrap analysis was based on 1000 replications [26].

2.3. Genome Features

Whole-genome-based approaches were used for the further analysis of taxonomic status of novel strain. The genomic DNA was extracted using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany). Whole genome shotgun sequencing of strains was performed using Macrogen (Seoul, Korea) using the Illumina HiSeq platform and assembled by SPAdes version 3.13.0 [27]. The Whole Genome Shotgun project of strain S-51^T was deposited in the NCBI repository (or DDBJ/ENA/GenBank) and the sequences are available at https://www.ncbi.nlm.nih.gov/bioproject (accessed on 28 April 2020) under accession numbers PRJNA626510 and SAMN14642770. The authenticity of the genome assembly was

checked by comparing 16S rRNA gene sequence using NCBI Align Sequences Nucleotide BLAST tool [28], and the potential contamination was checked using the ContEst16S algorithm [29]. The completeness of genome was checked using searches of conserved single-copy orthologous BUSCO V3.0.2 [30]. After analysis, the genome sequence was annotated using the NCBI Prokaryotic Genome Annotation Pipeline version 4.11 [31] and Rapid Annotations using Subsystems Technology (RAST) server version 2.0 [32]. Genome-based relatedness between strain S-51^T and closely related strains was determined using whole genome sequences based on average nucleotide identity (ANI) using the OrthoANIu algorithm [33]. DNA-DNA hybridization (DDH) was calculated using whole genome sequences in silico with the Genome-to-Genome Distance Calculator (GGDC version 2.1) using the blast method [34]. The average amino acid identity (AAI) was determined using a web server (http://enve-omics.ce.gatech.edu/aai/index (accessed on 18 July 2022)). In addition, a genomic feature map was constructed using CGView server [35]. Moreover, the annotation and analysis of secondary metabolite biosynthesis genes were carried out using anti-SMASH server version 5.0 [36]. The COG (Clusters of Orthologous Group) functional categories were assigned by searching against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [37]. Furthermore, CRISPR gene and Cas cluster were analyzed using the CRISPRCasFinder online server (https://crisprcas.i2bc.paris-saclay.fr/ (accessed on 18 July 2022)). The Venn diagram of whole genome orthologous genes was constructed using the Orthovenn2 web server [38] (https://orthovenn2.bioinfotoolkits.net/home (accessed on 18 July 2022)).

2.4. Physiology and Chemotaxonomy

The cell morphology of strain S-51^T, grown on R2A agar for 1 week at 28 °C, was examined by transmission electron microscopy (Talos L120C; FEI). Colony morphology was observed with the Zoom Stereo Microscope (SZ61; Olympus, Tokyo, Japan). Gram staining was performed as described previously [39]. Motility of strain S-51^T was determined in R2A medium containing 0.4% (*w*/*v*) agar. Oxidase activity was observed using 1% (*w*/*v*) tetra-methyl-*p*-phenylenediamine dihydrochloride. Catalase activity was assessed using 3% (*v*/*v*) hydrogen peroxide (H_2O_2). Growth at various temperatures from 4 to 40 °C on R2A agar plates was monitored for 10 days. Growth was determined on various media, including R2A agar, nutrient agar (NA; Oxoid, Hampshire, UK), sorbitol MacConkey agar (MA; Oxoid), tryptone soya agar (TSA; Oxoid), marine agar 2216 (Becton), potato dextrose agar (PDA; Becton), brain heart infusion agar (BHI; Oxoid), veal infusion agar (Becton), and Luria-Bertani agar (LBA; Oxoid). Salt tolerance was examined in R2A broth supplemented with NaCl (0–5%, at 0.5% intervals). The pH range was determined at 28 °C in R2A broth adjusted to pH 4–12 (in increments of 0.5 pH units) [40]. Hydrolysis of Tween 40, Tween 60, and Tween 80 was assessed using the method of Smibert and Krieg [41]. Anaerobic growth was monitored on the R2A agar plate at 28 °C for 10 days using the BD GasPak EZ Gas Generating Pouch System with nitrate as a final electron acceptor. Hydrolysis of xanthine, hypoxanthine, starch, chitin, CM-cellulose, tyrosine, and casein was tested as previously described [42]. A DNase activity assay was performed with DNase agar (Oxoid). Presence of spore was examined by staining with malachite green. For the nitrogen-fixation test, strain S-51^T was grown on N_2-free media with bromothymol blue (NfB) indicator [43]. Other biochemical and physiological tests were performed using the API ID 32GN and API 20NE test kits (bioMérieux; Marcy-l'Étoile, France). Enzyme activities were determined using an API ZYM kit (bioMérieux), as per the manufacturer's instructions.

