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Genetic Grouping of *Bradyrhizobium* Strains Compatible with Soybean (*Glycine max* L. Merr.) Harboring *Rj*–gene by AFLP–fingerprinting Analysis

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Soybean (*Glycine max* L. Merr.) cultivars form nodules on roots by infection with *Bradyrhizobium* strains. However, soybean cultivars harbor nodulation conditioning genes, Rj_2, Rj_3 and Rj_4 form ineffective nodules with some strains of *Bradyrhizobium.* These strains were classified into nodulation–types A, B and C through their compatibility with *Rj*–gene. The type B was incompatible with Rj_2Rj_3–cultivars while the type C was incompatible with Rj_4–ones. The type A was compatible with both Rj_2Rj_3– and Rj_4–cultivars. In this study, in order to examine the relationship between phenotype (nodulation type) and genotype of *Bradyrhizobium* strains for nodulation, genotyping of *Bradyrhizobium* strains of each nodulation type was performed and classified by AFLP (Amplified Fragment Length Polymorphism) fingerprinting. On this dendrogram, two clusters could be distinguished at a similarity level of 77%, and the cluster I was divided into 4 sub–clusters. The sub–cluster Ia had types A and B, and the sub–cluster Ib had types A, B and C. The cluster Ⅱ had types A and C. These results were similar to that of a RFLP analysis performed in our laboratory. Thus, *Bradyrhizobium* strains of each nodulation type unformed a specific cluster. However, types A and B formed one sub–cluster, and types A and C did another cluster. These findings indicated that type B was distantly related to type C in regard to the structure of genomic DNA, while type A located in an intermediate position between type B and type C.

Keywords: AFLP–fingerprinting, *Bradyrhizobium*, genetic grouping, *Ri*–gene, soybean

INTRODUCTION

Some soybean cultivars harboring *Rj*–gene(s) can nodulate selectively some specific serotypical strains of *Bradyrhizobium japonicum* and *B. elkanii* (Ishizuka *et al*., 1991a; 1991b; 1993a; 1993b; Saeki *et al*., 1999; Yamakawa *et al*., 1999). Compatibility between soybean and rhizobium might be determined by an antigenic determinant existing on the surface of the rhizobium. The relationship between the structure of the antigenic determinant and the compatibility has not been reported at present, but the structure of the lipopolysaccharide (LPS) containing *O*–antigen was reported from a study using *Rhizobium phaseoli* Tn*5* mutant that might involve successful gram–negative interactions with plant (Carlson *et al*., 1987). Thus, we thought that it was possible to clarify the genomic characteristics concerning to the compatibility of rhizobia with *Rj*–genotypic soybean cultivars by the genomic analysis. To present, genomic analyses of rhizobia were carried out using divers methods, and the classification was based on the family and species (Young *et al*., 1991; Young, 1992; Yanagi and Yamamoto, 1993), the compatibility between rhizobia and host plant species (Bjourson *et al*., 1992) and the analysis about the hereditary character among some serotype of rhizobia (Judd *et al*., 1993; Berkum and Fuhrmann, 2001; Saeki *et al*., 2004). However, study dealing with the relationship between the compatibility of soybeans harboring *Rj*–genes with *Bradyrhizobium* strains and the genomic structure of their strains were few (Saeki *et al*., 2000; Yamakawa and Eriguchi, 2005).

The compatibility between legumes and rhizobia (host specificity) underlies on the expressional responses of genes involved in rhizobial strains and species of host legumes (Lohrke *et al*., 1998; Stacey 1995; van Rhijin and Vanderleyden, 1995). However, it was unclear until now that which gene(s) exiting in *Bradyrhizobium* was concerned to the compatibility (*Rj*–genotype specificity) with *Rj*–genotype of soybean. The gene(s) determining *Rj*–genotype specificity was thought to be different from species–specific genes which have been reported until now, namely *nodD1, nodD2, nodY, nodS, nodU* and *nodZ* found in *Bradyrhizobium japonicum, B. elkanii* and *R. fredii* (van Rhijin and Vanderleyden, 1995). In other words, although there must be some relationships between the formation of effective nodule and several biochemical processes functioning in rhizobial cells, the genes related to *Rj*–genotype specificity were not clearly investigated. Therefore, the grouping of *Bradyrhizobium*

