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Bradyrhizobium iriomotense sp. nov., Isolated from a Tumor-Like Root of the Legume *Entada koshunensis* from Iriomote Island in Japan

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A polyphasic study was performed to determine the taxonomic position of strain EK05^T isolated from a root-outgrowth of *Entada koshunensis*, a legume available in Okinawa, Japan. Phylogenetic analysis of the 16S rRNA gene showed that the strain belongs to the genus *Bradyrhizobium*. Subsequent multilocus sequence analysis with ITS, *glnII*, *recA*, *gyrB*, and *atpD* sequences revealed that the isolate represents a distinct evolutionary lineage within the genus *Bradyrhizobium*. DNA-DNA hybridization indicated that strain EK05^T shares < 61% DNA relatedness with the type strains of all six recognized species of *Bradyrhizobium*, confirming that this strain is a novel species within the genus. Phylogenetic trees based on symbiotic loci, *nifH* and *nodC*, also placed strain EK05^T clearly in a novel branch. On the basis of its phylogenetic distinctiveness, we propose *Bradyrhizobium iriomotense* sp. nov. for strain EK05^T. The type strain is EK05^T (= NBRC 102520^T = LMG 24129^T).

Key words: *Bradyrhizobium*; *Bradyrhizobium iriomotense*; *Entada koshunensis*

Members of the genus *Bradyrhizobium* constitute an important group of nitrogen-fixing microbes that possess the remarkable ecological feature of being able to thrive in vastly contrasting habitats, *e.g.*, as free-living soil diazotrophs or aquatic oligotrophs, as rhizospheric bacteria or as endosymbionts of diverse legumes and non-legumes.[1,2] Throughout the years, scientists around the world have isolated a great number of bradyrhizobial strains from different climatic and geographical regions from a broad spectrum of host plants, such as *Glycine max*,[3] *Parasponia*,[4] *Arachis hypogaea*,[5] *Lupinus*,[6] *Aeschynomene*,[7] *Beta vulgaris*,[8] *Adenocarpus*, *Chamaecytisus*, *Spartocytisus*,[9] and *Desmodium*.[10] Diversity studies have been conducted to gain a better understanding of the taxonomy and distribution of bradyrhizobia, and to screen for strains that might be of use in agriculture and environmental management. An abundance of attention was directed towards the selection, identification, and development of new strains of bradyrhizobia with novel and useful characteristics.

Despite the large number of strains isolated through much scientific exploration, the number of named species under the genus *Bradyrhizobium* is still low. The genus currently includes six validly published species: *Bradyrhizobium japonicum*,[11] *Bradyrhizobium elkanii*,[12] *Bradyrhizobium liaoningense*,[13] *Bradyrhizobium yuanmingense*,[14] *Bradyrhizobium betae*,[8] and *Bradyrhizobium canariense*.[2] In the course of our investigation of bradyrhizobia associated with the legumes of Iriomote Island, Okinawa, Japan, we isolated a novel strain from the root-outgrowth of *Entada koshunensis*, a tropical woody climber belonging to mimoseae of the family Leguminosae. Although many members of mimoseae have been studied for rhizobial symbionts,[15–17] this legume, in spite of its availability in the tropics, is unreported in this regard. In the present study, polyphasic analysis, including phenotypic and molecular taxonomic approaches, revealed that the isolated strain represents a novel species of the genus *Bradyrhizobium*, for which we propose the name *Bradyrhizobium iriomotense* sp. nov.

Materials and Methods

Isolation of the strain, culture conditions, and

The DDBJ accession numbers for the *rrs*, ITS, *atpD*, *glnII*, *recA*, *gyrB*, *nifH*, *nodA*, and *nodC* sequences of strain EK05^T are AB300992-AB301000.

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Abbreviations: YMA, yeast mannitol agar; ITS, internal transcribed spacer; MLSA, multilocus sequence analysis; PGPR, plant growth promoting rhizobacteria

morphology study. Strain EK05^T was isolated from a tumor-like outgrowth of a root of the legume *Entada koshunensis*. The outgrowth was surface-sterilized with 70% ethanol for 2 min, followed by treatment with 3% aqueous sodium hypochlorite for 30 min. The tumor was washed 10 times in sterile water, cut with a sterile blade, and placed on modified YMA medium containing 0.5 g of yeast extract, 10 g of mannitol, 0.5 g of K_2HPO_4, 0.2 g of NaCl, 0.2 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $MgSO_4 \cdot 7H_2O$ per liter (pH 7.0). Cells were obtained after 7 d of incubation at 28 °C. The strain was purified by several subcultures. For long-term maintenance, a pure culture was suspended in 15% glycerol, transferred to sterilized serum tubes, and stored at −85 °C. Cell morphology was observed by light (Zeiss Axioskop2) and transmission electron microscopy (TEM). For the latter, cells from a fresh culture were stained with 1% phosphotungstic acid (negative staining, pH 7.0) and observed under a Hitachi H-7600 transmission electron microscope at an accelerating voltage of 100 kV.

DNA extraction. DNA extraction was carried out as described by Ausubel *et al.*[18] The genomic DNA was further purified by equilibrium ultracentrifugation in CsCl/ethidium bromide gradients by the method of Hamamoto and Nakase,[19] using an ultracentrifuge (Hitachi CS-210) at 400,000 × *g* for 16 h. The quality of DNA was verified by spectrophotometric determination of A_{260}/A_{280}, which was at least 1.8.

PCR and sequencing of the 16S rRNA gene. All PCR amplifications conducted in this study were performed using Ex *Taq* polymerase (Takara Shuzo, Kyoto, Japan) in a Gene Amp® PCR system 9700 (Applied Biosystems Japan, Tokyo, Japan). Sequencing was carried out with the BigDye ver. 3.1 Terminator Cycle Sequencing kit (Applied Biosystems Japan, Tokyo, Japan) using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The almost complete 16S rRNA gene was amplified and sequenced as described by Normand *et al.*[20]

Amplification of 16S-23S internal transcribed spacer and sequencing. The 16S-23S internal transcribed spacer (ITS) was amplified with the primers listed in Table 1. The following cycling conditions were used: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 2 min, 59 °C for 1 min and 72 °C for 1.5 min; and final extension at 72 °C for 7 min. The PCR product was sequenced according to the method of Willems *et al.*[21]

Amplification of atpD *gene and sequencing.* A 507-bp *atpD* fragment was amplified using the primer pair atpD-255F/atpD-782R (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). The following temperature profile was used: initial denaturation at 95 °C for 3.5 min; 30 cycles of 93.5 °C for 1 min, 55 °C for 40 s and 72 °C for 1 min; and final extension at 72 °C for 10 min. The product was purified and sequenced as described by Vinuesa *et al.*[22]

Table 1. List of Primers, PCR Conditions, and Sizes of PCR Products

Primer(s)	Sequence 5′-3′
ITS	
*SM	AAGTCGTAACAAGGTAGCC
BR3	GCTTTTCACCTTTCCCTCAC
*pH	CCTGCGGCTGGATCACCTCC
BR1	GTGGGTTTCCCCATTCGG
BR4	CGAACCGACGACCTCATGC
*BR4	GCATGAGGTCGTCGGTTCG
*BR5	CTTGTAGCTCAGTTGGTTAG
atpD	
atpD-255F	GCTSGGCCGCATCMTSAACGTC
atpD-782R	GCCGACACTTCMGAACCNGCCG
glnII	
glnII-12F	YAAGCTCGAGTACATYTGGCT
glnII-689R	TGCATGCCSGAGCCGTTCCA
recA	
recA-41F	TTCGGCAAGGGMTCGRTSATG
recA-640R	ACATSACRCCGATCTTCATGC
gyrB	
gyr21A	CAGGAAACAGGCTATGACCAARMG ICCNGSIATGTAYATHGG
gyQTKr	ACSAGCTTGTCCTTGGTYTG
nifH	
nifH-univ(F)	GCIWTITAYGGNAARGGNGG
nifH-univ(R)	GCRTAIABNGCCATCATYTC
nodC	
nodCF4	AYGTHGTYGAYGACGGATC
nodC1	CGYGACAGCCANTCKCTATTG

Degeneracy is indicated by standard conventions: K, G/T; M, A/C; R, A/G; S, C/G; W, A/T; Y, C/T; H, A/T/C; N, A/C/G/T.