For fatty acid analysis, cells of strain S-51^T and reference strains were harvested from identical culture condition (on R2A agar plates at 28 °C for 4 days). The cellular fatty acids were extracted using the MIDI protocol (Sherlock Microbial Identification System, version 6.0B) and analyzed with a gas chromatograph (HP 6890 Series GC System; Hewlett Packard). The fatty acids (%of totals) were identified using the TSBA6 database of the Microbial Identification System [44]. Polar lipids and isoprenoid quinones were extracted from freeze-dried cells according to the procedures described by Min-

nikin et al. [45]. The biomass was harvested from R2A agar plates incubated at 28 °C for 6 days. The polar lipids were analyzed with two-dimensional TLC (thin layer chromatography) using chloroform:methanol:water (65:25:4; *v*/*v*/*v*) in the first dimension and chloroform:methanol:aceteic acid:water (40:7.5:6:2; *v*/*v*/*v*/*v*) in the second direction.

3. Results and Discussion

3.1. Isolation of Uncultured Strain

In general, the isolation of bacterial strains involves enrichment culture, seral dilution, plating, and pure culture in this order using known media. However, in this study, a modified method using a transwell plate and diluted R2A medium was applied to isolate uncultured soil bacteria, including strain S-51^T, in the step of enrichment culture and in the step of plating, respectively.

3.2. Phylogenetic Analysis

The nucleotide sequence of 16S rRNA gene of strain S-51^T was deposited in the GenBank/EMBL/DDBJ database under the accession number KY117474. Preliminary comparisons with 16S rRNA gene sequences indicated that strain S-51^T showed top hits with previously uncultured bacterial clones in the NCBI GenBank database (99.65–97.72%) for non-type material sequences. The phylogenetic tree based on 16S rRNA gene sequences also revealed that strain S-51^T clustered with uncultured bacterium clones with strong bootstrap (99%) values (Figure 1). In addition, when the 16S rRNA gene sequence of strain S-51^T was analyzed with the EzBioCloud server for type-material sequences, it showed that strain S-51^T belonged to the family *Rhizobiaceae* and was most closely related to *Rhizobium naphthalenivorans* TSY03b^T (98.2% sequence similarity) and *Rhizobium selenitireducens* ATCC BAA-1503^T (98.1%). The sequence similarities of other members were <97.7%. As a result, the strain S-51^T showed 98.2–97.7% 16S rRNA gene sequence similarities to its closest members of the genus *Rhizobium*. These values lie within the range needed to specify a strain as a novel species of the genus [46].

Separate lineage and the strong bootstrap values with closest members (*Rhizobium naphthalenivorans* TSY03b^T and *Rhizobium selenitireducens* ATCC BAA-1503^T) formed in the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) trees displayed that strain S-51^T belonged to a novel species within the genus *Rhizobium* (Figures 2, S1 and S2).

Based on sequence similarities and phylogenetic analyses, *Rhizobium naphthalenivorans* KCTC 23252^T and *Rhizobium selenitireducens* KCTC 23231^T were selected for comparative analysis and were used as reference strains for physiology, biochemical, quinone, and fatty acid analyses.

3.3. Genome Analysis

The Whole-Genome Shotgun project of strain S-51^T was deposited at DDBJ/ENA/GenBank under the accession JABBGK000000000. The version described in this manuscript is version JABBGK010000000. The genome of strain S-51^T was 4,930,044 bp long. DNA G+C content calculated from whole genome sequence was 63.1 mol%. The genome contained 19 scaffolds, 327 subsystems, 254.0× genome coverage, and 1,092,255 N50 value (Table 1). The ANI, AAI, and dDDH values of strain S-51^T with the closely related type species of the genus *Rhizobium* were ≤82.6%, ≤83.6, and ≤25.3%, respectively (Table 2). These values were below the threshold values for ANI and AAI (95–96%) [47,48] and dDDH (70%) [34], suggesting that strain S-51^T is a species of a different type than those that exist within the genus *Rhizobium.*

