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ORIGINAL PAPER

Genetic diversity of rhizobia associated with *Vicia faba* in three ecological regions of China

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Abstract Great genetic diversity was revealed among 75 rhizobal isolates associated with *Vicia faba* grown in Chinese fields with AFLP, ARDRA, 16S rDNA sequencing, DNA–DNA hybridization, BOX-PCR and RFLP of PCR-amplified *nodD* and *nodC*. Most of the isolates were *Rhizobium leguminosarum*, and six isolates belonged to an unnamed *Rhizobium* species. In the homogeneity analysis, the isolates were grouped into three clusters corresponding to (1) autumn sowing (subtropical) region where the winter ecotype of *V. faba* was cultivated, (2) spring sowing (temperate) region where the spring ecotype was grown, and (3) Yunnan province where the intermediate ecotype was sown either in spring or in autumn. Nonrandom associations were found among the *nod* genotypes, genomic types and ecological regions, indicating an epidemic symbiotic gene transfer pattern among different genomic backgrounds within an ecological region and a relatively limited transfer pattern between different regions. Conclusively, the present results suggested an endemic population structure of *V. faba* rhizobia in Chinese fields and demonstrated a novel rhizobium associated with faba bean.

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Introduction

Faba bean (*Vicia faba L.*) is a leguminous crop cultivated in 57 countries around the world for as long as 6,000 years (Bond et al. 1985; Duke 1981; George 1999). Many botanical varieties have emerged in this species and they could be mainly categorized as winter and spring ecotypes (Ye 2003). *V. faba* forms nitrogen-fixing root nodules with *Rhizobium leguminosarum* bv. *viciae* (Jordan 1984). Previously, diversity of *V. faba* rhizobia has been investigated in various studies, and they were mainly for the rhizobial populations isolated from the same region (Laguerre et al. 1996, 2003) or for comparison with *R. leguminosarum* strains isolated from other legume species (Mutch and Young 2004; van Berkum et al. 1995). Comparison of rhizobial populations from different geographic regions was rarely studied (Mutch et al. 2003). There was no study of the rhizobia in association with faba bean ecotypes.

In the present study, we made a first comparative characterization of genomic and *nod* gene diversity of rhizobial populations associated with the spring, winter, and intermediate ecotypes (Ye 2003) of faba bean respectively in temperate region, subtropical region and plateau region in subtropics in China.

Vicia faba was introduced to China about 4,000–5,000 years ago according to archaeological remains (Bond 1985; Thompson and Kelly 1957; Ye 2003) and it has been cultivated in 25 Chinese provinces. The yield of dry faba bean in China covered about 46% of total world production in 2000–2002 (FAO). The faba bean producing area in China could be divided into two main ecological regions:

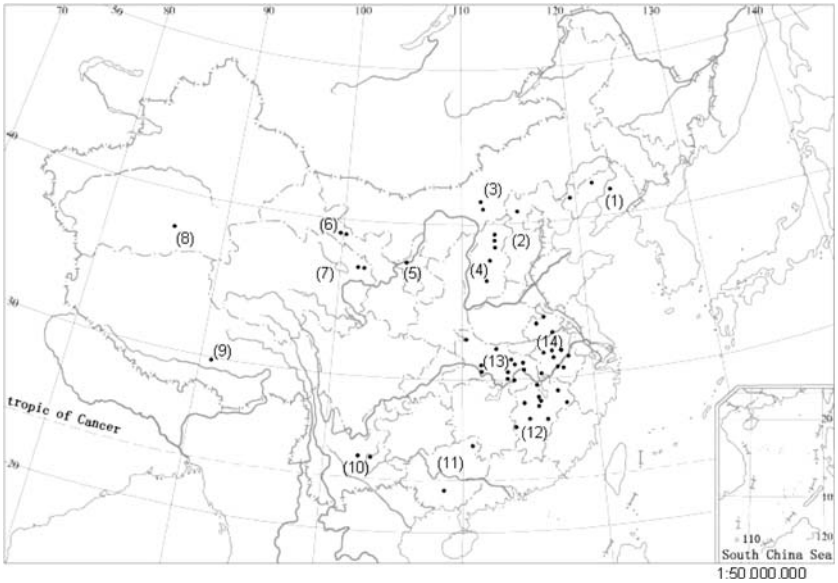

Fig. 1 Map of China showing the sampling sites. Sampling sites were marked with points. The *numbers in parenthesis* represent provinces of China: *(1)* Liaoning, *(2)* Hebei, *(3)* Inner Mongolia, *(4)* Shanxi, *(5)* Ningxia, *(6)* Gansu, *(7)* Qinghai, *(8)* Xinjiang and *(9)* Tibet are temperate provinces located in spring sowing region; *(10)* Yunan province is located in subtropical plateau region where intermediate ecotype of faba bean is sown either in autumn or spring; *(11)* Guangxi, *(12)* Jiangxi, *(13)* Hubei and *(14)* Anhui are subtropical provinces located in autumn sowing region

autumn sowing region where the faba bean belongs to winter ecotype and is sown in autumn; and spring sowing region where the spring ecotype faba bean is sown in spring (Ye 2003). In addition, Yunnan province was considered as an intermediate region where the intermediate ecotype of faba bean was sown in either spring or autumn (Ye 2003).

Since the successful spread of legume crops depends critically on their microsymbionts (Howieson et al. 2000; Lohrke et al. 1996; Mutch et al. 2004; Weir et al. 2004), diverse rhizobial communities associated with *V. faba* might have established in Chinese soils. Considering that the rhizobia of *V. faba* in Chinese soils have not been systematically studied and that the rhizobia-legume mutualism is essential in the sustainable agriculture (Graham and Vance 2000, 2003; Resh et al. 2002), we were interested in investigating the rhizobia associated with *V. faba* grown in Chinese fields. The aim of this work was to clarify the genetic diversity of the rhizobia nodulating different ecotypes of *V. faba* grown in different regions of China.

Materials and methods

Isolation of rhizobia

Root nodules were collected from *V. faba* plants grown on 56 sites of 14 Chinese provinces (Supplementary Table A; Fig. 1). Four subtropical provinces Anhui (11 sites), Jiangxi (10 sites), Hubei (11 sites) and Guangxi (2 sites) are located in the autumn sowing region. The nine temperate provinces, with 1–5 sites each, are located in the spring sowing region. Located in a plateau, the subtropical province Yunnan (two sites) is the intermediate ecological region where the faba bean is sown either in autumn or in spring. The Tibetan nodules were obtained with plant trapping method by inoculating the soil sample to the seedlings (Vincent 1970).

A standard procedure and YMA medium (Vincent 1970) were used to isolate rhizobia from root nodules (one nodule per plant). The isolates were purified by repeatedly streaking and their purity was checked by colony morphology and microscopic examination of cellular morphology. The nodulation ability was confirmed by inoculation of the isolates on *V. faba* seedlings (Vincent 1970). A total of 75 rhizobial isolates were obtained and all of them were fast-growing, acid producing bacteria (Jordan 1984). The isolates were incubated and stored as described previously (Liu et al. 2005).

AFLP genomic fingerprinting

The DNAs were extracted from bacteria, restricted by *Eco*RI and *Mse*I and ligated with *Eco*RI adapter and *Mse*I adapter according to the methods of Terefework et al. (2001). Primers *Eco*RI-gc (5′-GAC TGC GTA CCA ATT CGC-3′) and *Mse*I-cg (5′-GAT GAG TCC TGA GTA ACG-3′) with two selective nucleotides at the 3′ ends were used for selective PCR amplification (Terefework et al. 2001). The amplified fragments were separated by electrophoresis in 5% denaturing polyacrylamide gel, and the resulting gels were stained with silver staining method (Terefework et al. 2001). The Pearson correlation coefficient and UPGMA method were used to generate dendrogram from the AFLP patterns with the GelCompar program.

Genomic fingerprinting of bacteria by BOX-PCR

DNAs extracted with the same method (Terefework et al. 2001) were used as templates for repetitive extragenic

palindromic PCR with the BOXA1R primer (Koeuth et al. 1995) and the procedure of Nick et al. (1999). The amplified products (8 µl) were separated by electrophoresis in 2% (w/v) agarose gels containing ethidium bromide (0.5 µg ml^{-1}) and photographed under UV light. The Dice similarity coefficient and UPGMA method were used to generate dendrogram from the BOX-PCR patterns with the GelCompar program.