Amplification of the glnII *gene and sequencing.* A partial sequence of *glnII* (637 bp) was amplified using the primer pair glnII-12F/glnII-689R (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). The following temperature profile was used: initial denaturation at 95 °C for 3.5 min; 30 cycles of 93.5 °C for 1 min, 58 °C for 40 s and 72 °C for 1 min; and final extension at 72 °C for 10 min. The product was purified and sequenced as described by Vinuesa *et al.*[22]

Amplification of the recA *gene and sequencing.* A partial *recA* gene was PCR amplified using the primer pair recA-41F/recA-640R (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site), yielding a 508-bp product, excluding the primers. The temperature profile was the same as that of *glnII*-PCR. The product was purified and sequenced as described by Vinuesa *et al.*[22]

Amplification of the gyrB *gene and sequencing.* A partial *gyrB* gene was amplified using the primer pair gyr21A/gyQTKr (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). The product was a 809-bp fragment excluding the primers. The following temperature profile was used: initial denatu-

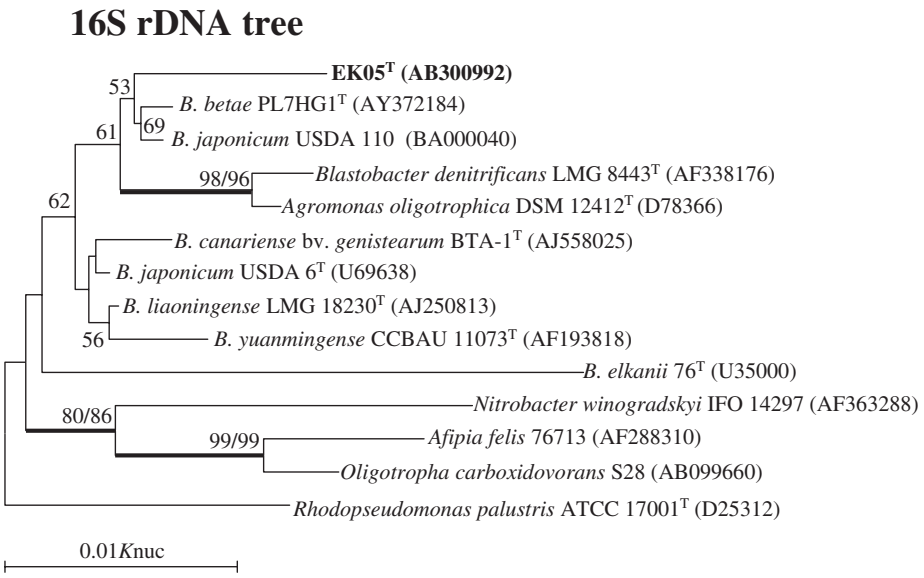

Fig. 1. Position of Strain EK05^T in the Phylogenetic Tree Based on the 16S rDNA (1289 bp) Sequences of Related Srains.
The tree was constructed by the neighbor joining method with the K2P distance correction model and 1,000 bootstrap replications. Bootstrap values (NJ/MP) above 50% are indicated at the nodes, and values greater than 70% are shown in bold lines. Bar, 0.01 K_{nuc} in nucleotide sequences.

ration at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and final extension at 72 °C for 10 min. The product was purified and sequenced as described by Sameshima *et al.*[23]

Amplification of the nifH *gene and sequencing.* A partial *nifH* fragment was amplified using primers nifH-univ(F)/nifH-univ(R) (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). A nested PCR protocol was designed by the method of Bürgmann *et al.*[24] The cycling conditions used for the reactions were as described by Widmer *et al.*,[25] except that annealing was performed for 8 s at 54 °C and for 30 s at 56 °C for the first reaction, and for 8 s at 51 °C and 30 s at 53 °C for the nested reaction to reduce amplification of nonspecific by-products.

Amplification of the nodC *gene and sequencing.* A partial *nodC* gene fragment of 934 bp was amplified using the primer pair nodCF4/nodC1 (Table 1 and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site). The following temperature profile was used: initial denaturation at 95 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and final extension at 72 °C for 10 min. The PCR product was sequenced according to the method of Laguerre *et al.*[26]

Sequence analysis and construction of phylogenetic trees. Sequence data were analyzed by ABI PRISM (Perkin-Elmer, Waltham, MA) sequence analysis program, and assembled using the ABI auto assembler (Perkin-Elmer). Homology searches were performed *via* BLAST (Basic Local Alignment Search Tool), either at NCBI (the National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST/) or at DDBJ (the DNA Data Bank of Japan) (http://www.ddbj.nig.ac.jp/). For the construction of trees, sequences of interest were extracted from the BLAST database and aligned with the CLUSTAL W function of the MEGA 3.1 computer program.[27] After alignment, the MEGA 3.1 computer program (default parameters) was used to construct phylogenetic trees by two different methods, neighbor-joining[28] and maximum parsimony.[29] The trees were bootstrapped with 1,000 replications of each sequence to evaluate the reliability of the tree topologies. For nucleic acid sequences (16S rDNA and ITS), the genetic distances used in NJ trees were Kimura two-parameter-model (K2P) distances.[30] For deduced amino acid sequences, the pairwise evolutionary distances used in the NJ trees were Poisson correction model distances[31] in MEGA 3.1. In MP analyses, the option "close-neighbor intercharge (CNI; search level 1) with initial tree by random addition (10 replications)" was in effect (MEGA version 3.1).

DNA relatedness determination. DNA-DNA hybridization was carried out according to the photobiotin microplate method, as described by Ezaki *et al.*[32] Hybridizations were performed on immunoplates (Nalge Nunc International, Rochester, NY) at 49.4 °C in a 2XSSC (1x SSC: 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) buffer containing 50% (v/v) formamide. DNA relatedness was expressed as a mean percentage of the homologous DNA binding value.