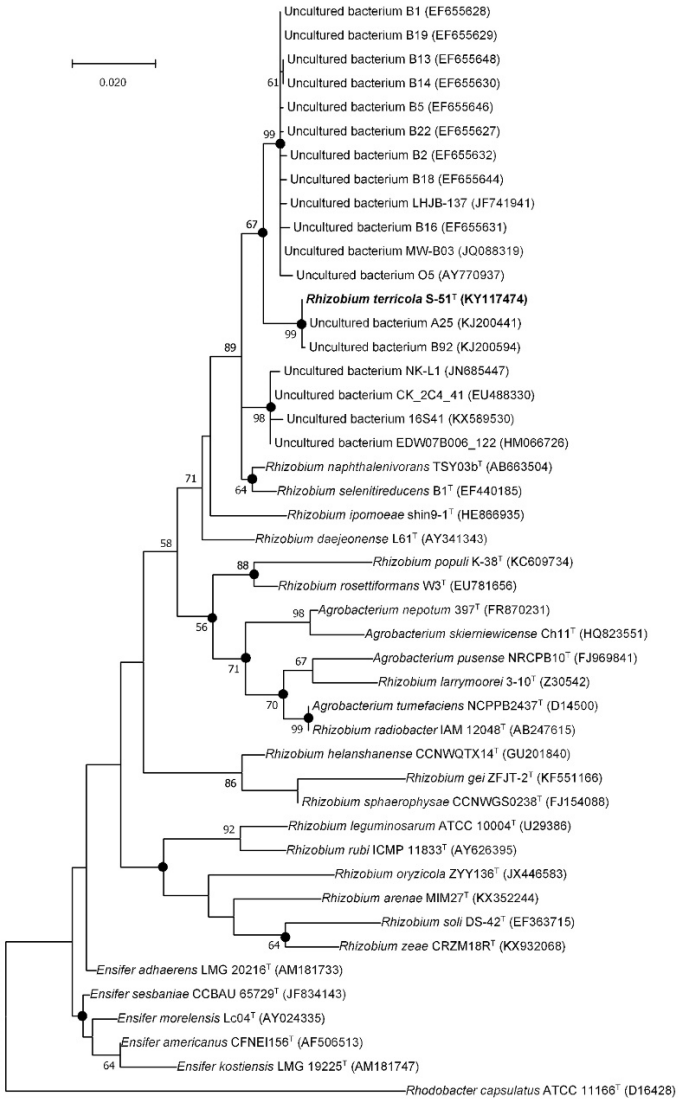

Figure 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strain S-51^T among closely related representatives of the family *Rhizobiaceae* with previously uncultured bacterium clones. Filled circles indicate nodes recovered by different tree-making algorithms (neighbor–joining and maximum–likelihood). The numbers at the nodes indicate the percentage of 1000 bootstrap replicates yielding this topology; only values > 50% are shown. *Rhodobacter capsulatus* ATCC 11166^T was used as an out-group. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions/nucleotide position.

Table 1. Genome features of strain S-51^T.

Genome Features	Value
Genome size (bp)	4,930,044
G+C content (mol%)	63.1
No. of contigs	19
N50	1,092,225
No. of subsystem	327
No. of proteins	4589
Total genes	4682
CDSs (total)	4629
Protein-coding genes	4589
Genes (RNA)	53
Complete rRNAs (5S, 16S, 23S)	3 (1, 1, 1)

Table 1. *Cont.*

Genome Features	Value
tRNAs	46
ncRNAs	4
Pseudo genes (total)	40
CDSs (without protein)	4589
Genome coverage	254.0×
CRISPR repeats	1
Cas cluster	0
Complete and single-copy BUSCOs (S)	143 (96.62%)
Complete and duplicated BUSCOs (D)	0 (0%)
Fragmented BUSCOs (F)	0 (0%)
Missing BUSCOs (M)	5 (3.38%)
Total BUSCO groups searched)	148 (100%)

