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Phylogeny and distribution of extra-slow-growing *Bradyrhizobium japonicum* harboring high copy numbers of RSα, RSβ and IS*1631*

Reiko Sameshima [a], Tsuyoshi Isawa [a], Michael J. Sadowsky [b], Tohru Hamada [c,1], Hiroaki Kasai [c], Arawan Shutsrirung [d], Hisayuki Mitsui [a], Kiwamu Minamisawa [a,*]

[a] *Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai 980-8577, Japan*
[b] *Department of Soil, Water and Climate, University of Minnesota, St. Paul, MN 55108-6028, USA*
[c] *Marine Biotechnology Institute, Kamaishi Laboratories, Kamaishi City, Iwate 026-0001, Japan*
[d] *Department of Soil Science and Conservation, University of Chiang Mai, Chiang Mai, Thailand*

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Abstract

We previously reported that extra-slow-growing *Bradyrhizobium japonicum* isolates obtained from three field sites in Japan, designated as HRS (highly reiterated sequence-possessing) strains, have high copy numbers of the insertion sequences RSα and RSβ. When strain collections in the USA, Japan, Korea, Thailand and China were examined by Southern hybridization using RSα, RSβ and IS*1631* as probes, HRS strains were found in the Japanese, Chinese, and American collections, but not in the Korean and Thai ones. Copy number analyses of RSα and RSβ, calibrated with the copy number of *rrs* (16S rRNA gene), indicated that the HRS stains can be divided into two major groups. Group A is comprised of members with a high copy number of RSα (mean ± S.D., 139 ± 27), a low number of RSβ (mean ± S.D., 30 ± 13) sequences, and extremely slow growth rates (mean doubling time ± S.D., 27 ± 9 h). In contrast, group B is comprised of strains with a high copy number of RSβ (mean ± S.D., 93 ± 6) and a lower number of RSα. These groupings of HRS strains were well correlated with phylogenetic clusters based on *rrs*, *gyrB* and serogroups (110/122 and 123/135). Growth rate of *B. japonicum* strains was also correlated exclusively with RSα copy number. The ecological, evolutionary and biotechnological implications of the findings are discussed.

Keywords: *Bradyrhizobium japonicum*; Diversity; Insertion sequence; Phylogeny; Slow growth

1. Introduction

Bradyrhizobium japonicum is a slow-growing, Gram-negative, nitrogen-fixing α-Proteobacterium that forms root nodules on soybeans [1,2]. We previously reported that 21 extra-slow-growing (ESG) *B. japonicum* strains indigenous to soils of Japan harbor high copy numbers of the insertion sequences (IS) RSα and RSβ [3–5]. We designated these isolates as HRS (highly reiterated sequence-possessing) strains of *B. japonicum* [3–5]. Genotypic and phenotypic characterizations suggested that the HRS strains were generated from non-HRS strains via transposition and recombination events that probably involve IS elements [3,5]. Nodulation genes of *B. japonicum* HRS strain were horizontally transferred into non-nodulating bacteria in soil and microcosm [6], suggesting that HRS strains have the potential for horizontal gene transfer in soil environments as a donor.

Soybean Bradyrhizobia (*B. japonicum* and *Bradyrhizobium elkanii*) are indigenous to several countries including the USA, China, South America, and Australia, although they are presumed to have originated in East Asia [1,7,8]. Phylogenetic studies based on nucleotide sequence analyses of *rrs* (the gene encoding 16S rRNA) have revealed that the soybean Bradyrhizobia cluster with a number of non-symbiotic bacteria, such as *Rhodopseudomonas*, *Nitrobacter* and *Afipia* [1,2,9]. This phylogenetic region of the α-Proteobacteria has been termed the 'BANA' domain [1,10]. While *B. japonicum* from soybean

* Corresponding author. Tel.: +81 (22) 217 5684; Fax: +81 (22) 263 9845.
E-mail address: kiwamu@ige.tohoku.ac.jp (K. Minamisawa).

[1] Present address: Amano Enzyme Inc., 4-179-35, Sue-cho, Kagamiga, Gifu 509-0108, Japan.

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nodules are phylogenetically dispersed within the BANA domain, *B. elkanii* strains form a compact cluster [1,9].

Previously, HRS strains have been found among *B. japonicum* cluster at three of six field sites tested in Japan [3,4,5,7]. The ESG HRS strains were dominant at the Nagakura field site, where paddy-upland rotation had been carried out for nearly 30 years [3,7]. ESG soybean Bradyrhizobia have also been isolated from Iowa [11,12], Nebraska [13] and China [14] and hyperreiterated DNA regions homologous to IS elements have been found in *B. japonicum* serocluster 123 strains indigenous to the upper Midwest U.S. [15].

In these studies we investigated the phylogeny and distribution of HRS strains isolated from geographically distinct regions in the world. In addition, we wanted to determine whether the soybean Bradyrhizobia from other regions of the world and previously reported as ESG were HRS strains in terms of copy numbers of RSα and RSβ.

2. Materials and methods

2.1. Bacterial strains and growth media

Bacterial strains and plasmids used in these studies are listed in Table 1. Seventy-six strains of *Bradyrhizobium* were obtained from culture collections in the USA, Korea, Thailand, and China. Bacteria were grown aerobically at 30°C in HM salts medium [16] supplemented with 0.1% arabinose and 0.025% (w/v) yeast extract (Difco, Detroit, MI, USA). For the indole-3-acetic acid (IAA) assay, strains were grown in the same medium supplemented with 0.01% (w/v) L-tryptophan [17]. YM medium [18] and TY medium [19] were also used for growth curve measurement.

2.2. Analysis of IAA

IAA production in culture supernatants was assayed as described previously [5,17] to distinguish *B. japonicum* from *B. elkanii*.