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strains based on their compatibility with soybeans harboring *Rj*–gene(s) could be impossible based on the utilization of the information on DNA sequences of the known rhizobial genes involved in the nodule formation. However, it was thought that some gene(s) involved in the compatibility between an *Rj*–genotype of soybean and some specific *Bradyrhizobium* strains could be made possible grouping tools into each nodulation type, by presuming the different sequences of putative *Rj–genotype specific nodulation* (*Rj–gsn*) gene reserved in the rhizobial strain (Tsurumaru *et al*., 2008).

Bacterial species are defined using a range of techniques, but since the work of Woese (1987) was published, there has been an interesting reliance on 16S rRNA gene sequence data to identify and classify bacteria. However, some evidences of gene transfer between species (Janssen *et al*., 1996; Sullivan *et al*., 1996; Eardly *et al*., 1996) indicated that 16S rRNA gene based phylogeny of rhizobial could be misleading. The 16S–23S rRNA intergenic gene spacer (IGS) sequence exhibits higher variability and discriminating power and has been used to identify genomic groups at the intraspecific level including various *Bradyrhizobium* strains (Doignon–Bourcier *et al*., 2000; Saeki *et al*., 2004). The amplification fragment length polymorphism (AFLP) technique (Vos *et al*., 1995) is a highly discriminating fingerprinting method, based on the selective PCR amplification of certain restriction fragments from a digested of total genomic DNA. This technique has been used to characterize various bacterial species (Aarts *et al*., 1998; Blears *et al*., 1998; Savelkoul *et al*., 1999; On *et al*., 2000). The AFLP analysis was compared to other techniques for the classification of *Bradyrhizobum* species, and a strong correlation between result obtained with AFLP and DNA–DNA hybridization (Jenssen *et al*., 1996; Willems *et al*., 2000; 2001) was reported. Recently, AFLP technique has been applied frequently to clarify the genotypic characterization and genomic diversity of *Rhizobiaceae* using the whole genome and shown to be the most discriminative one (Wolde–meskel *et al*., 2004; Chen *et al*., 2003; Gao *et al*., 2001; Terefework *et al*., 2001; Doignon–Bourcier *et al*., 2000).

The aim of the present work was to apply AFLP technique to the genomic grouping based on the sequence of genomic DNA of *Bradyrhizobium* strains concerned with the compatibility with soybeans harboring *Rj*–gene(s).

MATERIALS AND METHODS

Rhizobial strains and genomic DNA isolation

The rhizobial strains and their nodulation types used in this study are listed in Table 1. They were maintained on YMA (Vincent, 1970) plates at 4°C. *Sinorhizobium meliloti* MAFF303039, *Rhizobium sp*. Vigna MAFF303063 and *R. leguminosarum*. bv. *phaseoli* MAFF303035 were used as references without particular nodulation type. *Bradyrhizobium* or *Sinorhizobium/Rhizobium* strains were grown in HM liquid medium (Kuykendall, 1987) at 28°C, 100 rpm for 7 d or 5 d, respectively and harvested by centrifugation at 8,000 × g for 10 min at 4°C. The cell pellet was suspended in 189 μL of TE–buffer and lysed for 1 hr at 37°C by addition of 10 μL of 10% SDS solution and 1 μL of 20 mg mL^{-1} of proteinase K solution. From this lysate, genomic DNA isolation was carried out with IsoQuick according to the protocol (ORCA Research, Inc.) followed by RNase A (final concentration: 66 μg mL^{-1}) treatment for 30 min at 37°C, phenol chloroform extraction and ethanol precipitation (Moore, 1994).