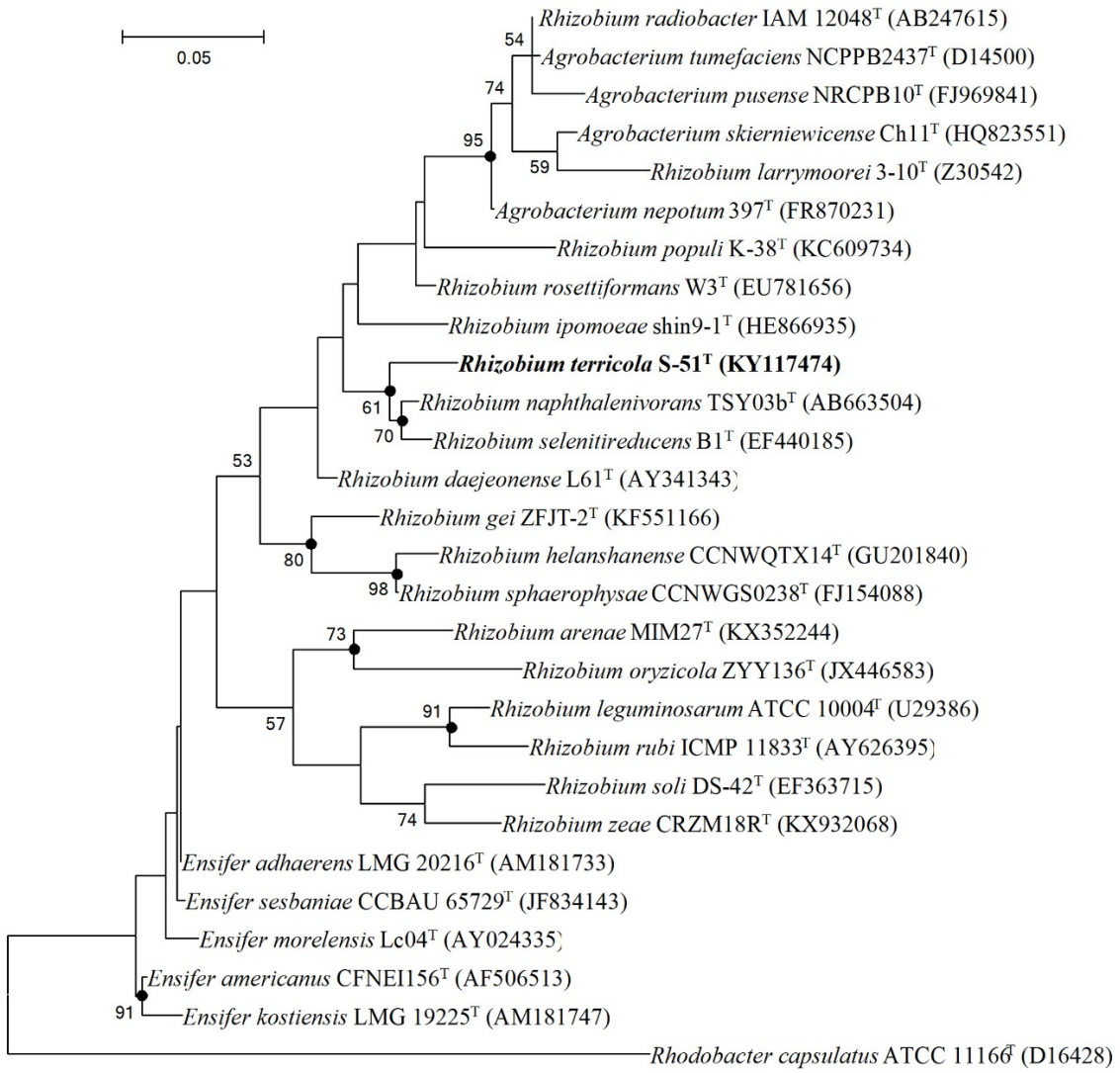

Figure 2. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strain S-51^T among closely related members of the family *Rhizobiaceae*. Filled circles indicate nodes recovered by all three treeing methods (neighbor-joining, maximum-likelihood, and maximum-parsimony). The numbers at the nodes indicate the percentage of 1000 bootstrap replicates yielding this topology; only values > 50% are shown. *Rhodobacter capsulatus* ATCC 11166^T was used as an out-group. GenBank accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide position.

Table 2. ANI, AAI, and dDDH values (%) of strain S-51^T and other members of the genus *Rhizobium*.

Strains	S-51^T		
	ANI	**AAI**	**dDDH**
Rhizobium naphthalenivorans NBRC 107585^T	80.7	81.5	23.0
Rhizobium selenitireducens ATCC BAA-1503^T	82.6	83.6	25.3
Rhizobium daejeonense CCBAU 10050^T	78.8	78.8	21.9
Rhizobium rubi NBRC 13261^T	74.3	53.3	19.7
Rhizobium pusense NRCPB10^T	75.2	68.3	20.9

Anti-SMASH analysis revealed that the genome of strain S-51^T consists of six biosynthetic gene clusters (BGCs), which are supposedly responsible for the biosynthesis of secondary metabolites such as hserlactones, terpene, bactetiocin, *N*-acyl amino acid, NRPS, and T1PKS (Table S1). Moreover, NRPS (non-ribosomal synthesized peptide) BGC showed a 17% similarity to entolysin (Table S1). The genomic map of strain S-51^T represents 3rRNAs, 50 tRNAs, and a tmRNA (Figure 3). In addition, 88 genes for the biosynthesis, transport, and catabolism of secondary metabolites and 1347 genes used for unknown functions were detected in COG functional categories (Figure 4). The Venn diagram was constructed based on whole-genome orthologous genes of strain S-51^T and the reference genome showed that the genome of S-51^T consisted of 75 unique genes and 2847 common genes for all four strains (Figure 5). The functions of these common genes may include carbohydrate metabolism for host plant recognition, lectin binding, and root attachment, membrane transport, secretion machinery for metabolites and signals important in plant-microbe interactions, nodulation (*nod*), and symbiotic N_2 fixation (*nif* and *fix*). Interestingly, the number of unique genes in the reference strains is reversely proportional to the values of ANI, AAI, and dDDH in order. Furthermore, various N_2-fixation regulatory proteins have been detected in the genome of strain S-51^T (Table S2).