2.3. PCR amplification of IS1631

Since HRS strains of *B. japonicum* from field sites in Japan contain IS*1631* [3], a PCR amplification method done using a IS*1631*-specific primer was developed to screen strains harboring this IS element. Bacterial cells were harvested at the late-exponential phase of growth, washed with 1 ml NaCl (1% w/v), and centrifuged at 18 500×*g* for 3 min at 25°C. The pellet was resuspended in 200 μl distilled H_2O and frozen at −20°C. Cell lysates were prepared by mixing 40 μl of cell suspensions with a 10 μl of proteinase K solution (1 mg ml^{-1}), 50 μl BL buffer [40 mM Tris (hydroxymethyl) aminomethane 1% Tween 20 (Wako Pure Chemical Industry, Inc., Osaka, Japan), 0.5% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan), and 1 mM EDTA, pH 8]. Cell mixtures were incubated at 60°C for 20 min, and then 95°C for 5 min, and centrifuged for 10 min at 18 500×*g* at 25°C. The supernatant contained crude DNA, which was used as a template for PCR amplification [20]. The IS*1631*-specific primers, 5′-CCATCGACAACCGCATCTACCGCTACAAG-3′, 5′-CGTCCGTCGAACTCAAGCAGATGGCAATG-3′, corresponded to positions 1543–1571 and 2579–2551 of IS*1631*, respectively, isolated from *B. japonicum* HRS strain T2 (DDBJ accession no. AB011021) [3]. The 10-μl reaction mixture contained 0.2 μM of each primer, 200 μM of each of the four dNTPs, 1.5 mM $MgCl_2$, 1×PCRx Amplification Buffer (PCRx Enhancer System, Life Technologies, Inc., USA), 1×PCRx Enhancer Solution (PCRx Enhancer System, Life Technologies, Inc., USA), and 0.2 U ExTaq polymerase (Takara Shuzo, Kusatsu, Japan). PCR conditions were: 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min chain extension at 72°C for 25 cycles, followed by a 10-min elongation step at 72°C. To verify the template DNA in the lysate from each *Bradyrhizobium* strains was of PCR quality, *rrs* was amplified using primer pairs of R27f 5′-CAGGAAACAGCTATGACCAGAGTTTGATCCTGGCTCAG-3′ and U339r 5′-TGTAAAACGACGGCCAGTCTGCTGCCTCCCGTAGGAG-3′. The 10-μl reaction mixture contained 0.2 μM of each primers, 200 μM of each of the four dNTPs, 1×PCRx ExTaq Buffer, and 0.2U ExTaq polymerase (Takara Shuzo, Kusatsu, Japan). The same PCR temperature program was used as described above. The PCR products were electrophoresed on TAE agarose gels [21].

2.4. DNA isolation and hybridization

Total genomic DNA isolation and hybridization was carried out as described previously [5]. Total genomic DNAs (200 ng/lane) were digested with *Xho*I, *Bam*HI or *Eco*RI. Hybridization probes for full-length RSα, RSβ, and IS*1631* were prepared as *Bam*HI fragments from plasmids pαHD7, pT14HD4 and pT27HD5, respectively (Table 1). For the *rrs* probe, a fragment was prepared by PCR amplification from cell lysate of

B. japonicum strain USDA110 using primer pairs R27f 5′-CAGGAAACAGCTATGACCAGAGTTTGATCCTGGCTCAG-3′ and U785r 5′-TGTAAAACGACGGCCAGTACTACC(AC)GGGTATCTAATCC-3′.

2.5. Estimation of copy numbers of RSα, RSβ and IS1631

B. japonicum possess single or two copies of rRNA gene region on chromosome [22]. Thus, we first determined copy numbers of rRNA gene region of tested strains by *rrs*-specific hybridization, and estimated copy numbers of RSα, RSβ and IS*1631* on the chromosome. Southern hybridization membranes (Hybond-N+, Amersham, Tokyo,

Table 1
Bacterial strains used in this study and detection of *B. elkanii* by IAA production test and IS*1631*-harboring *B. japonicum* by PCR amplification

Strain	Relevant characteristics	Source or reference
Bradyrhizobium strain from soybean nodules		
USDA135	USA, Isolate from alkaline soil	[a]
USDA479, USDA480, USDA482, USDA486	Luisiana State, USA, Isolates from alkaline soil	[a]
USDA490	South Dakota, USA, Isolate from alkaline soil	[a]
RJ19FYZ	North Dakota, USA, Isolate from alkaline soil	[a]
USDA24, (USDA31), USDA38, USDA117,		[b]
USDA122, USDA140, USDA142, USDA143,		[b]
USDA184, USDA8-o, USDA8-t		[b]
(61A101c), 61A118b, 61A124a, 61A148		[b]
YCK225, YCK226, YCK227, YCK228, YCK229,	Milyang soils of Kyeongnam province, Korea	[c]
YCK230, YCK231, YCK233, YCK234, YCK235,	Milyang soils of Kyeongnam province, Korea	[c]
YCK236, YCK237, YCK238	Milyang soils of Kyeongnam province, Korea	[c]
YCK240, YCK241, YCK242, YCK243, YCK261,	Korea	[c]
YCK263, YCK264, YCK266, YCK269	Korea	[c]
YCK295, YCK297, YCK298, YCK299, YCK300,	Namjeju soils of jeju province, Korea	[c]
YCK301	Namjeju soils of jeju province, Korea	[c]
YCK331, YCK332, YCK333, YCK334, YCK335,	Suweon soils of Kyeonggi province, Korea	[c]
YCK371, YCK372, YCK374, YCK375	Iksan soils of Chonbuk province, Korea	[c]
NA6059, NA6062	Ban Khong Kaow, Hang Dong District, Chiang Mai, Thailand	[d]
NA6080, NA6086, NA6090	Bang Kard 1, San Pa Tong District, Chiang Mai, Thailand	[d]
NA6154, (NA6167)	Ban Kard 2, San Pa Tong District, Chiang Mai, Thailand	[d]
(NA6265)	Mae Jo, San Sai District, Chiang Mai, Thailand	[d]
NA6545	San Ma Hah Pol, Mae Tang District, Chiang Mai, Thailand	[d]
TAL211, (TAL213), TAL216	Thailand	[b]
THA2, THA5, THA6, THA7	Thailand	[b]
2281	Heilongjiang Province, China	[e]
NC4a, NC6a, NC3a*, NC32a*	Nakazawa, Niigata, Japan	[3–5,7]
NK2, NK8, NK5*, NK6*	Nagakura, Niigata, Japan., A field site of paddy-upland rotation	[7]
T7, T9, T2*, T22*	Tokachi, Hokkaido, Japan	[7]
Reference strain for IS copy number		
USDA110		[f]
(USDA83)		[g]
Plasmids		
pαHD7	pCNTR containing RSα (1.2kb) in *B. japonicum* HRS isolate NK5	[7]
pT14HD4	pCNTR containing RSβ (1.4kb) in *B. japonicum* HRS isolate T2	[7]
pT27HD5	pCNTR containing IS*1631*(2.7kb) in *B. japonicum* HRS isolate T2	[7]