Amplified 16S rDNA restriction analysis (ARDRA) and sequencing of 16S rDNA

Primers fD1 and rD1 and the PCR procedure described by Weisburg et al. (1991) were used to amplify almost complete 16S rDNA. The PCR products (5 µl) were digested separately by the restriction endonucleases *Alu*I, *Mbo*I, *Msp*I and *Hae*III. The restriction patterns were separated by electrophoresis in 3% (w/v) of agarose gels supplied with ethidium bromide (0.5 µg ml^{-1}) and photographed under UV light. The ARDRA patterns were analyzed as mentioned in BOX-PCR analysis.

Based on the results of BOX-PCR and 16S rDNA PCR-RFLP, representative strains were chosen for direct sequencing of the 16S rDNA PCR products (Vinuesa et al. 1998) with the same primers. Clustal W software (Jeanmougin et al. 1998; Thompson et al. 1994) was used to align the acquired sequences and the related sequences obtained from the GenBank database by blast searching. A neighbor-joining tree was reconstructed using the Jukes–Cantor distances and bootstrapped with 1,000 replications with program of MEGA3.1 (Kumar et al. 2004).

DNA–DNA hybridization

A phenol–chloroform extraction protocol (Marmur 1961) was used to isolate and to purify total DNAs from the *V. faba* rhizobia. The G + C content of *V. faba* rhizobia was determined by the thermal melting profile method (De Ley 1970) using *Escherichia coli* K12 as standard. DNA–DNA relatedness was determined by the initial renaturation rate method (De Ley et al. 1970).

Restriction fragment length polymorphism (RFLP) of PCR amplified nodulation genes

For most test bacteria, primers NBA12 and NBF12′ and the procedure described by Laguerre et al. (1996) were used to amplify the complete *nodD* gene. In several isolates, this primer pair was not functional and primers NBA12 and Y6 were used instead to amplify an internal *nodD* fragment (Mutch and Young 2004; Zézé et al. 2001). Primers NodC-for540 and NodCrev1160 and PCR procedure of Sarita et al. (2005) were used to amplify a 640 bp fragment of *nodC* gene. The PCR products (5 µl) were digested separately with the restriction endonucleases *Msp*I, *Hinf*I, *Alu*I, *Mbo*I and *Dde*I (for *nodD*) or with *Afa*I, *Dde*I, *Hae*III, *Hinf*I, *Msp*I and *Taq*I (for *nodC*). The electrophoresis and subsequent analysis of restriction fragments were the same as in ARDRA.

Homogeneity analysis

The relationships among ecological regions, genomic types and *nod* genotypes were estimated by homogeneity analysis with the HOMALS 1.0 program in SPSS 11.0 package (by Data Theory Scaling System Group, Faculty of Social and Behavioral Sciences, Leiden University, The Netherlands). In this analysis, the ecological region, *nod* genotype and genomic type were treated as three variables. Three levels were contained in variable of ecological region as mentioned in the section of rhizobia isolation: Yunnan, subtropical region, and temperate region. Thirteen levels corresponding to the 13 *nod* genotypes were included in the variable of *nod* genotype. The 12 genomic types were 12 levels in the variable of genomic type. The result of homogeneity analysis was presented in a two-dimension figure, in which different levels of the variables were grouped.

Results and discussion

Genomic diversity of *V. faba* rhizobia

AFLP, BOX-PCR, ARDRA, sequencing of 16S rDNA and DNA–DNA hybridization were used to estimate the genomic diversity of the rhizobial populations in the present study.

AFLP analysis

In studies of rhizobial diversity, similarities of 50 and 60% have been used to define the AFLP groups. However, these similarity levels did not produce meaningful taxonomic groups in many cases (Andronov et al. 2003). In the present study, AFLP analysis was applied to a subset of isolates because other 18 isolates were obtained later and added in subsequent analyses. Among the test isolates, no identical AFLP patterns were found, demonstrating that the method was powerful to differentiate closely related bacteria. The similarity level of 38% was used in this study to define the AFLP groups because the reference strains for different *R. leguminosarum* biovars were separated and the isolates formed clearly separated groups at this similarity. Four AFLP groups were drawn (Table 1 and Supplementary Fig. A) among the isolates. *R. leguminosarum* bv. *viciae* USDA 2370^T (van Berkum et al.