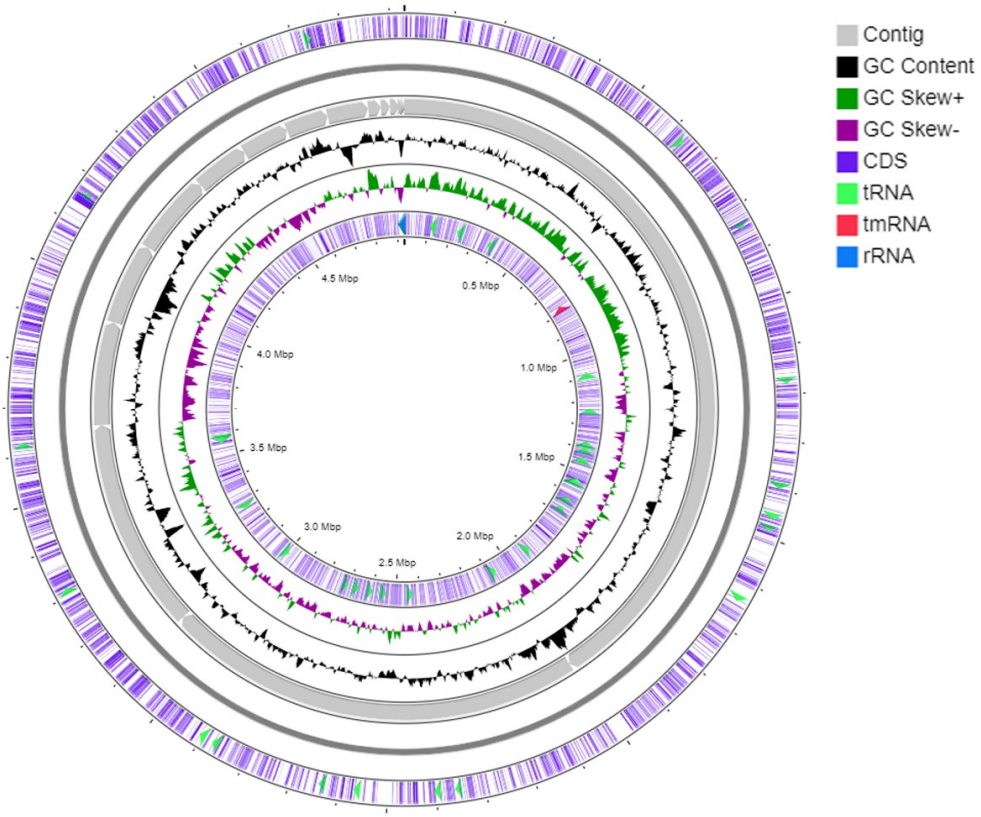

Figure 3. Graphical genome map of strain S-51^T. RNA genes (tRNAs light green, rRNAs blue, tmRNA red), GC content, and GC skew.

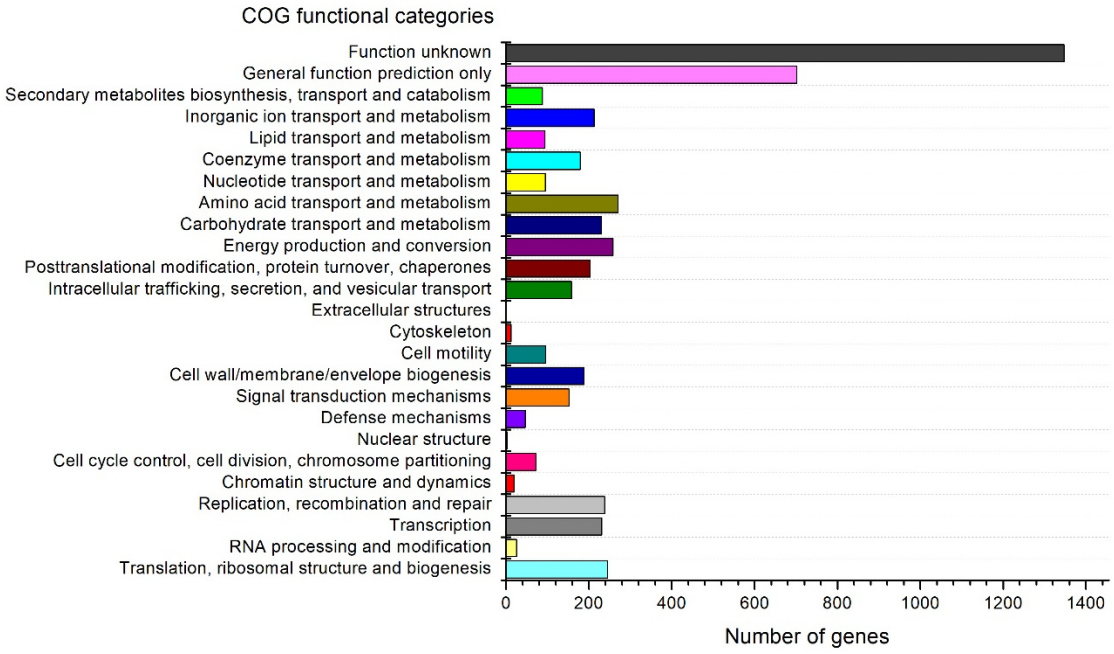

Figure 4. COG functional classification of proteins in strain S-51^T genome.