Asterisks indicate HRS strains that have been previously characterized to possess high copy numbers of RSα and RSβ [3–5,7]. Strains in parentheses, USDA31, 61A101c, NA6167, NA6265, TAL213, and USDA83 were *B. elkanii* based on IAA production assay [5,17]. Underlined strains were *B. japonicum* strains harboring IS*1631* detected by the PCR amplification (see text). Species name '*liaoningense*' for the Chinese strain 2281 was proposed by Xu et al. ([14], see text).
[a]M. Sadowsky, University of Minnesota, USA.
[b]M. Abe, Kagoshima University, Japan.
[c]Ui-Gum KANG, National Yeongnam Agricultural Experiment Station, Korea.
[d]Arawan Shutsrirung, Chiang Mai University, Thailand.
[e]H. Fan, Chinese Academy of Agriculture Sciences, Beijing, China.
[f]H.H. Keyser, U.S. Department of Agriculture, Beltsville, MD, USA.
[g]E.W. Triplett, University of Wisconsin, Madison, WI, USA

Japan) were exposed on Imaging Plate (Fuji Photo Film, Co. Ltd., Tokyo, Japan), and hybridization signals were detected by an imaging analyzer FLA-2000 (Fuji Photo Film, Co. Ltd., Tokyo, Japan). Signal intensities of the RSα-, RSβ- and *rrs*-specific hybridizing bands in strains were compared with that of USDA110, which possesses 12, six and one copies of RSα, RSβ and *rrs*, respectively [23]. Signal intensities of IS*1631*- and *rrs*-specific hybridization of tested strains were compared with that of USDA83, which contains four copies and one copy of IS*1631* and *rrs*, respectively. The copy number of the *rrs* on chromosome was determined by digestion of total

DNA with *Eco*RI, *Bam*HI and *Hin*dIII, these restriction sites are absent in the amplified region of *rrs*.

Chromosome-based copy numbers of RSα (N_a), RSβ (N_b) and IS*1631* (N_i) were calculated according to the one or two *rrs* copy numbers of tested strains as the follows, respectively: $N_a = 12N_{rrs}A_sR_{110}A_{110}^{-1}R_s^{-1}$; $N_b = 6N_{rrs}B_sR_{110}B_{110}^{-1}R_s^{-1}$; $N_i = 4N_{rrs}I_sR_{83}I_{83}^{-1}R_s^{-1}$ (N_{rrs}, copy number of *rrs* in a tested strain, which is generally a single copy [22]; A_s, total radioactivity of RSα-specific hybridization in a tested strain; R_s, total radioactivity of *rrs*-specific hybridization in a tested strain; A_{110}, total radioactivity of RSα-specific hybridization in USDA110; R_{110}, total radioactivity of *rrs*-specific hybridization in USDA110; B_s, total radioactivity of RSβ-specific hybridization in a tested strain; B_{110}, total radioactivity of RSβ-specific hybridization in USDA110; I_s, total radioactivity of *rrs*-specific hybridization in a tested strain; I_{83}, total radioactivity of IS*1631*-specific hybridization in USDA83; R_{83}, total radioactivity of *rrs*-specific hybridization in USDA83. The coefficients of 12, 6 and 4 in the formula indicate copy numbers of RSα, RSβ and IS*1631* in the standard strains, USDA110 or USDA83, respectively.)

2.6. *Sequencing of 16S rRNA and* gyrB

The *rrs*-specific primers, pr0R: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 9Rev: 5′-AAGGAGGTGATCCAGCC-3′ were used to amplify about 1300 bp of *rrs*. PCR conditions were: 1 min denaturation at 94°C, 1 min annealing at 58°C, and a 2-min chain extension at 72°C for 35 cycles. A 980-bp-long partial fragment of *gyrB* was amplified using primers gyr21A: 5′-CAGGAAACAGGCTATGACCAARMGICCNGSIATGTAYATHGG-3′ and gyQTKr: 5′-ACSAGCTTGTCCTTGGTYTG-3′. DNA amplification cycles were: 1 min denaturation at 94°C, 1 min annealing at 58°C, and a 2-min chain extension at 72°C for 35 cycles.

Amplified DNA fragments were separated on agarose gels, and purified using QIAquick Gel Extraction Kit (Qiagen, Germany). Direct sequencing was performed by using the PCR products as templates and the PCR primers with a ABI PRISM 310 DNA sequencer and ABI PRISM Big Dye Terminator Sequencing Kit (PE Applied Biosystems, USA).

Determined sequences were unified the lengths, and multiple sequence alignment, genetic distance calculation with Kimura's two-parameter method, bootstrap resamplings, and phylogenetic tree constructions by neighbor-joining method [24] were performed using Clustal W (DDBJ, Mishima, Japan, http://www.ddbj.nig.ac.jp/).

2.7. *Determination of mean generation time and statistical analysis*

Broth cultures of exponential phase of *Bradyrhizobium* strains were inoculated, in duplicate, into 5 ml of fresh HM medium to adjust a cell turbidity of 0.01 at 660 nm. Cultures were incubated at 30°C under the aerobic condition with shaking. The turbidity of each culture was measured every 6–8 h. The mean generation time was calculated from values interpolated from the growth curves at the beginning and the end of the exponential phase of growth. The correlation of generation times with IS copy numbers were investigated by simple linear regression analysis, and significance of the relationships were determined by *t*-test using JMP software (SAS Institute Inc., NC, USA). Growth curve of representative strains were measured in triplicate, into 6 ml of fresh medium (HM, YM or TY medium) to adjust a cell turbidity of 0.01 at 660 nm. Cultures were incubated at 30°C under the aerobic condition with shaking. The turbidity of each culture was measured about every 24 h.

2.8. *Nucleotide sequence accession number*

The sequences of *rrs* and *gyrB* genes ascertained in this work were deposited under the following accession numbers to DDBJ/EMBL/GenBank database. *rrs*: AB070561 (strain NA6167), AB070562 (strain NA6545), AB070563 (strain NA6059), AB070564 (strain NA6062) AB070565 (strain NA6090) AB070566 (strain YCK295) AB070567 (strain NK2) AB070568 (strain NK5) AB070569 (strain NK6) AB070570 (strain T2) and AB070570 (strain USDA135). *GyrB*: AB070580 (*Rhizobium leguminosarum*), AB070581 (*Rhodopseudomonas palustris* ATCC17001), AB070582 (*Rhodopseudomonas* sp. B29 [25]), AB070583 (*Blastobacter denitrificans* NCIMB 12292T), AB070584 (*Bradyrhizobium elkanii* USDA76T), AB070585 (*B. elkanii* USDA94), AB070586 (*B. japonicum* ATCC10324), AB070587 (*B. japonicum* ATCC35281), AB070588 (*B. japonicum* USDA110), AB070589 (*B. japonicum* USDA135), AB070590 (*B. liaoningense* 2281T), AB070591 (strain NA6167), AB070592 (strain NA6090), AB070593 (strain NA6545), AB070594 (strain YCK295), AB070595 (strain NK2), AB070596(strain NK5), AB070597 (strain NK6), AB070598 (strain NC32a), and AB070599 (strain T2).