Physiological and biochemical tests. The phenotypic characterization (*i.e.*, the utilization of sole carbon and energy sources, sole nitrogen sources, resistance to

antibiotics, tolerance to NaCl, temperature and pH ranges for growth, and some physiological and biochemical reactions) was done as described by Gao *et al.*[33] The type strains of *B. japonicum*, *B. liaoningense*, *B. yuanmingense*, *B. betae*, *B. canariense*, and *B. elkanii* were used as references. The following antibiotics were used to test for resistance: erythromycin (100 µg/ml), ampicillin Na (100 µg/ml), polymyxin B (300 µg/ml), kanamycin sulphate (50 µg/ml), tetracyline (10 µg/ml), and chloramphenicol (50 µg/ml). A catalase test, an oxidase test, nitrate reduction, and a test for 3-ketolactose production from lactose oxidation were performed according to Smibert and Krieg.[34] Nitrogen fixation was tested in jellified Winogradsky's N_2-free mineral medium, as described by Islam *et al.*[35]

Chemotaxonomic characterization. The G+C content was determined by HPLC (Hitachi LaChrom L-7100) separation as described by Tamaoka and Komagata.[36] For cellular fatty acid analysis, strain EK05^T and the reference bradyrhizobial strains were grown on modified yeast extract-mannitol broth (YMB, composition described above) for 7 d at 28 °C. The fatty acids were analyzed with the Sherlock MIDI Microbial Identification System (MIDI, Newark, DE).[37,38] The analyses were based on the conversion of fatty acids to fatty acid methyl esters by mild acidic methanolysis. The methyl ester derivatives thus produced were detected by gas-liquid chromatography, followed by data analysis with Sherlock MIS software.

Legume nodulation test. A plant-inoculation study was conducted to evaluate the nodule-eliciting capability of isolate EK05^T in the model plant *Macroptilium atropurpureum*. The seeds were scarified and surface-sterilized with concentrated sulfuric acid for 15 min, rinsed with sterile water, vortexed with 3% sodium hypochlorite for 10 min, and then washed with copious amounts of sterilized distilled water. They were laid on 1% agar plates and incubated in the dark at 25 °C overnight. Thereafter, they were transferred to an incubator, and were kept at 25 °C for 3 d on a light/dark (16 h:8 h) cycle. The germinated seedlings were planted in small pots with B&D medium,[39] and were inoculated with a 100-µl suspension ($OD_{600} = 0.2$) of strain EK05^T and *B. japonicum* USDA 110 (control). The plants were incubated in the above environment and observed for 8 weeks to confirm nodulation.

Results and Discussion

Analysis of the 16S rRNA gene

Phylogenetic trees based on 16S rDNA (Fig. 1) were constructed including the type strains of the genus *Bradyrhizobium*. When compared with the BLASTN tool, the 16S rDNA sequence of strain EK05^T showed 99.0% similarity to that of *B. japonicum* USDA110, 98.4% similarity to that of *B. betae* PL7HG1^T, 98.5% similarity to that of *B. canariense* BTA-1^T, 98.5% similarity to that of *B. japonicum* USDA6^T, 98.6% similarity to that of *B. liaoningense* LMG 18230^T, 98.1% similarity to that of *B. yuanmingense* CCBAU 11073^T, and 96.2% similarity to that of *B. elkanii* USDA 76^T. In the 16S rDNA based tree, we also included representative strains of *Afipia*, *Agromonas*, *Blastobacter*, and *Nitrobacter* of the alpha subdivision of the *Proteobacteria*, because it has been reported that these genera intermingled with bradyrhizobia in *rrs* phylogeny.[21,40–44] The taxonomic positions of the genera *Agromonas* and *Blastobacter* are still controversial. The two genera, despite mingling within the genus *Bradyrhizobium* in the 16S rDNA based tree, are phenotypically very different from all the species of *Bradyrhizobium*. The members of *Agromonas* are free-living soil diazotrophs with close relations with *Blastobacter*. On the other hand, the genus *Balstobacter* was established artificially by combining aquatic bud- and cluster-forming bacteria in a single taxon.[43] Our investigation showed that strain EKO5^T distinctly belonged to *Bradyrhizobium*, and hence we did not consider the above non-bradyrhizobial genera in subsequent analyses.

Analysis of ITS sequence

The 16S-23S internal transcribed spacer (ITS) has become popular for determining relatedness among closely related bradyrhizobial strains, which are otherwise difficult to distinguish from one another due to the highly conserved nature of their 16S rRNA gene.[21,42] Strain EK05^T did not group with any validly published species of the genus *Bradyrhizobium* when the NJ and MP phylogenetic trees were inferred based on the ITS sequences (Fig. 2). With BIONUMERICS version 3.0 software, the ITS sequences were aligned with a gap penalty of 800%, and the sequence similarities were calculated without gap penalties. In order to improve the quality of the alignment, photosynthetic bradyrhizobia were not included in the analysis. The ITS sequence of strain EK05^T was 94.5% similar to that of *B. japonicum* USDA110, 92.6% to that of *B. betae* PL7HG1^T, 92.8% to that of *B. canariense* BTA-1^T, 91.8% to that of *B. japonicum* USDA6^T, 92.6% to that of *B. liaoningense* LMG 18230^T, 94.1% to that of *B. yuanmingense* CCBAU 11073^T, and 89.1% to that of *B. elkanii* USDA 76^T. Willems *et al.*[43] have hypothesized that two bradyrhizobial strains with $< 95.5\%$ ITS sequence similarity usually belong to separate genospecies.

Analyses of housekeeping loci glnII, recA, atpD, *and* gyrB

The phylogenies of a gene do not necessarily represent the phylogenies of a species, and therefore the *ad hoc* committee on reevaluation of species definition of bacteria[45] suggested analyzing about five genes of diverse chromosomal loci to supplement DNA-DNA reassociation data. In another meeting of