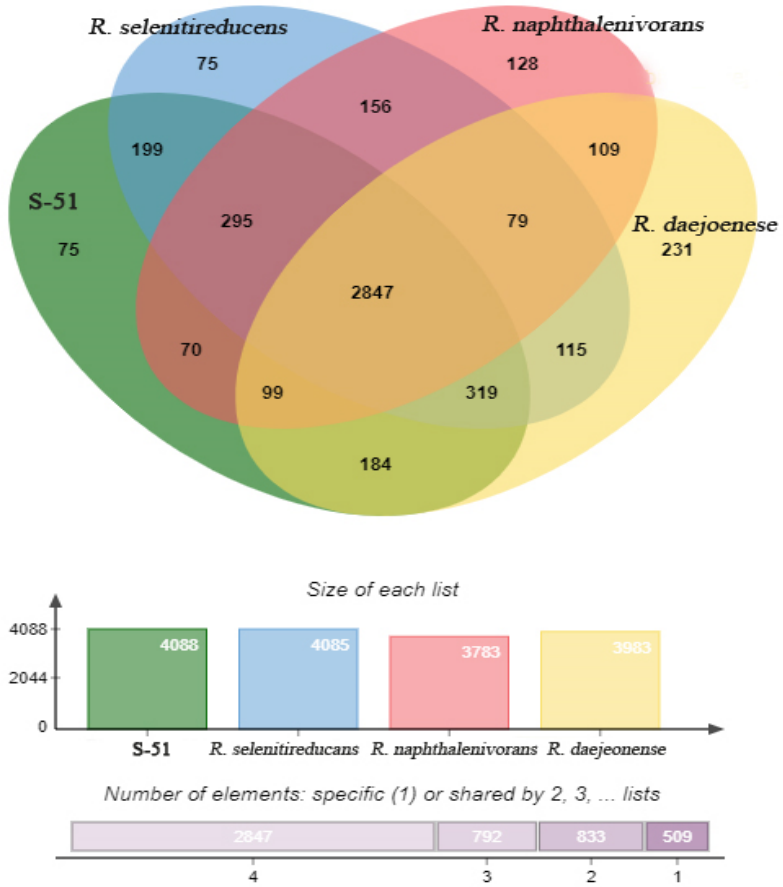

Figure 5. Venn diagram of whole-genome orthologous genes in S-51^T and three type species of reference strains (*Rhizobium selenitireducens*, *Rhizobium naphthalenivorans*, and *Rhizobium daejeonense*). The numbers in the diagram indicate overlapped conserved genes or non-overlapped unique genes in each species.

3.4. Physiology and Chemotaxonomic Characteristics

Cells of strain S-51^T (Figure S3) were rod-shaped, Gram-stain-negative, aerobic, and motile. Cells were 1.2–1.9 μm long and 0.6–0.8 μm wide. Colonies on R2A agar were straw-color, circular, entire, and convex. It could grow well on R2A agar, but would be difficult to grow on other media. The differential phenotypic characteristic features of strain S-51^T are presented in Table 3 with the closest reference of the genus *Rhizobium*; highest salt tolerance (5.0%), weakly assimilation of l-alanine, adipic acid and propionic acid, positive activity of *β*-glucosidase, and negative activity of *β*-galactosidase clearly distinguished strain S-51^T from closely related type strains. The free-living strain S-51^T was able to grow in the nitrogen-free media, revealing the ability to fix atmospheric nitrogen by discoloration of the NfB indicator [43].

Table 3. Differential phenotypic characteristics of strain S-51^T with closely related species of the genus *Rhizobium*. Strains: 1, S-51^T; 2, *Rhizobium naphthalenivorans* KCTC 23252^T [8]; 3, *Rhizobium selenitireducens* KCTC 23231^T [14]. DNA G+C content was calculated using whole genome sequences. All the data were from this study, except the data in parentheses. +, positive; w, weakly positive; −, negative.

Characteristic	1	2	3
Isolation source	Forest soil	(Sediment)	(Bioreactor)
Maximum growth temperature (°C)	37	37	40
pH range	5.5–11.0	6.0–9.0	6.0–9.5
Highest salt tolerance (%, *w*/*v*)	5.0	3.0	1.5
Nitrate reduction	+	−	+
Hydrolysis of			
Urea	+	+	−
CM-cellulose	−	−	+
Enzyme activities			
Acid phosphatase	w	+	+
Alkaline phosphatase	w	+	+
Esterase (C4)	w	+	+
Naphthol-AS-BI-phosphohydrolase	−	+	−
α-glucosidase	+	+	−
β-galactosidase	−	+	+
β-glucosidase	+	−	−
Assimilation from:			
Adipic acid	w	−	−
Capric acid	−	+	+
D-maltose	+	+	−
D-mannitol	+	−	+
D-mannose	+	+	−
D-sorbitol	+	−	+
L-alanine	w	+	+
L-fucose	+	−	+
L-histidine	−	−	+
L-rhamnose	+	−	+
Malic acid	+	+	−
Potassium gluconate	−	+	−
Propionic acid	w	−	−
DNA G + C content (mol%)	63.1	61.2	63.5

The major cellular fatty acids of strain S-51^T were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), cyclo-$C_{19:0}$ $\omega8c$ and $C_{18:0}$ (Table 4). The major fatty acids of closely related type strains were similar. However, the compositions of primary and secondary fatty acids showed characteristic differences for strain S-51^T compared to other references. The only respiratory quinone was ubiquinone-10 (Q-10), the same as the reported major respiratory quinone of the genus *Rhizobium*. The principal polar lipids for strain S-51^T were phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylcholine (PC),

and unidentified glycolipid (GL). The presence of unidentified glycolipid (GL) as a major polar lipid, along with the presence of unidentified aminophospholipid (APL) and other minor polar lipids (L1-L3), distinguished strain S-51^T from other closely related type strains (Figure 6) [6,14].