3. Results

3.1. *IS*1631*-specific PCR amplification*

Previous studies suggested that HRS strains possessing high copy numbers of RSα and RSβ are confined to the *B. japonicum* subgroup of BANA domain [3–5,7]. To determine if the Bradyrhizobia used in this study, which came from geographically distinct regions of the world, were *B. japonicum* or *B. elkanii*, we assayed IAA production in culture [1,5,16,17]. Five strains, USDA31, 61A101c, NA6167, NA6265 and TAL213, produced IAA in culture indicating that they were members of *B. elkanii*,

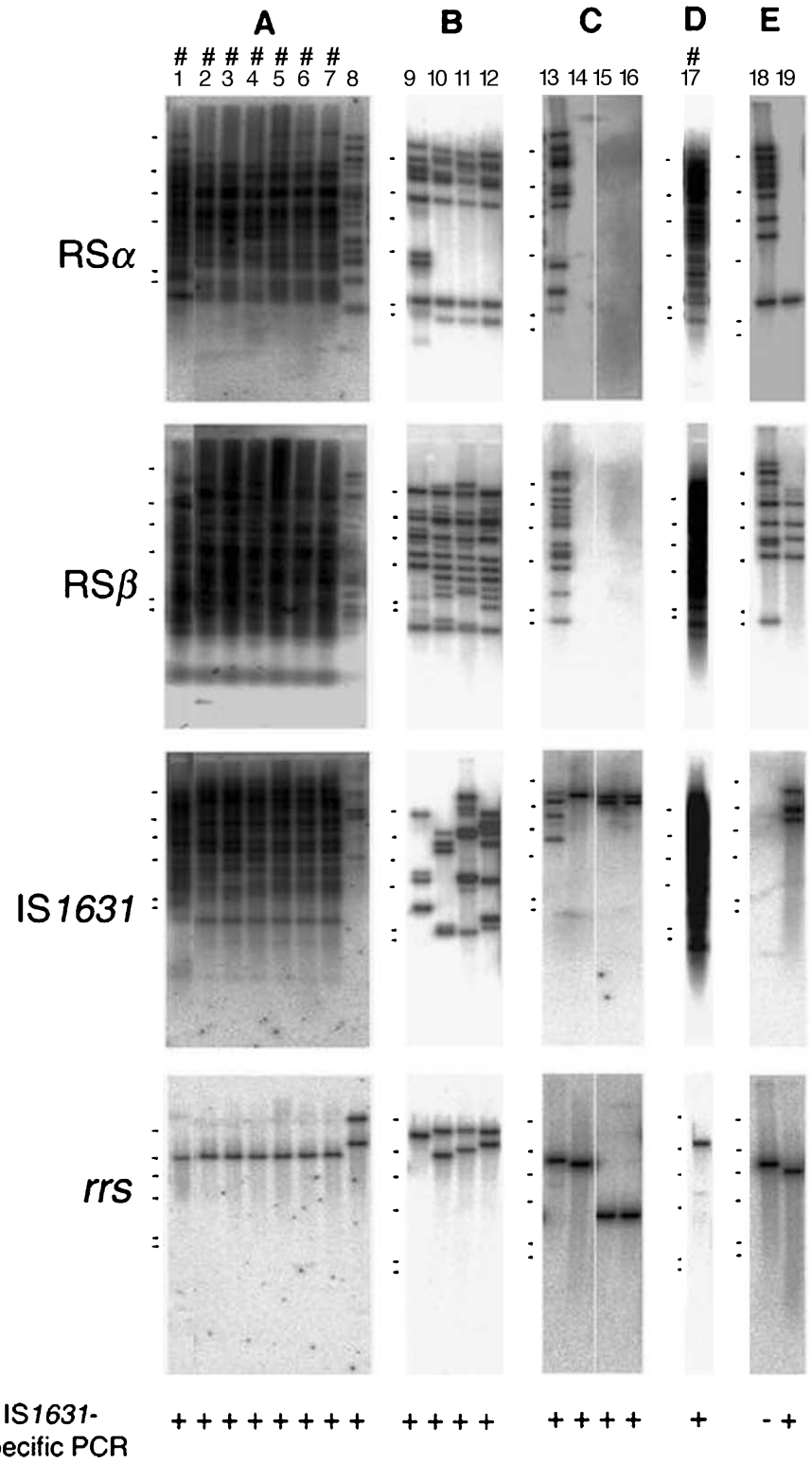

Fig. 1. Southern hybridization of *Bradyrhizobium* strains possessing IS*1631* from USA (A), Korea (B), Thailand (C), China (D) and reference strains (E) with RSα, RSβ, IS*1631*, and *rrs* as probes. A: Each lane contained total DNA digested with *Xho*I (RSα and RSβ) and *Eco*RI (IS*1631* and *rrs*) from the following strains: USDA135 (lane 1), USDA479 (lane 2), USDA480 (lane 3), USDA482 (lane 4), USDA486 (lane 5), USDA490 (lane 6), RJ19FYZ (lane 7), and 61A124a (lane 8). B: Each lane contained *Eco*RI-digested total DNA from the following Korea strains: YCK230 (lane 9), YCK295 (lane 10), YCK297 (lane 11), and YCK334 (lane 12). C: Each lane contained *Eco*RI-digested total DNA from the following Thailand strains: NA6545 (lane 13), NA6090 (lane 14), NA6059 (lane 15), and NA6062 (lane 16). D: Lane 17 contained *Eco*RI-digested total DNA from ESG strain 2281 from China [14]. E: Each lane contained *Eco*RI-digested total DNA from *B. japonicum* USDA110 (lane 18) and *B. elkanii* USDA83 (lane 19), which were used for calibration of copy numbers of the IS elements. The copy numbers of RSα and RSβ in USDA110 are regarded as 12 and six copies respectively according to the previous report [23]. USDA83 possessed four copies of IS*1631* (see text). #: HRS strains harboring extremely high copy numbers of RSα, RSβ, and IS*1631*. Size markers indicating the migration of the λ/*Hin*dIII ladder (23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb) are depicted on the left of each panel.

while the remaining 71 strains of IAA non-producers were classified as *B. japonicum*. These later strains were used for further analyses (Table 1).