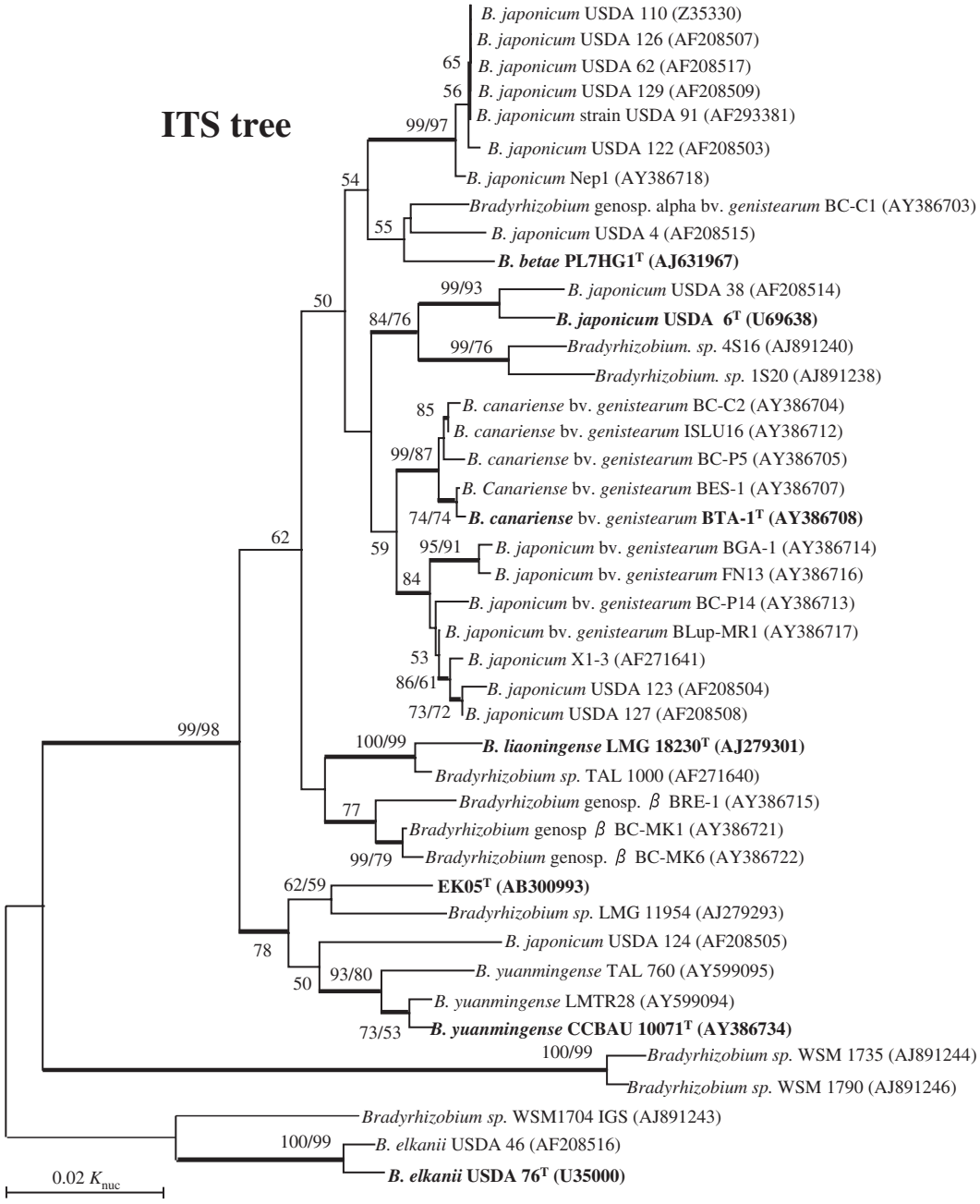

Fig. 2. Position of Strain EK05^T in the Phylogenetic Tree Based on the ITS (16S-23S rDNA) Sequences (672 bp) of Related Strains of *Bradyrhizobium*.

The tree was constructed by the neighbor joining method with the K2P distance correction model and 1,000 bootstrap replications. Bootstrap values (NJ/MP) above 50% are indicated at the nodes, and values greater than 70% are shown in bold lines. Bar, 0.02 K_{nuc} in nucleotide sequences.

the subcommittee on taxonomy of rhizobia and agrobacteria, held at Toulose, France in July 2004, a consensus was formed that multilocus sequencing is more convenient and reliable for rhizobial taxonomy than DNA-DNA hybridization in proposing new species.[46] In accordance with this, we attempted to carry out a multilocus sequence analysis (MLSA) targeting the housekeeping loci *glnII* (encoding a glutamine synthetase), *recA* (encoding the recombinase A protein), *gyrB* (encoding DNA gyrase subunit B), and *atpD* (encoding the β subunit of ATP synthase) due to the availability of the corresponding databases for other bradyrhizobial strains. Previously, these core house-keeping loci were used in comparative analysis in the taxonomic characterization of rhizobia.[23,47–51] It has been reported that these protein coding-loci are unlinked in the genome and that they provide independent genealogies for the inference of a reliable phylogenetic tree.[52,53]

Sequences of our target house-keeping genes for all the strains were not available in the Genbank, which is

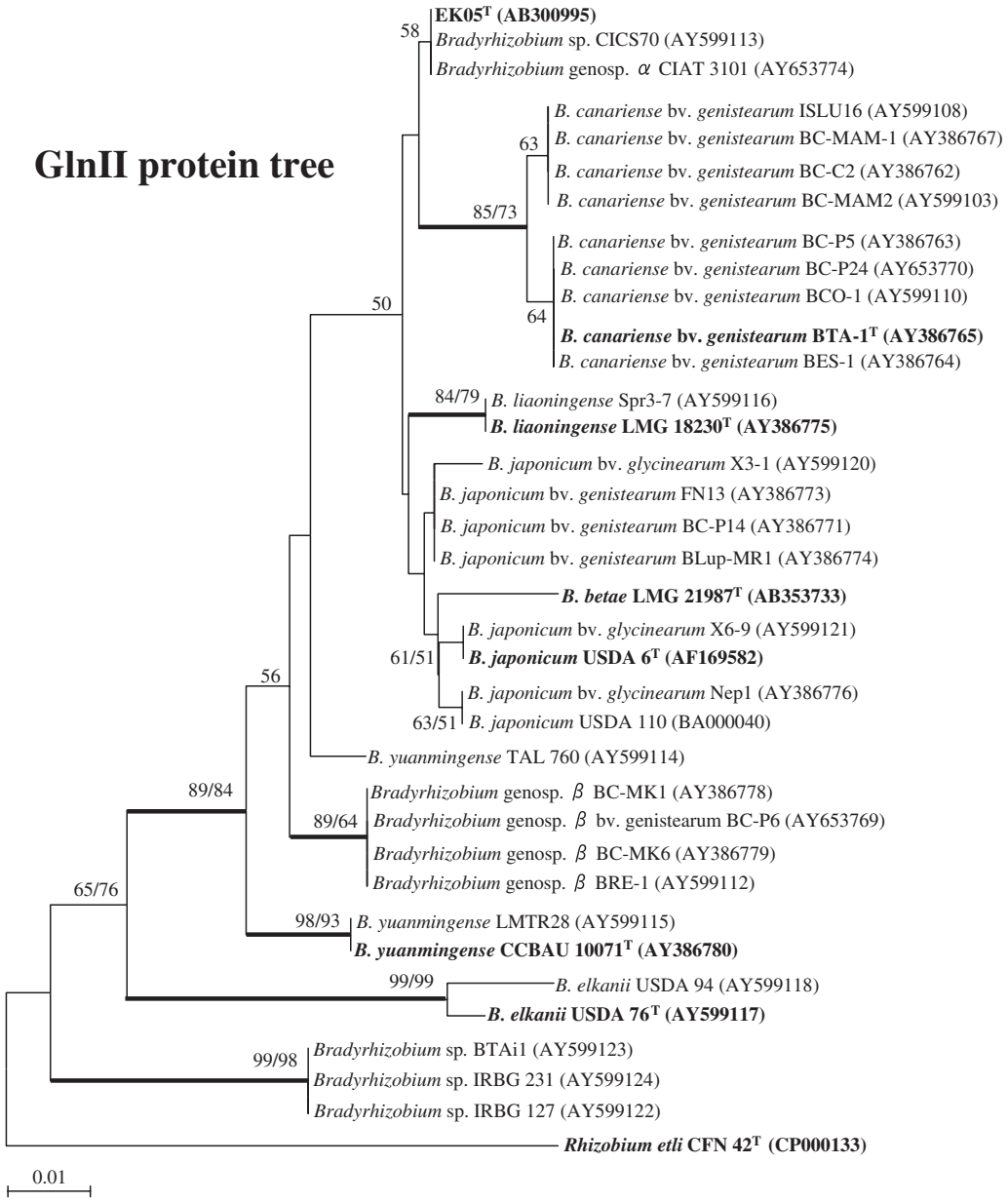

Fig. 3. Comparative NJ Analysis of Amino Acid Sequences of Partial GlnII Protein (173 amino acids) from *Bradyrhizobium iriomotense* sp. nov. EK05^T and Related Strains from GenBank.