Table 1. Nodulation types and sources of rhizobium strains

Nodulation type	Rhizobium species[a]	Strain(s)	Source[b]
A	*B. japonicum*	Is–2, Is–11, Is–17, Is–29, Is–35, Is–41 Is–55, Is–77, Is–80	1
	B. elkanii	USDA110	3
		USDA76	3
B	*B. japonicum*	Is–1, Is–66, Is–74, Is–76, Is–79,	1
		A1017	2
		USDA6, USDA122	3
	B. elkanii	USDA31, USDA33	3
C	*B. japonicum*	Is–21, Is–34, Is–61, Is–111	1
	B. elkanii	USDA61	3
–	*R. leguminosarum* bv. *Phaseoli*	MAFF303035	2
–	*R.* sp. Vigna	MAFF303063	2
–	*S. meliloti*	MAFF303039	2

[a] Rhizobium species; *B.: Bradyrhizobium, R.: Rhizobium, S.: Shinorhizobium.* [b] Source; 1, Isolates from the nodules of soybean plant grown in the field of the National Institute of Agricurtural Sciences (NIAS), Tsukuba. 2, Culture collection of Ministry of Agriculture, Foresty and Fisheries (MAFF). 3, Culture collection of United States. Department of Agriculture (USDA). Nodulation types of *B. japonicum* and *B. elkanii* strains were identified by the method of Ishizuka *et al*. (1991a).

AFLP fingerprinting

AFLP fingerprinting was carried out according to AFLP™ Microbial Fingerprinting Protocol (Applied Biosystems, USA). Enzyme master mix for restriction–ligation reaction was prepared as the following: 10 μL of 10 × T4 DNA ligase buffer with ATP (50 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg mL^{-1} bovine serum albumin), 10 μL of 0.5 M NaCl, 100 units of MseI, 500 units of EcoRI, 100 units of T4 DNA Ligase and sterile distilled water added to bring the total volume to 100 μL.

The restriction–ligation was carried out in a sterile 0.5–mL microcentrifuge tube. A 11 μL volume of restriction–ligation mixture containing 0.01 μg per 5.5 μL of genomic DNA, 1.0 μL of 10 × T4 DNA ligase buffer with ATP, 1.0 μL of 0.5 M NaCl, 0.5 μL of 1.0 mg mL^{-1} BSA, 1.0 μL of MseI adaptor, 1.0 μL of EcoRI adaptor, 1.0 μL of enzyme master mix was incubated for 2 h at 37°C, and diluted by addition of 189 μL of $TE_{0.1}$ buffer (20 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) to each restriction–ligation reaction. These restriction–ligation samples were stored at −20°C.

For pre–selective amplification, 4.0 μL of restriction–ligation sample were used as template DNA in 20 μL of reaction mixture containing 0.5 μL of AFLP EcoRI preselective primer, 0.5 μL of AFLP MseI preselective primer, 15.0 μL of AFLP Amplification Core Mix. A volume of 20 μL of mineral oil was layered over the mixture which was then stored at 4°C. The PCR reactions were performed on the thermal cycler (Program Temp Control System PC–800, ASPEC) using the following cycle profile, cycle 1: 2 min at 72°C; cycle 2–21: 20 sec at 94°C, 30 sec at 56°C and 2 min at 72°C and kept at 4°C.

For selective amplification, the 10.0 μL of preselective amplification product was diluted by 190 μL of $TE_{0.1}$ buffer. A 3 μL volume from the resulting dilution was used as template DNA in 20 μL of reaction mixture containing 1 μL of MseI–0 primer (5 μM), 1 μL of dye–labeled EcoRI–0 primer (1 μM), 15 μL of AFLP core mix. The PCR reactions were performed on the thermal cycler using the following cycle profile, cycle 1: 20 sec at 94°C, 30 sec at 66°C and 2 min at 72°C, cycle 2–10: 20 sec at 94°C, 30 sec at 65°C and 2 min at 72°C decreasing the annealing temperature of 1°C each cycle, cycle 11–30: 20 sec at 94°C, 30 sec at 56°C and 2 min at72°C, cycle 31: 30 min at 60°C and kept at 4°C.