PCR analysis, done using IS*1631* specific primer sets, indicated that the 1.0-kb PCR product was exclusively present in the authentic HRS strains NK5, NK6 and NC32a [3], and that no PCR product was observed in non-HRS strains (data not shown). The identity of the PCR product as IS*1631* was verified by digestion patterns using internal restriction sites *Pst*I, *Pvu*II, and *Sac*II.

PCR analyses of the strains in Table 1 indicated that IS*1631*-harboring strains were present at a frequency of 24% (17 of 71 strains) in the *Bradyrhizbium* strains in the collections [eight of 22 USA strains (with prefixes USDA, RJ and 61A), four of 37 Korean strains (with prefix YCK), four of 16 strains Thailand strains (with prefixes NA, TAL and THA) and one strain from China (with no prefix)].

3.2. Hybridization with RSα, RSβ, IS1631, and rrs probes

The *Bradyrhizobium* strains exhibiting positive PCR amplification with IS*1631*-specific primers were hybridized with RSα, RSβ, and IS*1631* gene probes (Fig. 1). Seven strains (USDA135, USDA479, USDA480, USDA482, USDA486, USDA490 and RJ19FYZ) from alkaline soils in US had extremely numerous hybridizing bands specific for the probes, although a single copy of *rrs* was observed in these strains (Fig. 1A). The Korean strains harboring IS*1631* showed several copies of RSα and RSβ, which was similar to the non-HRS strain USDA 110 (Fig. 1B and E). Three of four strains harboring IS*1631* from the Thailand collection (NA6059, NA6062 and NA6090) contained no copies of RSα and RSβ (Fig. 1C), although *B. japonicum* strains isolated from soybean nodules in Japan always possess RSα and RSβ [4]. The ESG (ESG) strain 2281 from China showed high copy numbers of RSβ as well as RSα [3], and had a single copy of *rrs* gene (Fig. 1D).

3.3. Genome-based copy numbers of IS elements

Table 2 summarizes estimated copy numbers of three IS elements and *rrs* per genome among *Bradyrhizobium* strains from USA, Korea, Thailand and China, as compared with the HRS strains found in Japan [3,4]. Copy numbers of RSα and RSβ allowed division of the HRS Bradyrhizobia into two major groups. Group A is characterized by a higher copy number of RSα (mean ± S.D., 139 ± 27) than RSβ (30 ± 13), while Group B is characterized by a higher copy number of RSβ than RSα. Group B can be divided into two subgroups, with subgroup B1 showing higher copy numbers of RSβ (93 ± 6), and subgroup B2 strains have lower copy numbers of RSβ (55 ± 5), although the copy numbers of RSα are similar in both subgroups (43 ± 3 and 37 ± 1, respectively). When copy numbers of the three IS elements were compared, copy number of RSβ was correlated with that of IS*1631*, although RSα showed no correlation with RSβ and IS*1631* (Table 3). Results of this study indicate that despite the large number of IS elements in these Bradyrhizobia (Table 2), the copy number of *rrs* in the HRS strains was not numerous.

3.4. Phylogeny of HRS strains based on rrs and gyrB

To determine the phylogenetic position of the HRS strains, we sequenced the *rrs* genes of representative strains and constructed phylogenetic trees with relatively close organisms in database using uniformed length (1295 ~ 1304 bp) of the sequences (Fig. 2A). The soybean Bradyrhizobia, including 'standard strains' and those strains tested in this work, were classified into three clusters; *B. elkanii*, *B. japonicum* cluster 1 (BJ1) and *B. japonicum* cluster 2 (BJ2). The HRS strains in Group A having higher copy numbers of RSα (Table 2), NK5 and NK6, belonged to cluster BJ1, whereas HRS strains of Group B having higher copy numbers of RSβ, strain T2 and the newly identified HRS strains USDA135 and 2281, fell into cluster BJ2 (Fig. 2A).

Although *rrs* sequence analysis is a standard method used for the modern taxonomic analysis of bacteria, this molecule evolves very slowly such that the resultant phylogenetic analysis is not always useful to distinguish closely related strains [26]. To increase more resolution, we determined the sequence of the *gyrB* gene [26] in representative strains and some relatively close organisms, and constructed phylogenetic trees using uniformed length (669 ~ 675 bp) of the sequences (Fig. 2B). Although *gyrB* encodes a protein and GC content biases could have substantial impacts on sequence placement [27], we consider the bias of this tree is small enough to ignore [28] because GC contents of sequences used in this study were small differences, ranging from 59.6% to 65.3%. The topology of the clusters based on *gyrB* slightly differed from that of the *rrs*-based tree in that the BJ2 cluster of *rrs* tree was divided into two groups based on *gyrB* analysis. Interestingly, the *gyrB* topology is similar to phylogenetic tree constructed using ITS (internally transcribed spacer region) sequence data [29]. This result suggests that strains in BJ2 (Fig. 2A) are diverse in terms of molecular phylogeny based on *gyrB* gene. However, BJ1 and *B. elkanii* clusters were well conserved (Fig. 2B), which corresponded to those in the *rrs* tree (Fig. 2A). These two phylogenetic trees show that HRS strains were derived from a wide range of *B. japonicum* strains.

It should be noted that Xu et al. [2,14] proposed the species name *B. liaoningense* for ESG strain 2281. We did not use the species name for 2281 in this paper, because the strain fell into the same cluster with *B. japonicum* USDA135 in both phylogenetic tree. Van Berkum et al. suggested that the 135 serogroup should continue at this time to be considered as a member of *B. japonicum* [29].

Table 2
Copy numbers of RSα, RSβ, IS*1631* and *rrs* gene, generation time, phylogenetic position and serotype correlated with grouping of *B. japonicum*