The tree was constructed by the Poisson correction distance model in the MEGA v.3.1 software package. Numbers at nodes indicate percentage bootstrap values from 1,000 replications. Only values (NJ/MP) above 50% are indicated, and values greater than 70% are shown in bold lines. Bar, 0.01 substitutions per site.

why we could not use the same set of strains to construct all the phylogenetic trees. However, we could use the same set of strains to construct the phylogenetic trees based on GlnII and RecA. In both trees (Figs. 3, 4), strains of *B. elkanii*, *B. canariense*, and *B. yuanmingense* (except for strain TAL 760) clustered, with high bootstrap support. Relative analyses between these two trees clearly showed an incongruence in topologies of the strains. In the GlnII-tree, strain EKO5^T showed proximity towards *Bradyrhizobium* genospecies α and *B. canariense*, whereas in the RecA-tree, strain EKO5^T remained distant from *Bradyrhizobium* genospecies α and *B. canariense*. Topological incongruence among the phylogenetic trees of different marker genes of a single strain is not uncommon,[48,49,54] and such incongruence of topologies based on GlnII and RecA of bradyrhizobial strains have also been reported by other authors.[9]

In the GyrB-tree (Fig. 5), the *B. elkanii* strains formed a monophylectic group with high bootstrap support. Like those in other phylogenetic trees, the type strain (ATCC 10324^T) and strain USDA 110 of *B. japonicum* formed paraphylectic groups. On the other hand, our isolate, strain EKO5^T, did not cluster robustly with any of the recognized species of *Bradyrhizobium*. It was noted that some *B. japonicum* strains clustered with the type strains of *B. liaoningense* and some *B. japoni-*

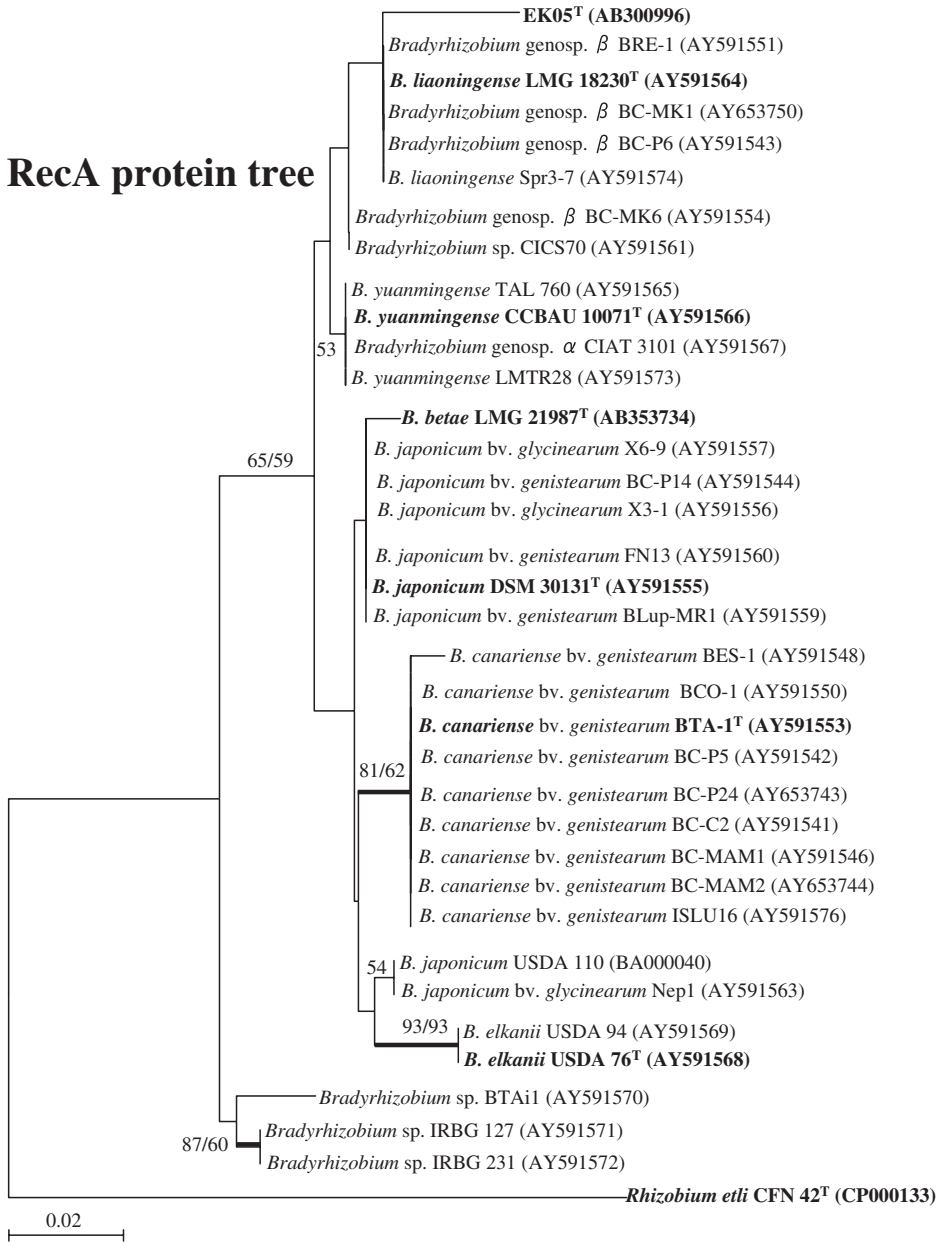

Fig. 4. Comparative NJ Analysis of Amino Acid Sequences of Partial RecA Protein (169 amino acids) from *Bradyrhizobium iriomotense* sp. nov. EK05^T and Related Strains from GenBank.

The tree was constructed by the Poisson correction distance model in the MEGA v.3.1 software package. Numbers at nodes indicate percentage bootstrap values from 1,000 replications. Only values (NJ/MP) above 50% are indicated, and values greater than 70% are shown in bold lines. Bar, 0.02 substitutions per site.

cum strains clustered with that of *B. yuanmingense*. We assumed that the sequence data of these so-called *B. japonicum* strains were submitted to the databank before the strains were identified properly, based on delicate taxonomic examination. We could not amplify the partial *atpD* fragment of *B. betae* LMG 21987^T. Probably the primer pair (atpD-255F/atpD-782R) that we used or the reaction conditions that we imposed in PCR was not appropriate for this strain. Hence, without *B. betae* LMG 21987^T, we conducted analysis based on the *atpD* gene (tree not shown). In the 465-bp fragment, strain EKO5^T possessed 94.2%, 93.8%, 93.1%, 93.1%, and 91.0% *atpD* sequence similarity to those of the type strains of *B. yuanmingense*, *B. japonicum*, *B. canariense*, *B. elkanii*, and *B. liaoningense* respectively.