For loading and electrophoresis on the ABI PRISM 310, the loading sample was prepared by mixing 1.0 μL of selective amplification product, 24.0 μL of deionized formamide and 1.0 μL of GeneScan–500 [ROX] size standard, heating at 95°C for 5 min and chilling quickly on ice. An electrophoresis of the loading sample was carried out on ABI PRISM 310 Genetic Analyzer (Applied Biosystem, USA) equipped with capillary column.

Numerical analysis of banding patterns

After electrophoresis, data on the peak height and the fragment size of selective PCR products were collected by GeneScan 2.1 (Applied Biosystems, USA) soft ware. In the detected fragments, fragments of more than 5% of the maximum peak height and 50 to 400 bp in the size were selected. After the size of PCR products measured by capillary chromatograph was imported into the personal computer installed Microsoft Excel 2001 software (Microsoft Co. JAPAN) which was a spreadsheet application, the number of common bands between pair of each rhizobium strains based on AFLP pattern was analyzed by the basic function (COUNT IF). The proportion of the selected fragments common to the strains compared (F_{xy}) was calculated by using the formula $F_{xy}=2n_{xy}/(n_x+n_y)$ proposed by Nei and Li (1979) in which nx and ny are the total number of selected fragments observed in the strains x and y, respectively and nxy is the number of selected fragments shared by them. The distance matrix (D) corresponding to the genetic distance was calculated as follow; $D=1-F_{xy}$. A dendrogram was constructed from D using the unweighted pair–group method algorithm (UPGMA) contained in the computer program Phylip 3.3 (developed by J. Felsenstein, Biology Department, Indiana University, in 1990).

RESULTS AND DISCUSSION

In the present study, the AFLP technique was based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. This technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity (Vos *et al.*, 1995). In several reports, the AFLP technique was used to study the overall genomic diversity of closely related rhizobia and was described as a good tool for distinguishing rhizobial strains belonging to many different species and genera (van Berkum and Fuhrmann, 2001; Wolde–meskel *et al.*, 2004: Biondi *et al.*, 2003; Doignon–Bourcier *et al.*, 2000; Chen *et al.*, 2003; Terefework *et al.*, 2001). Therefore, in the AFLP technique, the identification of the fragment length is thought to be done without problem. However the amplification based on PCR of the fragment digested by restriction enzymes was not always done in the same way as described in this work.

The amplified fragments by a specific PCR were separated and detected by ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). This system allows analyzing the fragment size and strength by a capillary electrophoresis using a size marker mixed with the PCR product. In this system, the fragment of 50–500 bp size can be detected, but the amplification of the fragment beyond 400 bp was unstable and extremely decreased less than the number of fragments expected from the combination of specific primers used to adjust the number of amplification fragments to 50–100 ones in *E. coli*. Because of this reason, the DNA of *Bradyrhizobium* strains was used as the target of this study. Also, a possible reason for this incongruence could be that the digestion by the restriction enzymes was insufficient. However, the reproducibility of the system was not observed even when the same digestion product was used as a template for amplification. Therefore, it is not clear whether this cause is related to a technical or machine problem. However, the

amount of mixture of the sample and the amount of primer seem to be fixed, but the mean of the fluorescent strength is greatly different for every experiment, influencing the number of bands and the multiple pattern frequency.

So, we compared with the AFLP analysis results of 4–8 replicates about the same DNA extraction of each strain as shown as an example of Is–1 in Table 2, and the only AFLP pattern (rep–6 in the case of this strain, Is–1) detected in larger than 90% similarity and which counted most number of fragments (Table 2 and Table 3) was selected as the representative one within those replicates. The AFLP patterns of all strains selected as above–mentioned method were compared with each other and the genetic distance line (Table 4, in upper light side) of each strain was calculated.

As for these results, a dendrogram (Fig. 1) was constructed by the UPGMA cluster analysis from the comparison of AFLP patterns of each strain shown in Table 1, based on the genetic matrix of Table 5 calculated from data of Table 4. The phylogenic tree (Fig. 1) was divided into cluster I and Ⅱ at a similarity level of 77%. Furthermore, the cluster I was separated into 4 sub–clusters. The subclusters Ia, Ib and cluster Ⅱ are characteristics in terms of nodulation type of the strains. The sub–cluster Ia was occupied with nodulation type A, type B and type C as 25% 75% and 0%, respectively. The sub–cluster Ib contained 38%, 50% and 12% of nodulation type A, type B and type C, respectively. However, in the cluster Ⅱ, the presence rates of the nodulation type A, type B and type C were 62%, 0% and 38%, respectively.