Group strain	IS*1631*-specific PCR	Estimated copy number				Generation time (h)	Cluster in *rrs*-phylogenetic tree	Serotype[a]
		RSα	RSβ	IS*1631*	*rrs*			
Group A: higher copy numbers of RSα								
BJ1								
NK5	+	176	47	51	1	24.9 ± 3.3	BJ1	110
NK6	+	110	18	23	1	26.4 ± 3.5	BJ1	122-129
NC3a	+	139	23	4	1	29.2 ± 0.9		110
NC32a	+	132	30	3	1	27.7 ± 0.0	BJ1	110
Mean ± S.D.		139 ± 27	30 ± 13			27.1 ± 8.7		
Group B: higher copy numbers of RSβ								
Subgroup 1								
USDA135	+	64	91	50	1	12.3 ± 0.3	BJ2	135
USDA479	+	41	107	43	1	12.9 ± 0.3		135
USDA480	+	42	115	42	1	14.2 ± 0.4		135
USDA482	+	43	108	38	1	14.8 ± 0.5		135
USDA486	+	38	96	32	1	18.0 ± 0.6		135
USDA490	+	37	79	33	1	16.7 ± 0.7		135
RJ19FYZ	+	34	63	32	1	14.5 ± 0.2		135
2281	+	48	86	99	1	16.1 ± 0.0	BJ2	
Mean ± S.D.		43 ± 3	93 ± 6			14.7 ± 0.3		
Subgroup 2								
T2	+	38	50	83	2	12.0 ± 0.6	BJ2	123-J5033
T22	+	35	59	91	2	20.4 ± 1.2		123-J5033
USDA123	+	23[b]	42[b]		2[c]	10.7 ± 0.0	BJ2	123
Mean ± S.D.		37 ± 1	55 ± 5			13.6 ± 1.3		
Group C: non-HRS strain with IS*1631*								
YCK295	+	5	6	1	2	17.59 ± 0.3	BJ2	
YCK297	+	6	9	5	2			
YCK334	+	6	9	4	2			
NA6545	+	6	7	2	1	10.7 ± 0.2	BJ1	
NA6059	+	0	0	3	1		BJ2	
NA6062	+	0	0	2	1		BJ2	
NA6090	+	0	0	2	1		BJ2	
61A124a	+	8	10	3	2	18.7 ± 0.2		
Mean ± S.D.		6 ± 3	8 ± 4			15.6 ± 1.6		
Group D: non-HRS strains without IS*1631*								
YCK225	–	4	4	0	1	11.4 ± 0.3		
NK2	–	13	7	0	1	8.5 ± 0.1	BJ1	122-129
NK8	–	18	6	0	1			110
NC4a	–	7	6	0	1	8.3 ± 0.0		122-129
NC6a	–	12	5	0	1			110
T7	–	19	3	0	2	8.5 ± 0.0	BJ2	J5033
T9	–	17	3	0	2			123-129-J5033
Mean ± S.D.		11 ± 3	5 ± 1			9.0 ± 0.5		
Group E: reference strains to estimate copy number of IS elements								
USDA110	–	12	6	0	1	9.7 ± 0.3	BJ1	
USDA83	+	2	4	4	1			

[a]Serotypes determined previously [4].
[b]Determined previously [4].
[c]Data from Ham et al. [11].

3.5. *Correspondence of copy numbers of IS elements to phylogenetic cluster, serogroup, and generation time*

The grouping of Bradyrhizobia, characterized by copy numbers of IS elements, is shown in Table 2. The grouping of strains with regards to HRS groups (A or B) showed good correspondence between phylogenetic position, serogroup, and generation time (Table 2, and summarized in Fig. 3). The strains in HRS group A have higher copy numbers of RSα, belong to phylogenetic cluster BJ1, and are in serogroups 110 and 122. On the other hand, strains in HRS group B, having higher copy numbers of RSβ, belong to phylogenetic cluster BJ2, and correspond to serotypes 135 and 123 that are in subgroups 1 and 2, respectively.

Table 2 also indicates that group A HRS strains grew very slowly (mean generation time ± S.D., 27.1 ± 8.7 h) in HM medium. The HRS group B strains and those harbor-