In fact, because of the conserved nature of the 16S rRNA gene of *Bradyrhizobium*, many strains have 16S rDNA sequence divergences of only 0.1 to 2.0%, insufficient to distinguish their phylogenetic placement. For example, the type strains of *B. japonicum* and *B. liaoningense* differ by only 3 nucleotides. This difference was nearly 3-fold less than the differences observed among a majority of *B. japonicum* serotypes.[55,56] A further complicating factor of these

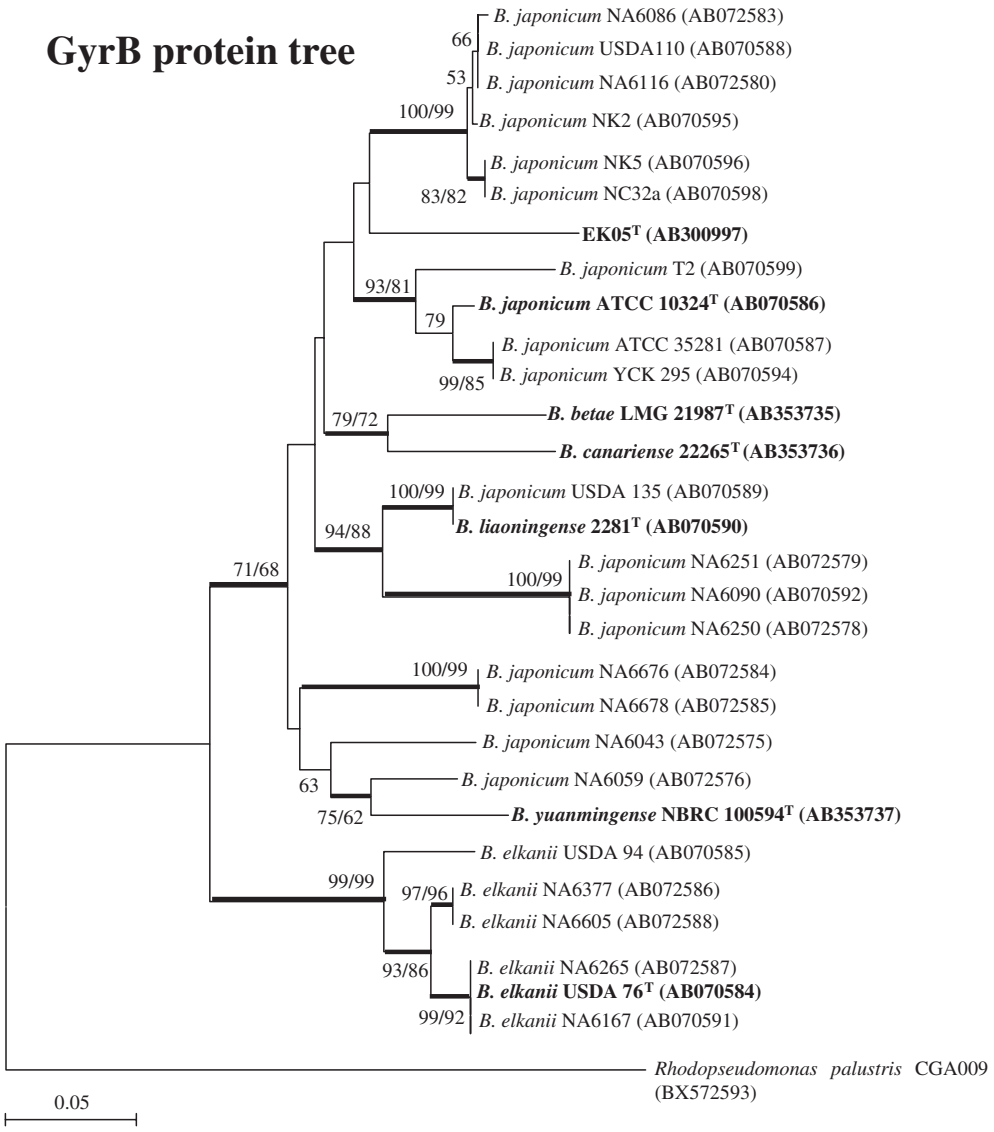

Fig. 5. Comparative NJ Analysis of Amino Acid Sequences of Partial GyrB Protein (256 amino acids) from *Bradyrhizobium iriomotense* sp. nov. EK05^T and Related Strains from GenBank.

The tree was constructed by the Poisson correction distance model in the MEGA v.3.1 software package. Numbers at nodes indicate percentage bootstrap values from 1,000 replications. Only values (NJ/MP) above 50% are indicated, and values greater than 70% are shown in bold lines. Bar, 0.05 substitutions per site.

organisms is their very slow growth rate, which made standard phenotypic characterization much difficult. Therefore, although a great number of *Bradyrhizobium* strains have been recovered from numerous legume hosts to date, the strains have not been fully characterized in order to assign formal species status. Often the strains were misidentified, or referred to only as *Bradyrhizobium* sp., with the host plant usually listed in parentheses.[57] Therefore, in some cases, artificial incongruence of topology resulted when phylogenetic trees were inferred based on the sequences of different loci (*e.g.*, *gyrB*, *glnII*, and *recA*) of these poorly identified strains. For example, it was found that the 16S rRNA gene sequence of *B. japonicum* USDA 135 and that of *B. liaoningense* USDA 3622^T were identical. It was also reported that the antigens of the later strain showed cross-reaction with antisera elicited against the antigens of strain *B. japonicum* USDA 135.[55] Here, in our analysis, we also found that these two strains clustered together with high bootstrap support in the phylogenetic tree based on GyrB. We think that *B. japonicum* USDA 135 should be considered to be *B. liaoningense*. Similarly, in previous studies, strains USDA 110 and USDA 6^T of *B. japonicum* were found to be distinguishable by DNA homology.[55] These two strains clustered separately in all the phylogenetic trees we constructed here. Obviously, strain USDA 110 deserves reclassification as a separate species.

Although the topology of strain EKO5^T varied in different protein trees, however, its phylogenetic distinctiveness remained intact in all the trees constructed in the present study. We also constructed a phylogenetic

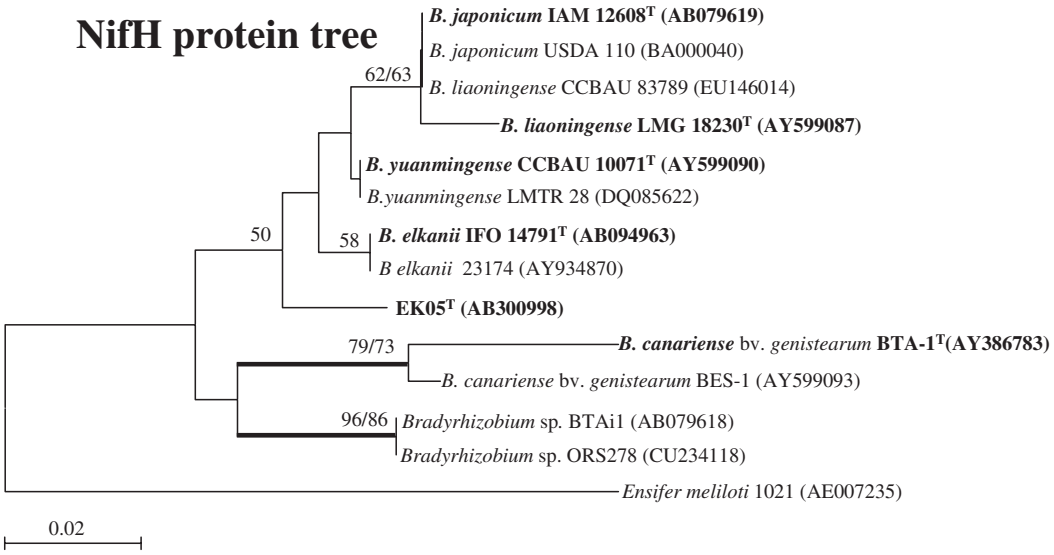

Fig. 6. Comparative NJ Analysis of Amino Acid Sequences of Partial NifH Protein (86 amino acids) from *Bradyrhizobium iriomotense* sp. nov. EK05^T and Related Strains from GenBank.