This result appears to be similar with the result analyzed by the RFLP fingerprinting method in our labora-

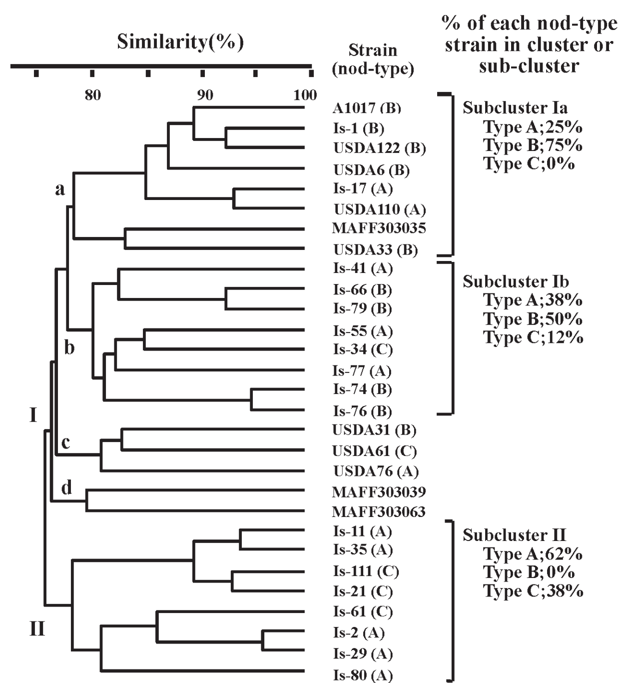

Fig. 1. Dendrogram constructed by the UPGMA cluster analysis from AFLP patterns. I: cluster I; Ⅱ: cluster Ⅱ; a, b, c and d are sub–clusters Ia, Ib, Ic and Id, respectively.

Table 2. The total number of peaks and the number of comigrating bands between replication of *Bradyrhizobium japonicum* Is–1 based on AFLP analysis

Replication	Number of peak	Rep–1	Rep–2	Rep–3	Rep–4	Rep–5	Rep–6	Rep–7	Rep–8
Rep–1	45		84	85	84	94	89	94	81
Rep–2	39	25		79	78	88	83	88	75
Rep–3	40	35	26		79	89	84	89	76
Rep–4	39	32	27	31		88	83	88	75
Rep–5	49	31	36	30	34		93	98	85
Rep–6	44	34	30	32	38	35		93	80
Rep–7	49	29	33	30	32	43	33		85
Rep–8	36	27	29	27	28	33	30	33	

Numbers above the diagonal represent the total number (n_x+n_y)of bands between pair of replication (x and y). Numbers below the diagonal represent the number of shared fragments (n_{xy}) estimated as the number of common bands.

Table 3. Similarity between replication data of Is–1 based on AFLP analysis

Replication	Rep–1	Rep–2	Rep–3	Rep–4	Rep–5	Rep–6	Rep–7	Rep–8
Rep–1		0.6	0.82	0.76	0.66	0.76	0.62	0.67
Rep–2			0.66	0.69	0.82	0.72	0.75	0.77
Rep–3				0.78	0.67	0.76	0.67	0.71
Rep–4					0.77	0.92	0.73	0.75
Rep–5						0.75	0.88	0.78
Rep–6							0.71	0.75
Rep–7								0.78
Rep–8								

Similarity was callculated from $n_{xy}/(n_x+n_y)$ based on data of Table 2.