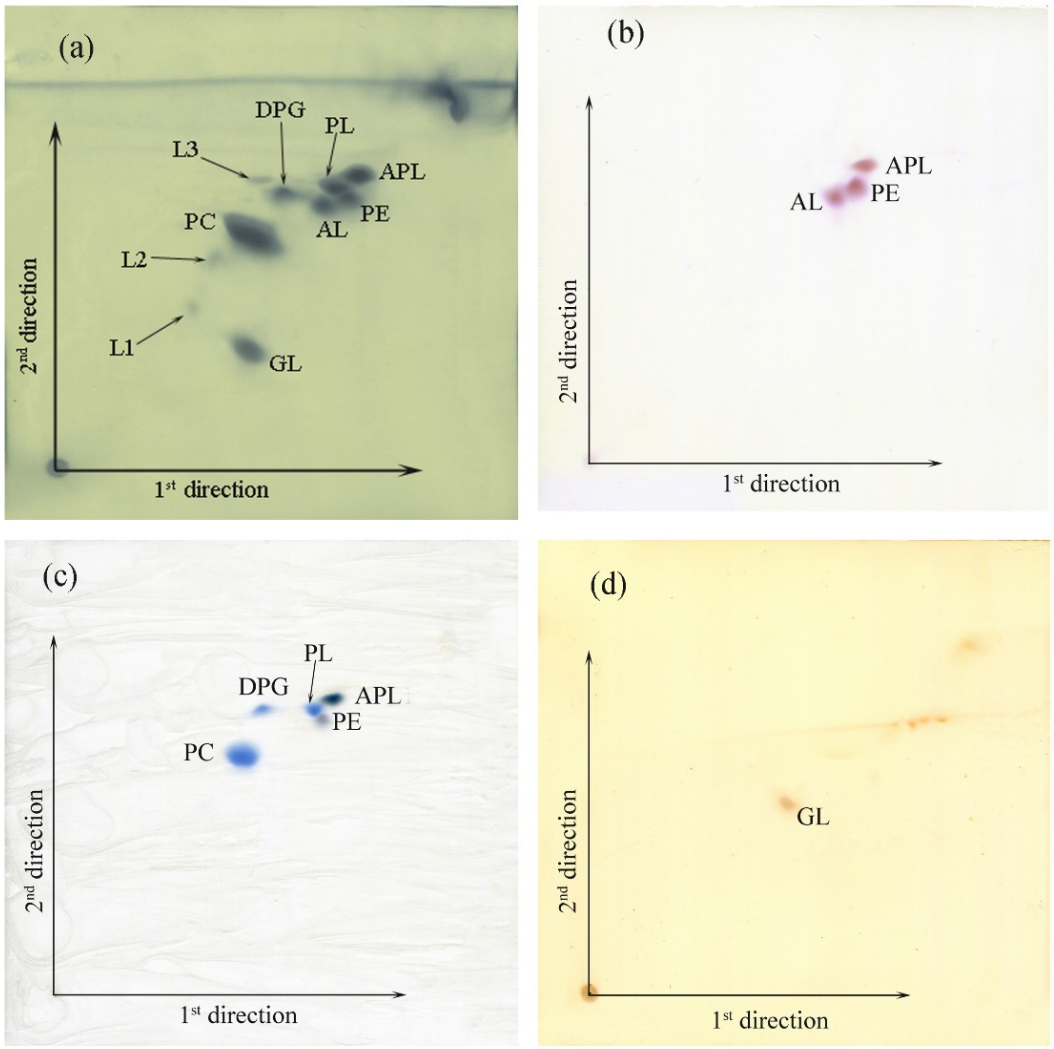

Figure 6. Thin-layer chromatograms of the polar lipids from strain S-51^T. The components were seen by staining with 5% molybdophosphoric acid in ethanol and heating them at 180 °C for 15 min (**a**); ninhydrin at 110 °C for 15 min (**b**); molybdenum at room temperature (**c**); and α-naphthol-sulphuric acid at 110 °C for 15 min (**d**). Abbreviations: PE, phosphatidylethanolamine; PL, unidentified phospholipid; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; GL, unidentified glycolipid; AL, unidentified aminolipid; APL, aminophospholipid; L1–L3, unidentified polar lipids.

Table 4. Cellular fatty acid profiles (% of total) of strain S-51^T and closely related reference strains. Strains: 1, S-51^T; 2, *Rhizobium naphthalenivorans* KCTC 23252^T; 3, *Rhizobium selenitireducens* KCTC 23231^T. All data were obtained from this study. TR, trace amount (<0.5%); –, not detected.