The tree was constructed by the Poisson correction distance model in the MEGA v.3.1 software package. Numbers at nodes indicate percentage bootstrap values from 1,000 replications. Only values (NJ/MP) above 50% are indicated. Bar, 0.02 substitutions per site.

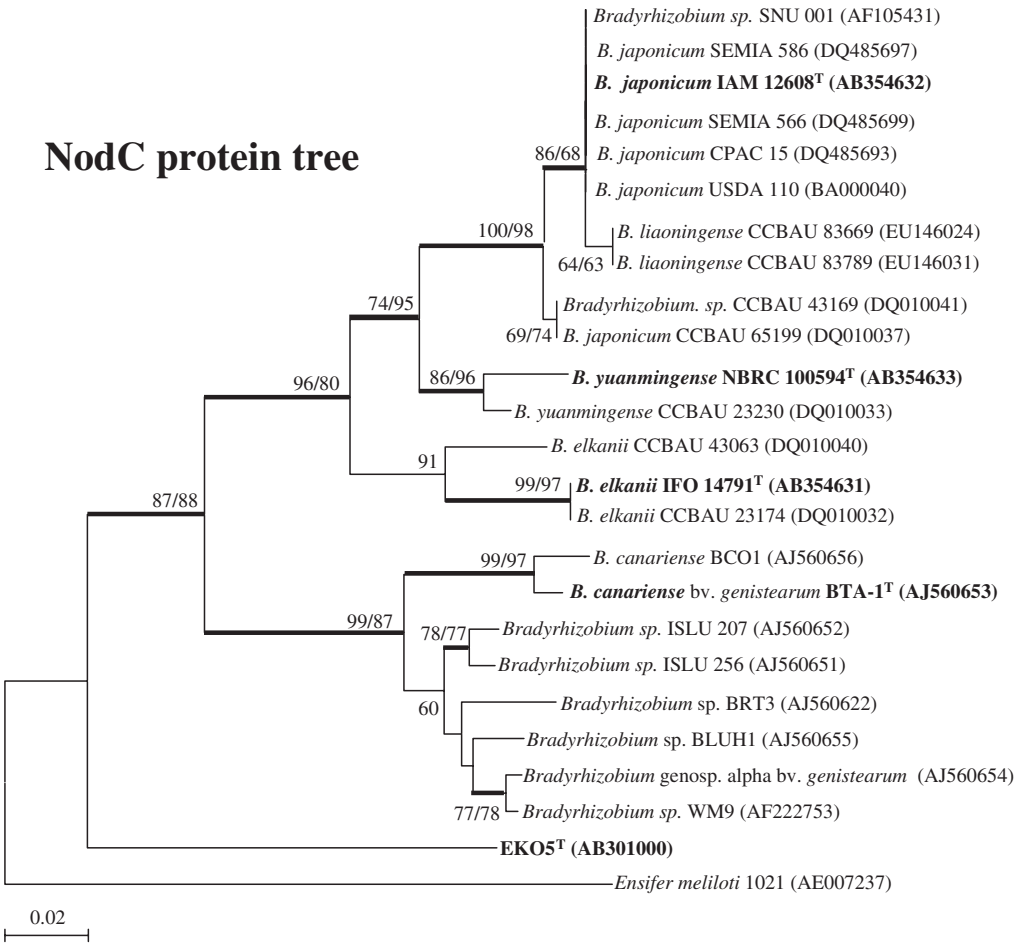

Fig. 7. Comparative NJ Analysis of Amino Acid Sequences of Partial NodC Protein (151 amino acid) from *Bradyrhizobium iriomotense* sp. nov. EK05^T and Related Strains from GenBank.

The tree was constructed by Poisson correction distance model in MEGA v.3.1 software package. Numbers at nodes indicate percentage bootstrap values from 1,000 replications. Only values (NJ/MP) above 50% are indicated, and values greater than 70% are shown in bold lines. Bar, 0.02 substitutions per site.

tree with concatenated amino acid sequences of GlnII, RecA, and GyrB proteins (Supplemental Fig. 2; see *Biosci. Biotechnol. Biochem.* Web site). We noticed that the branch length of EKO5^T was almost equal to that of other type strains, indicating a distinct position of EKO5^T. From our analysis, we agreed with other authors[43,55,58] that the present classification of bradyrhizobial species is insufficient to accommodate numerous strains that show significant variation at the genetic level. A reclassification based on DNA-DNA homology together with analyses of a fixed set of carefully chosen house-keeping genes may come to be used to minimize the current topological incongruence among different gene trees. We also think that proper caution is required during data submission to Genbank so that newly recovered bradyrhizobial strains are not affiliated to any bradyrhizobial species based solely on 16S rRNA or ITS sequences.

Analyses of symbiotic loci nifH *and* nodC

To shed light on the pattern of nitrogen-fixation genes present in strain EK05^T with respect to those in other lineages of the genus *Bradyrhizobium*, we partially sequenced two commonly used representative symbiotic loci, *nifH* and *nodC*. We failed to amplify these two loci from *B. betae* LMG 21987^T. Hence, we could not include this strain in the analyses based on *nifH* and *nodC*. It would be reasonable to point out that *B. betae* was isolated from a tumor of *Beta vulgaris*, which is a non-legume.[8] This species has not yet been reported to be able to fix nitrogen or to elicit nodules in legume plants. In two bradyrhizobial strains (*e.g.*, the *Bradyrhizobium* strains BTAi1 and ORS278), the canonical *nodABC* genes were not found when the full genome of these strains was sequenced recently.[59] Therefore, it is possible that *B. betae* LMG 21987^T possesses a different kind of symbiotic apparatus, or that the strain is devoid of the above attributes. Our strain, EK05^T, remained unclustered, and appeared in a distinct branch both in the NifH- and the NodC-phylogenetic tree (Figs. 6, 7). Such discrete branching probably indicates an independent nitrogen-fixing evolutionary history of strain EKO5^T.

DNA relatedness and DNA base composition content analyses

The results of DNA-DNA hybridization between strain EK05^T and the recognized bradyrhizobial species (Table 2) indicated that strain EK05^T possesses significantly low DNA relatedness, in the range of 9–61%, justifying the placement of this isolate into a novel species of the genus *Bradyrhizobium*.[60] The G+C content of the EK05^T isolate was 61.2%. This value, although within the reported GC-range of the family *Bradyrhizobiaceae*, is little bit lower than those of its closest 16S rRNA phylogeny-neighbors, *B. japonicum* USDA 110 and *B. betae* PL7HG1^T, which have G+C contents of 64.0% and 63.7% respectively.

Table 2. Results of DNA-DNA Hybridization Experiments

	Strain EKO5^T
B. japonicum IAM 12608^T	16 (34)
B. japonicum USDA 110	37 (20)
B. betae LMG 21987^T	50 (61)
B. canariense LMG 22265^T	39 (53)
B. yuanmingense NBRC 100594^T	41 (40)
B. liaoningense LMG 18230^T	9 (16)
B. elkanii IFO 14791^T	12 (32)

Values are percentage results from hybridization at 49.4 °C. Reciprocal values are given in parentheses.