Table 4. The total number of peaks and the number of comigrating bands between pair of each rhizobium strains based on AFLP analysis

Strain	Number of peak	Nod–type	Is–2	Is–11	Is–17	Is–29	Is–35	Is–41	Is–55	Is–77	Is–80	USDA 76	USDA 110	A1017	Is–1	Is–66	Is–74	Is–76	Is–79	USDA 6	USDA 31	USDA 33	USDA 122	Is–21	Is–34	Is–61	Is–111	USDA 61	MAFF 303035	MAFF 303039	MAFF 303063
Is–2	44	A		106	109	91	114	106	109	107	73	93	108	125	103	106	109	127	99	105	127	137	109	104	103	95	112	105	139	110	114
Is–11	62	A	54		127	109	132	124	127	125	91	111	126	143	121	124	127	145	117	123	145	155	127	122	121	113	130	123	157	128	132
Is–17	65	A	52	66		112	135	127	130	128	94	114	129	146	124	127	130	148	120	126	148	158	130	125	124	116	133	126	160	131	135
Is–29	47	A	64	60	58		117	109	112	110	76	96	111	128	106	109	112	130	102	108	130	140	112	107	106	98	115	108	142	113	117
Is–35	70	A	56	116	74	62		132	135	133	99	119	134	151	129	132	135	153	125	131	153	163	135	130	129	121	138	131	165	136	140
Is–41	62	A	52	62	70	54	68		127	125	91	111	126	143	121	124	127	145	117	123	145	155	127	122	121	113	130	123	157	128	132
Is–55	65	A	58	72	80	56	80	88		128	94	114	129	146	124	127	130	148	120	126	148	158	130	125	124	116	133	126	160	131	135
Is–77	63	A	66	78	72	70	90	72	84		92	112	127	144	122	125	128	146	118	124	146	156	128	123	122	114	131	124	158	129	133
Is–80	29	A	50	46	34	50	46	36	40	54		78	93	110	88	91	94	112	84	90	112	122	94	89	88	80	97	90	124	95	99
USDA76	49	A	42	54	56	42	62	50	56	56	34		113	130	108	111	114	132	104	110	132	142	114	109	108	100	117	110	144	115	119
USDA110	64	A	48	70	112	54	74	66	76	66	30	56		145	123	126	129	147	119	125	147	157	129	124	123	115	132	125	159	130	134
A1017	81	B	60	76	82	66	80	84	96	86	44	72	82		140	143	146	164	136	142	164	174	146	141	140	132	149	142	176	147	151
Is–1	59	B	52	58	88	54	64	62	70	66	32	54	94	82		121	124	142	114	120	142	152	124	119	118	110	127	120	154	125	129
Is–66	62	B	52	68	88	56	72	68	66	68	38	48	88	78	96		127	145	117	123	145	155	127	122	121	113	130	123	157	128	132
Is–74	65	B	52	68	64	52	80	84	88	78	38	56	62	82	62	64		148	120	126	148	158	130	125	124	116	133	126	160	131	135
Is–76	83	B	64	78	86	70	86	88	92	84	42	72	84	148	86	88	80		138	144	166	176	148	143	142	134	151	144	178	149	153
Is–79	55	B	54	64	64	54	68	74	82	72	42	56	62	78	58	60	102	70		116	138	148	120	115	114	106	123	116	150	121	125
USDA6	61	B	62	76	86	62	82	72	76	82	48	54	82	92	86	98	68	98	64		144	154	126	121	120	112	129	122	156	127	131
USDA31	83	B	60	74	88	64	80	76	86	70	42	78	84	98	78	80	84	100	74	76		176	148	143	142	134	151	144	178	149	153
USDA33	93	B	60	70	90	60	78	76	82	70	42	72	86	96	86	84	68	102	62	80	110		158	153	152	144	161	154	188	159	163
USDA122	65	B	48	60	90	50	64	64	72	64	32	48	92	84	98	108	64	88	58	90	80	82		125	124	116	133	126	160	131	135
Is–21	60	C	56	98	60	62	102	60	64	66	48	54	58	78	52	62	70	78	62	72	70	62	54		119	111	128	121	155	126	130
Is–34	59	C	52	64	76	50	72	72	86	76	36	56	74	88	76	64	72	90	62	76	76	76	64	58		110	127	120	154	125	129
Is–61	51	C	54	92	58	60	92	60	60	68	48	48	60	72	50	60	66	72	64	70	62	50	50	96	64		119	112	146	117	121
Is–111	68	C	80	80	82	84	86	74	82	82	48	62	80	98	76	74	82	98	72	82	90	82	72	76	84	70		129	163	134	138
USDA61	61	C	52	60	60	52	64	60	64	66	32	70	66	76	64	66	60	86	54	68	94	80	70	54	66	50	74		156	127	131
MAFF303035	95	–	68	92	92	70	94	80	94	88	46	76	96	114	88	92	78	116	78	92	104	124	90	78	84	76	94	86		161	165
MAFF303039	66	–	40	64	64	42	70	54	60	60	26	52	62	86	60	58	54	92	48	68	72	80	62	52	66	52	68	64	94		136
MAFF303063	70	–	50	62	74	54	68	60	74	70	36	66	74	90	76	74	64	88	62	72	80	88	60	58	76	54	78	70	100	80	