Fatty Acid	1	2	3
Saturated			
$C_{16:0}$	1.2	1.7	1.9
$C_{18:0}$	6.5	10.2	7.9
$C_{19:0}$ cycloω8*c*	7.9	6.9	4.6
$C_{19:0}$ 10-methyl	TR	0.9	TR

Table 4. *Cont.*

Fatty Acid	1	2	3
Unsaturated			
$C_{18:1}\omega 9c$	2.3	–	1.1
$C_{20:1}\omega 7c$	2.0	0.6	1.3
Hydroxy			
$C_{18:0}$ 3OH	2.0	3.3	2.9
Summed features *			
2†	4.4	–	3.9
3	0.6	3.1	1.3
8	71.9	72.3	73.3

* Summed features represent groups of two or three fatty acids that could not be separated using the MIDI system and include both peaks with discrete ECLs as well as those where ECLs are not reported separately. Summed feature 2† is listed as ECL 10.928 and/or $C_{12:0}$ aldehyde, summed feature 3 is listed as $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$, and summed feature 8 is listed as $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$. † One or more of an unknown fatty acid of ECL (equivalent chain length) 10.928 or $C_{12:0}$ aldehyde.

4. Conclusions

Based on the above discussed genomic, phylogenetic, phenotypic, and chemotaxonomic characteristic differences, strain S-51^T represents a novel member in the genus *Rhizobium*, for which the name *Rhizobium terricola* sp. nov. is proposed. In particular, it has 75 unique genes different from the closest reference strains, although two strains share many more genes. The presence of unidentified glycolipid (GL) and unidentified aminophospholipid (APL) as major polar lipids is also distinguished from other known closely related type strains. In addition, strain S-51^T is differentiated by isolation source, wider pH range, and higher salt tolerance than the closest two type strains, which may derivate uncultivable property.

So far, many microbiologists have isolated a great number of cultivable bacteria and characterized them for identification, mechanism, interaction, and function, but these bacteria are only tiny portion of all bacteria known through metagenomic data. Therefore, the isolation of uncultured strains including strain S-51^T may increase the possibility of new functions or improve functions in various areas.

Description of Rhizobium terricola sp. nov.

Rhizobium terricola [ter.ri'co.la. L. fem. n. *terra* soil; L- suff. *-cola* (from L. masc. or fem. n. *incola*) inhabitant, dweller; N.L. n. *terricola* a dweller of soil, referring to the isolation of the type strain from soil].

Cells (0.6–0.8 μm wide and 1.2–1.9 μm long) are non-spore-forming, aerobic, rod-shaped, motile, and Gram-stain-negative. No growth occurs on NA, LBA, BHI, PDA, LBA, MA, and veal infusion agar, but poorly grows on TSA and marine 2216 agar. Cells are able to fix nitrogen. Colonies on R2A agar are straw colored, entire, convex, and circular. Colony size of the cells are 0.5–1 mm on R2A agar for seven days at 28 °C. Cells grow optimally in the absence of NaCl, but tolerate up to 5.0% (*w*/*v*). Cells grow at 15–37 °C (optimum, 25–32 °C) and pH 5.5–11.0 (optimum, 7.0–9.5). Catalase and oxidase tests are positive. Urea and aesculin are hydrolyzed, but casein, tyrosine, xanthine, hypoxanthine, CM-cellulose, starch, DNA, chitin, Tween 80, Tween 60, and Tween 40 are not. The flexirubin test is negative. Nitrate is reduced to nitrite, but nitrite is not reduced to nitrogen. The glucose is not fermented. The PNPG (4-nitrophenyl-*β*-D-galactopyranoside) is positive. The type strain shows the following enzyme activities: positive for esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, *α*-glucosidase, and *β*-glucosidase; weakly positive for alkaline phosphatase, esterase (C4), and acid phosphatase; negative for lipase (C14), *α*-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase, and *α*-fucosidase. In API 20NE and ID 32GN tests, 3-hydroxybutyric acid, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, D-saccharose, D-sorbitol, inositol, lactic acid, L-arabinose, L-fucose, L-proline, L-rhamnose, malic acid, sodium acetate, and valeric acid are assimilated;

adipic acid, L-alanine, and propionic acid are weakly assimilated. The sole respiratory quinone is Q-10. The principal cellular fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), cyclo-$C_{19:0}$ $\omega8c$ and $C_{18:0}$. The major polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylcholine, and unidentified glycolipid. The DNA G+C content of the type strain is 63.1 mol%.

The type strain, S-51^T (=KACC 19117^T = KEMB 9005-539^T = NBRC 112711^T), was isolated from forest soil, geographically located at Suwon, Gyeonggi-do, Republic of Korea (37°18′5″ N and 127°1′56″ E). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome sequence of strain S-51^T are KY117474 and JABBGK000000000, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14090733/s1: Figure S1: Neighbor–joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain S-51^T among closely related members of the family *Rhizobiaceae*; Figure S2: Maximum–parsimony tree based on 16S rRNA gene sequences showing the phylogenetic position of strain S-51^T among closely related members of the family *Rhizobiaceae*; Figure S3: Transmission electron microscopic image of strain S-51^T grown on R2A agar. Table S1: The distribution of biosynthetic gene clusters in strain S-51^T; Table S2. N_2-fixation regulatory proteins in S-51^T genome.

Author Contributions: R.H.D., D.K.C. conceived, designed and conducted all the experiments. D.-U.K. interpreted the data. J.K. (Jungmin Kim) and J.K. (Jaisoo Kim) coordinated and supervised the study. R.H.D., D.K.C. and D.-U.K. analyzed all the data and prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

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