Phenotypic characterization

In an N_2-fixation test, strain EKO5^T formed a diffused growth layer stretching from the surface to a certain depth of the tube containing Winogradsky's N_2-free soft gel mineral medium. This growth indicated the ability of strain EKO5^T to fix nitrogen under free-living microaerobic conditions. In a sole carbon source assimilation test, strain EK05^T and *B. japonicum* differed based on the utilization of lactose, maltose, sucrose, L-sorbose, and citrate. The two strains also differed with regard to their sensitivity to chloramphenicol and tetracycline; and in their ability to reduce nitrate. On the other hand, strain EK05^T could be distinguished from *B. betae* by the utilization of maltose, L-sorbose, and citrate as sole carbon source; an inability to use DL-α-alanine as sole nitrogen source; the sensitivity to chloramphenicol; the inability to grow in the presence of NaCl (1.0%); and the range of growth pH. Although some phenotypic features were shared in common, strain EK05^T could be distinguished from each of the recognized species of *Bradyrhizobium* (Table 3).

The fatty acid profile of isolate EK05^T was similar to that of the genus *Bradyrhizobium*. The profile was characterized by a predominance of $C_{18:1\omega7c}$ (80.13%) and $C_{16:0}$ (14.71%). Other fatty acids present at levels above 1% were 11 methyl $C_{18:1\omega7c}$ (2.01%) and $C_{16:1\omega7c}$ and/or iso-$C_{15:0}$ 2OH (2.02%). A comparison of the fatty acid profiles as between strain EK05^T and the recognized species of bradyrhizobia is summarized in Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site.

It was difficult to culture the host plant, *Entada koshunensis*, in our laboratory. Hence, we chose *Macroptilium atropurpureum*, a well-known promiscuous host for *Bradyrhizobium* strains, to conduct a plant-inoculation study. After 3 weeks of incubation, strain EK05^T was found to elicit nodules. The cross section of the nodules was pinkish, indicating effective nodulation. The time required to induce nodules in the plants inoculated with strain EK05^T was noticeably shorter than that with the positive control (*B. japonicum* USDA 110). In the positive control, it took 5 to 6 weeks for the nodules to become visible. A shorter time to establish nodulation probably indicates the somewhat better symbiotic effectiveness of strain EK05^T at least with

Table 3. Characteristics Useful in Distinguishing *B. iriomotense* EK05^T from Recognized Bradyrhizobial Species

Strains: 1, EK05^T (this study); 2, *B. japonicum* USDA 110 (this study unless indicated); 3, *B. japonicum* IAM 12608^T (this study unless indicated); 4, *B. betae* LMG 21987^T (this study unless indicated); 5, *B. canariense* LMG 22265^T (this study unless indicated); 6, *B. yuanmingense* NBRC 100594^T (this study unless indicated); 7, *B. liaoningense* LMG 18230^T (this study unless indicated); 8, *B. elkanii* IFO 14791^T (this study unless indicated). +, growth; −, no growth; w, weak; v, variable; nr, not reported.

Characteristics	1	2	3	4	5	6	7	8
C source								
D-Fructose	+	+	+[a]	w[c]	+	−	v[c]	+
Lactose	−	+	+	−	−	−	−	v
Maltose	−	w	+	+	−	v[b]	−	+
Sucrose	−	w	+	−	−	v[b]	−	+
L-Sorbose	−	+	w[a]	+	−	−	−	w[c]
Citrate	−	+	+	+	+	+	+	+
N source								
Glycine	−	−	+	nr	−	−	−	−[a]
DL-α-Alanine	−	−	−	+	−	+	−	+
Resistance to:								
Erythromycin (100 μg ml^{-1})	+	+	+	v[b]	v[b]	−	−	+
Kanamycin (50 μg ml^{-1})	−	−	−	−	−	+	+	−
Chloramphenicol (50 μg ml^{-1})	−	+	+	+	+	+	−	+
Tetracycline (10 μg ml^{-1})	−	+	+	−	+	+	+	+
NaCl (1.0%) in YMB	−	−	−[a]	+	−	−	−	+
NO_3 reduction	−	+	+	−	−	+	−	+[a]
Growth characteristics:								
pH 4.5	+	+	−	−	+	−	+	+
pH 10	−	−	+[a]	+	−	−	+[a]	v[b]
Colony size (mm) after 7 d of incubation in YMA	0.2–1.0	1.0–2.0	1.0–2.0	0.2–0.5	1.0–1.5	1.0–2.0	0.2–0.5	1.0–1.5
G+C content (mol %)	61.2	64.0[c]	62.8[c]	63.7[c]	63.8[b]	63.0[d]	64.2[b]	63.9[d]

[a]Our own data are not consistent with those reported previously.
[b]Data from Vinuesa *et al.*[2)]
[c]Data from Rivas *et al.*[8)]
[d]Data from Yao *et al.*[14)]

the legume *Macroptilium atropurpureum*, but further study is required to produce a clear picture of the symbiotic potential of this novel strain.

In conclusion, in the present study we performed a taxonomic investigation of a potential bradyrhizobial isolate and found that it qualified as a novel species status. We conducted multilocus sequence analysis using eight gene partitions (six informational/housekeeping loci, *rrs*, ITS, *glnII*, *recA*, *gyrB*, and *atpD*, and two *sym* loci, *nifH* and *nodC*. Our analyses are in line with the recommendations proposed by the *ad hoc* committee for the re-evaluation of species definition in bacteriology.[45)] The information found in this study should be helpful in future classification of bradyrhizobial strains. The investigation should also be supportive of future ecological and agricultural studies, since bradyrhizobia are among the most well-known plant growth-promoting rhizobacteria (PGPR).

Description of Bradyrhizobium iriomotense *sp. nov.*

Bradyrhizobium iriomotense (i.ri.o.mo.te.n'se N.L. neut. adj. *iriomotense*, referring to Iriomote Island of Okinawa, the province in Japan where the bacterium was first isolated).

The species grows slowly and forms opaque, white, circular, convex, and granular-textured colonies on YMA plates within 5–6 d. The cells are about 0.6–0.7 × 1.2–2.1 μm and motile by a single subpolar flagellum (Supplemental Fig. 3; see *Biosci. Biotechnol. Biochem.* Web site). They are aerobic, Gram-negative, non-sporulating, and rod-shaped. They produce much extracellular mucilage. The strain uses D-fructose, D-glucose, D-mannose, D-galactose, D-xylose, D-ribose, D-arabinose, and adonitol, but not lactose, maltose, sucrose, L-sorbose, melibiose, raffinose, D-trehalose, citrate, fumeric acid, or succinic acid as sole carbon and energy sources. The species uses L-threonine, L-leucine, L-phenylalanine, L-aspartic acid, and L-glutamic acid, but not DL-α-alanine or glycine, as sole carbon and nitrogen sources. Growth is observed at 15–32 °C, but not at 10 °C or 37 °C. The pH range is 4.5–9.0. Growth is not seen on YMB medium containing 1.0% NaCl. The 3-ketolactose is not produced from lactose oxidation; moreover, the species is oxidase- and catalase-positive. Nitrate reduction is negative. The species tolerates erythromycin (100 μg/ml), ampicillin Na (100 μg/ml), and polymyxin B (300 μg/ml), but is sensitive to kanamycin (50 μg/ml), chloramphenicol (50 μg/ml), and tetracycline (10 μg/ml). The G+C content of the type strain is 61.2 mol %. As determined by 16S rDNA sequence analysis, the strain is closely related to members of the genus *Bradyrhizobium*.

The type strain, EK05^T (= NBRC 102520^T = LMG 24129^T), was isolated from a tumor-like root-outgrowth of the legume *Entada koshunensis*.

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