Numbers above the diagonal represent the total number ($n_x + n_y$)of bands between pair of *Bradyrhizobium* strains (x and y). Numbers below the diagonal represent the number of shared fragments (n_{xy}) estimated as the number of comigrating bands.

Table 5. Matrix of pairwise genetic distance between rhizobium strains based on AFLP analysis

Strain	Nod–type	Is–2	Is–11	Is–17	Is–29	Is–35	Is–41	Is–55	Is–77	Is–80	USDA 76	USDA 110	A1017	Is–1	Is–66	Is–74	Is–76	Is–79	USDA 6	USDA 31	USDA 33	USDA 122	Is–21	Is–34	Is–61	Is–111	USDA 61	MAFF 303035	MAFF 303039	MAFF 303063
Is–2	A		0.49	0.52	0.08	0.51	0.51	0.47	0.38	0.32	0.55	0.56	0.50	0.51	0.52	0.52	0.50	0.45	0.41	0.53	0.56	0.56	0.46	0.50	0.43	0.29	0.50	0.51	0.64	0.56
Is–11	A			0.48	0.45	0.12	0.50	0.43	0.38	0.49	0.51	0.44	0.52	0.45	0.46	0.47	0.46	0.45	0.38	0.49	0.55	0.53	0.20	0.47	0.19	0.38	0.51	0.41	0.50	0.53
Is–17	A				0.48	0.45	0.45	0.38	0.44	0.64	0.51	0.13	0.29	0.31	0.51	0.44	0.42	0.47	0.32	0.42	0.43	0.31	0.52	0.39	0.50	0.38	0.52	0.43	0.51	0.45
Is–29	A					0.47	0.50	0.50	0.36	0.34	0.56	0.51	0.49	0.49	0.54	0.48	0.46	0.47	0.43	0.51	0.57	0.55	0.42	0.53	0.39	0.27	0.52	0.51	0.63	0.54
Is–35	A						0.48	0.41	0.32	0.54	0.48	0.45	0.50	0.45	0.41	0.47	0.44	0.46	0.37	0.48	0.52	0.53	0.22	0.44	0.24	0.38	0.51	0.43	0.49	0.51
Is–41	A							0.31	0.42	0.60	0.55	0.48	0.49	0.45	0.34	0.41	0.39	0.37	0.41	0.48	0.51	0.50	0.51	0.40	0.47	0.43	0.51	0.49	0.58	0.55
Is–55	A								0.34	0.57	0.51	0.41	0.44	0.48	0.32	0.34	0.38	0.32	0.40	0.42	0.48	0.45	0.49	0.31	0.48	0.38	0.49	0.41	0.54	0.45
Is–77	A									0.41	0.50	0.48	0.46	0.46	0.39	0.40	0.42	0.39	0.34	0.52	0.55	0.50	0.46	0.38	0.40	0.37	0.47	0.44	0.53	0.47
Is–80	A										0.56	0.68	0.64	0.58	0.60	0.60	0.63	0.50	0.47	0.63	0.66	0.66	0.46	0.59	0.40	0.51	0.64	0.63	0.73	0.64
USDA76	A											0.50	0.50	0.57	0.51	0.45	0.45	0.46	0.51	0.41	0.49	0.58	0.50	0.48	0.52	0.47	0.36	0.47	0.55	0.45
USDA110	A												0.24	0.30	0.52	0.43	0.43	0.48	0.34	0.43	0.45	0.29	0.53	0.40	0.48	0.39	0.47	0.40	0.52	0.45
A1017	B													0.21	0.50	0.41	0.39	0.49	0.28	0.45	0.43	0.21	0.56	0.36	0.55	0.40	0.47	0.43	0.52	0.41
Is–1	B														0.50	0.45	0.39	0.49	0.20	0.45	0.46	0.15	0.49	0.47	0.47	0.43	0.46	0.41	0.55	0.44
Is–66	B															0.44	0.46	0.15	0.46	0.43	0.57	0.51	0.44	0.42	0.43	0.38	0.52	0.51	0.59	0.53
Is–74	B																0.10	0.43	0.35	0.40	0.45	0.42	0.45	0.37	0.45	0.34	0.46	0.35	0.41	0.40
Is–76	B																	0.49	0.32	0.40	0.42	0.41	0.45	0.37	0.46	0.35	0.40	0.35	0.38	0.42
Is–79	B																		0.45	0.46	0.58	0.52	0.46	0.46	0.40	0.41	0.53	0.48	0.60	0.50
USDA6	B																			0.47	0.48	0.29	0.40	0.37	0.38	0.36	0.44	0.41	0.46	0.45
USDA31	B																				0.38	0.46	0.51	0.46	0.54	0.40	0.35	0.42	0.52	0.48
USDA33	B																					0.48	0.59	0.50	0.65	0.49	0.48	0.34	0.50	0.46
USDA122	B																						0.57	0.48	0.57	0.46	0.44	0.44	0.53	0.41
Is–21	C																							0.51	0.14	0.41	0.55	0.50	0.59	0.55
Is–34	C																								0.45	0.34	0.45	0.45	0.47	0.41
Is–61	C																									0.41	0.54	0.48	0.56	0.55
Is–111	C																										0.43	0.42	0.49	0.43
USDA61	C																											0.45	0.50	0.47
MAFF303035	–																												0.42	0.39
MAFF303039	–																													0.41
MAFF303063	–																													