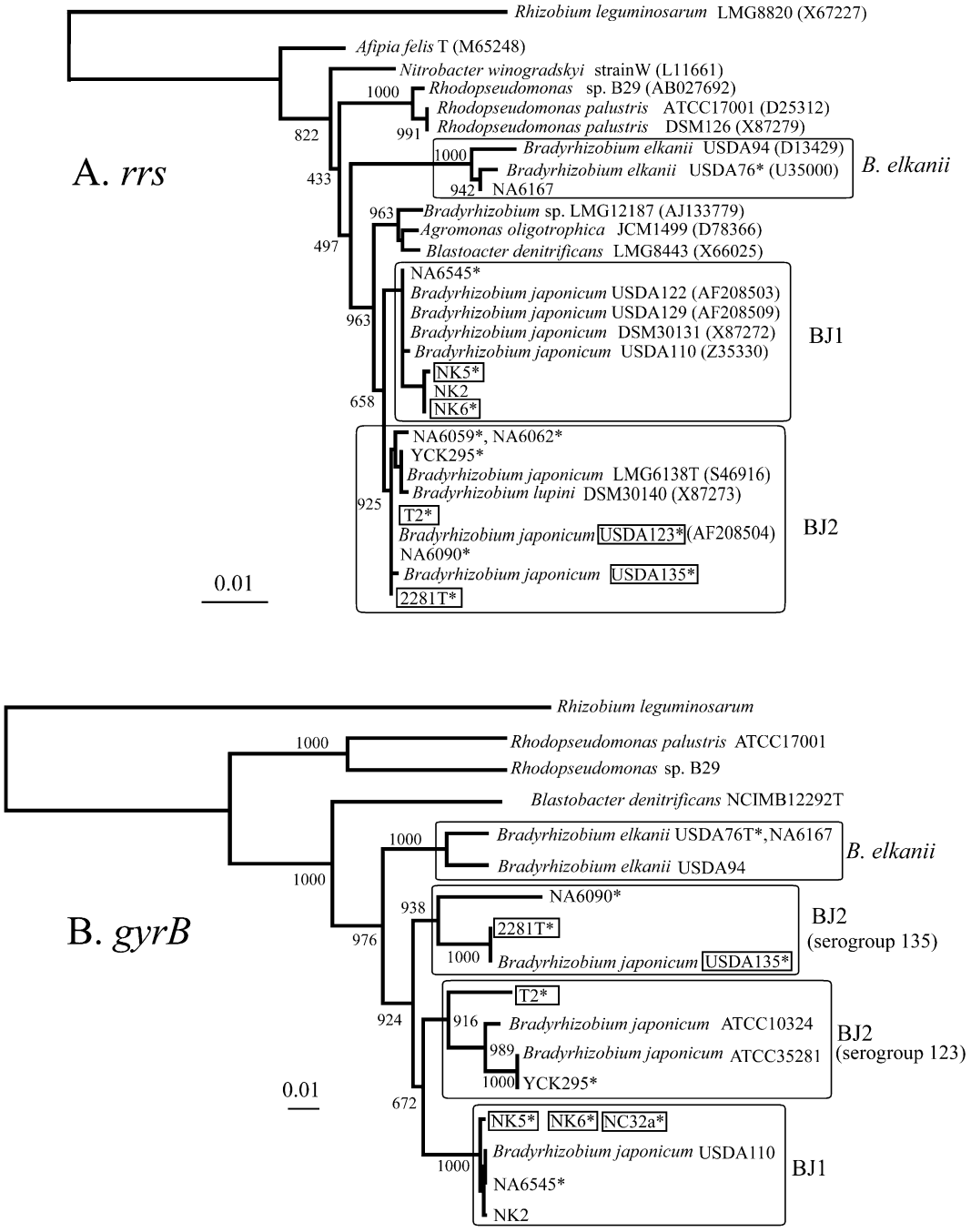

Fig. 2. Phylogenetic tree showing relatedness of *Bradyrhizobium* spp. strains and their relatives in α-Proteobacteria by neighbor-joining grouping of the aligned sequences of the *rrs* (A) and *gyrB* (B) genes. Bootstrap values are shown at nodes. Asterisk and closed box show IS*1631*-possessing *Bradyrhizobium* strains, and HRS strains possessing high copy numbers of RSα and RSβ, respectively (Table 2). Bar indicates 0.01 base substitutions per nucleotide. Accession numbers of reference bacteria are shown in parentheses. *Rhodopseudomonas* sp. strain B29 is photosynthetic nitrogen-fixing bacteria from rice [25].

ing IS*1631* also showed slower growth (13.6∼15.6 h) than the reference strains without IS*1631* (9.0±0.5 h) in HM medium. To test whether these traits are true in other media, we compared growth curves between HRS and non-HRS strains in conventional YM and TY media. The growth rates of HRS strains (USDA123, T2, USDA135, 2281, NK6 and NC32a) in YM medium were apparently lower than those of non-HRS strains (NK2 and T7) as well (Fig. 3). In particular, HRS strains of group A and B subgroup 1 showed very weak growth in YM medium. When the representative strains of group A and non-HRS strains were inoculated to rich TY medium, the two strains of group A (NK6 and NC32a) did not grow at all, although non-HRS strains (NK2 and NC4a)

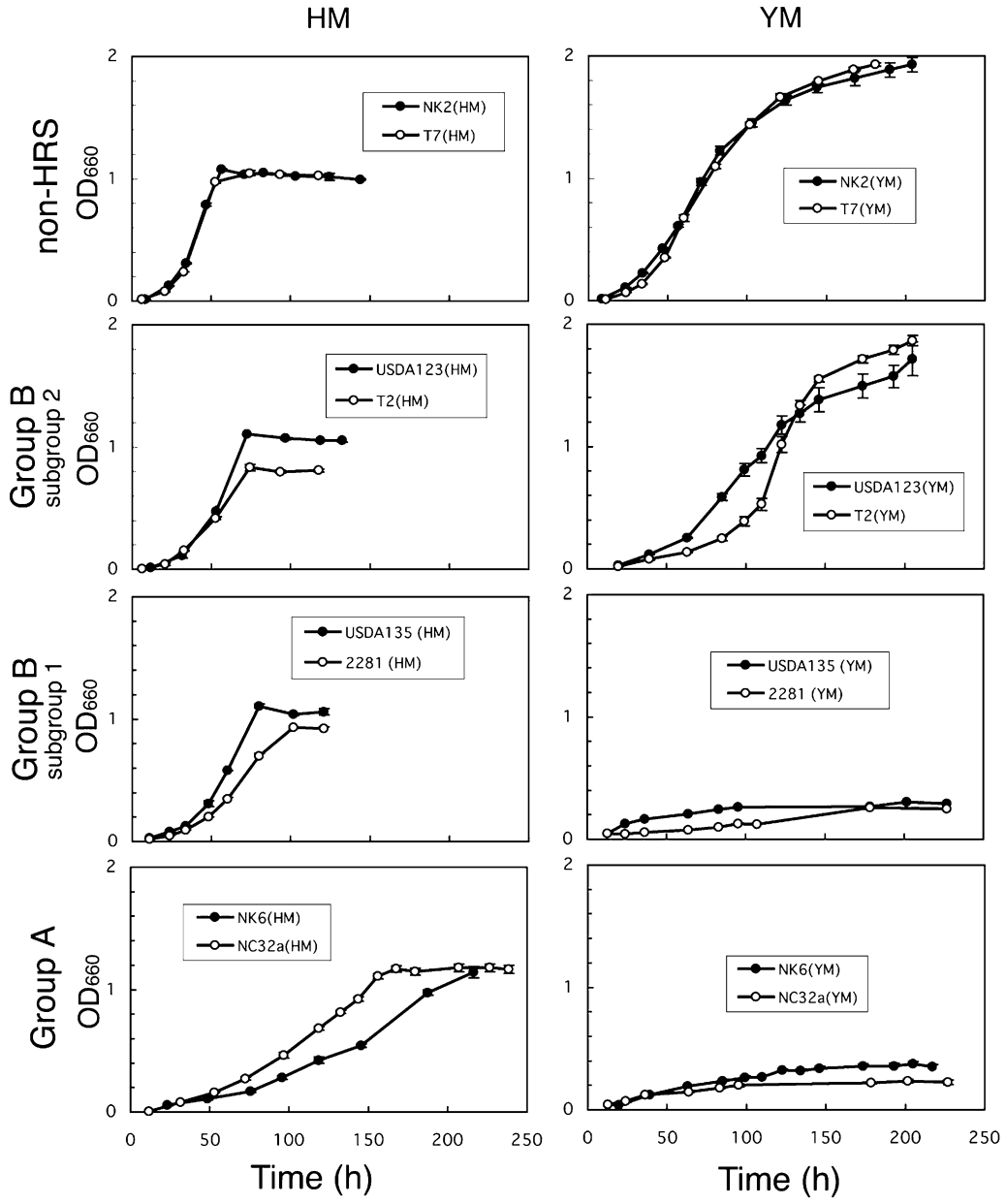

Fig. 3. Growth curves of representative HRS and non-HRS strains in HM and YM media. Strains NK2 and T7 were used as non-HRS strains. HRS strains were examined for each group (Table 2): Group B subgroup 2: USDA123 and T2; Group B subgroup 1: USDA135 and 2281; Group A: NK6 and NC32a.

grew normally (data not shown). Group A HRS strains, possessing high copy numbers of RSα, consistently showed extra-slow growth in three media tested, although growth characteristics of group B HRS strains depended on the media to some extent. Thus, the slow growth of HRS strains was a general feature rather than lack of catabolism of specific substrates as carbon and energy sources.