Data represent the genomic distances ($D = 1 - F_{xy}$) estimated from the F_{xy} values $\{F_{xy} = n_{xy}/(n_x + n_y)\}$ using each data shown in Table 2 by the method proposed by Nei and Li (1979).

tory (Yamakawa and Eriguchi, 2005). Because the genomic DNA of the strains used in this study was digested with the same restriction enzyme *Mse*I used during our RFLP analysis, the dendrogram obtained in this work might have showed almost same result as RFLP of previous paper (Yamakawa and Eriguchi, 2005). However, it could be speculated that the fingerprinting analysis with genomic DNA may lead to similar dendrograms even if it is made by any techniques. When the dendrogram (Fig. 1) was observed without referring to the relationship between strains and the nodulation type, it became clear that *B. elkanii* USDA31, USDA76 and USDA61 except for *B. elkanii* USDA33 were concentrated on the sub–cluster Ic. This result indicated that the genomic structures of *B. japonicum* and *B. elkanii* strains were largely different. Moreover, this clarified that AFLP analysis was a discriminative method to characterize species within the *Bradyrhizobium* genus.

The results of fingerprintings of AFLP and RFLP based on the genomic DNA sequence of *Bradyrhizobium* strains of each nodulation type showed that any nodulation type didn't form a specific cluster. However, the nodulation types A and B formed one sub–cluster, while types A and C formed another one. These findings indicated that the type B was distantly related to the type C in regard to the structure of genomic DNA, while the type A located in a intermediate position between type B and type C. Also, the compatibility between *Rj* genotype of soybean cultivars and nodulation type of *Bradyrhizobium* strains could be controlled by several genes scattered in the overall genome of *Bradyrizobium* strain and not by a specific gene region conserved in the genome as shown in a previous report (Turumaru *et al.*, 2008).

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