Moreover, the correlation of generation time in HM medium with RSα copy numbers were observed by simple linear regression analysis, although there was no significant correlation between generation times and copy numbers of RSβ and IS*1631* (Table 3).

4. Discussion

We previously showed that HRS strains of *B. japonicum* isolated in Japan had slow-growing phenotypes in culture [5,7]. Since ESG soybean Bradyrhizobia from Iowa [11,12], Nebraska [13] and China [14] have also been reported, we wanted to address whether these Bradyrhizobia fell into the category of HRS strains, with high copy numbers of RSα and RSβ. Results of the IS copy number studies reported here clearly indicate that these Bradyrhizobia belonged to group B of HRS strains (Table 2, Fig. 4). Similarly, Judd and Sadowsky [30] isolated a serocluster 123-specific hyperreiterated DNA region, HRS1, which

Table 3
Correlation of copy numbers of IS elements and generation time

	(1) RS	(2) RS	(3) IS*1631*	(4) Generation time
(1) Copy number of RS	1			
(2) Copy number of RS	0.13	1		
(3) Copy number of IS*1631*	0.17	0.66*	1	
(4) Generation time (h)	0.81*	0.04	0.10	1

Correlation matrix was examined from data of 22 strains from Table 2 by simple linear regression analysis. Figures followed by asterisk indicate statistical significance ($P < 0.01$).

corresponds to ISB20 in HRS strain T2 from Tokachi, Hokkaido, Japan [3]. This IS element is reiterated in the ESG, alkaline-tolerant strain USDA135, as well as in other members of serocluster 123 [15]. While, different IS probes have been used to characterize *B. japonicum* strains in the USA and Japan, this work consistently showed that ESG soybean Bradyrhizobia from USA, Japan and China fell into the category of HRS strains. Moreover, results of our studies show that they were clearly classified into two groups (A and B) in terms of IS copy number, serogroup and phylogeny (Table 2, Fig. 4). In this work, HRS strains of *B. japonicum* were not found in soybean Bradyrhizobia from Korea and Thailand, suggesting that the generation and adaptation of HRS strains might be dependent on local soil conditions and soybean cultivation. This idea is further strengthened by our observations that group A HRS strains occupy soybean nodules with a frequency over 50% in water-lodged soils during the field of paddy-upland rotation in Japan [3,7] and HRS strains of serogroup 135 are more frequently isolated from alkaline soils [11–13]. These soil conditions may be required for the generation and adaptation of HRS strains in the field.

HRS strains of *B. japonicum* provide an insight to bacterial evolution with respect to generation of genetic diversity in soil and symbiotic bacteria. Since IS copy number generally increases bacterial evolution [31], HRS strains have been probably generated from non-HRS strains by transposition and recombination events [4]. The presence and correlation of HRS strains with phylogeny and serocluster (Fig. 2, Fig. 4) suggest that generation of HRS strains occurred after the strains diverged into each phylogenetic position or serogroup.

Recently, whole structures of many bacterial genomes have been rapidly published. It is likely that amounts of IS elements on bacterial genomes are associated with genome structure instability, in particular gene orders within operons [32]. IS element often shuffle operon (and/or genome) structures, and fluctuate gene expression during evolution [31,33,34]. Thus, the presence of a high copy number of IS elements in HRS strains may affect several bacterial functions. The correlation of extra-slow growth with IS copy numbers (Table 3, Fig. 3) suggests that genes required for growth in culture may be impaired by large amounts of IS elements although the complex mechanisms of slow growth rate are not yet understood [35]. The slow growth of HRS strains is not due to a simple loss of specific carbon utilization abilities, because the HRS strains grew slower than non-HRS strains in the media tested (Table 2, Fig. 3).

HRS strains have been isolated from normal nodules of

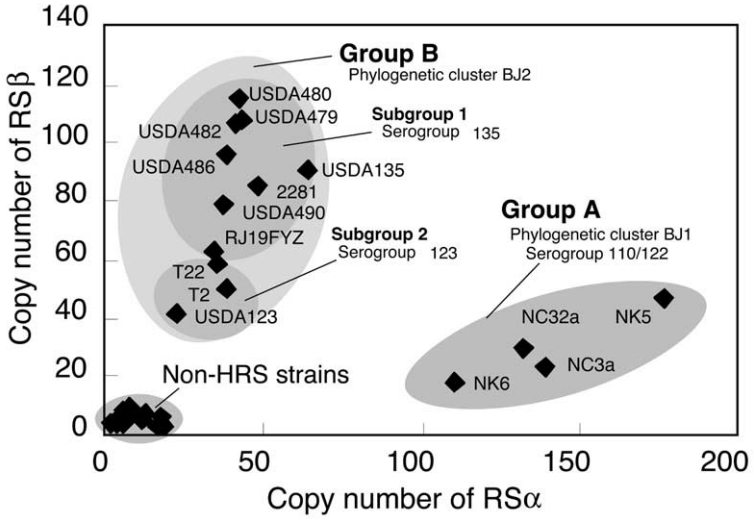

Fig. 4. Schematic presentation of relatedness of *B. japonicum* HRS strains between RSα and RSβ copy numbers, phylogenetic cluster, and serogroup. According to these features, HRS strains of soybean Bradyrhizobia were classified into groups A and B. Group A HRS strains occupy soybean nodules with a frequency over 50% in water-lodged soils during the field of paddy-upland rotation in Japan [3,7]. In group B, HRS strains of serogroup 135 are more frequently isolated from alkaline soils in USA [11–13]. Group B HRS strains are found in Japan, China as well as USA (see text).

field-grown soybeans [4,5,7]. *Macroptilium atropurpureum* was well nodulated with ESG strain NK5 (Group A HRS strain), and the nodulated legumes normally grew without nitrogen supply in a experiment of horizontal gene transfer [6]. Thus, the symbiotic phenotypes of HRS strains are probably similar to that of non-HRS strains. Because of the slow growth of HRS strains in YM medium commonly used for rhizobial works (Fig. 3), the existence of HRS strains have been probably overlooked during isolation processes of *Bradyrhizobium* spp. in the environments.

Interestingly, while *B. japonicum* is generally thought to have the ability to denitrify from nitrate to N_2 under anaerobic conditions [36,37], the group B HRS strains USDA135 and USDA123 have completely or partially lost the ability to denitrify, respectively [36,37]. Thus, other phenotypes of HRS strains are also different from those of 'classical' strains of *B. japonicum*. Taken together, our results suggest that HRS strains might provide an important resource for phenotypic diversity to explore desired features in *B. japonicum* without genetically engineered Bradyrhizobia.

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