1995) and bv. *trifolii* 162 × 68 were single strains distantly related to the isolates.

Using AFLP and other techniques, Andronov et al. (2003) showed that diversity in *R. galegae* bv. *orientalis* corresponds well with the host plant variation. However, the AFLP groups were not clearly related to the host plant ecotypes in our study, because AFLP groups 1, 3 and 4 were isolated from different ecotypes of faba bean, which was similar to the results of Wolde-meskel et al. (2004) with rhizobia of *Acacia* spp. and *Sesbania sasban.*

BOX-PCR

BOX-PCR was attractive for its high-resolution and good reproducibility, and had been widely used to reveal the genetic diversity of closely related bacterial strains (Cho et al. 2000; Healy et al. 2005; Rademaker et al. 2000; Vinuesa et al. 1998). In this study, 75 isolates were used and they were divided into 12 BOX clusters at similarity level of 80% (Table 1; Fig. 2). Similar to the AFLP analysis, USDA 2370^T and 162 × 68 were ungrouped strains. BOX clusters 4, 6 and 12 contained isolates originated from two ecological regions. The remaining clusters contained isolates originated from either subtropical region or temperate region (Table 1). In this study, great genomic diversity was revealed in *V. faba* rhizobia with BOX PCR. In the case of BOX clusters 4 and 5, the isolates were completely the same as AFLP groups 1 and 2, respectively. Two and five BOX clusters were distinguished in AFLP groups 4 and 3, respectively.

ARDRA and sequencing analysis

The 75 isolates and 19 reference strains of defined species in *Rhizobium*, *Sinorhizobium, Mesorhizobium, Bradyrhizobium* and *Agrobacterium* were used. Three rDNA types were defined in the isolates (Table 1) and they were grouped in the genus *Rhizobium* (Supplementary Fig. B). Except CCBAU 85003 and 6 isolates in AFLP group 2, the remaining 68 isolates were found in rDNA type 1 together with *R. leguminosarum* USDA 2370^T and 162 × 68. CCBAU 85003 formed rDNA type 2 that was most similar to *R. leguminosarum*. The 6 isolates in AFLP group 2 were defined as rDNA type 3 that had RFLP patterns with similarity of 90% to those of *R. leguminosarum* and *R. tropici* type strains. The ARDRA relationships were supported by the phylogeny of 16S rDNA (Fig. 3). A total of 18 strains representing the AFLP groups and BOX clusters were used in sequence analysis. Most of the isolates had 99.7–100% of 16S rDNA sequence similarities with published *R. leguminosarum* biovars. Two isolates of AFLP group 2 had 99.5% of similarities with *R. etli*, 99.2–99.3% with *R. leguminosarum* biovars, and 98.7–95.5% with other *Rhizobium* species.

DNA–DNA hybridization

This analysis was performed only for the AFLP group 2 (BOX cluster 5) because these isolates were quite different from *R. leguminosarum*. The G + C mol% (Tm) of these isolates was about 62%, within the range of *Rhizobium* (Jordan 1984). In the DNA–DNA hybridization, DNA relatedness more than 70% was detected among the isolates within AFLP group 2 (BOX group 5), while the representative isolate CCBAU 33202 had 14 and 19% of DNA relatedness with *R. leguminosarum* USDA 2370^T and *R. etli* CFN42^T. These results indicated that the AFLP group 2 (BOX cluster 5) represented a genomic species different from both species.

Based upon the results above, the AFLP group 2 (BOX cluster 5) was a genomic species that might represent a novel species in *Rhizobium*. The other genomic groups were *R. leguminosarum* as indicated in RFLP and phylogenetic analysis of 16S rDNA (Supplementary Fig. B and Fig. 3). However, they were all easily distinguished from USDA 2370^T and 162 × 68 with high-resolution method AFLP or BOX-PCR. This finding was coincident with van Berkum et al. (1995) that the faba bean rhizobia were genetically distinguishable from the *R. leguminosarum* strains isolated from other hosts. Our results in this study confirmed that most of the *V. faba* rhizobia were *R. leguminosarum* as documented earlier (Bond et al. 1985; Jordan 1984; Mutch et al. 2003; Mutch and Young 2004; Young et al. 2003), but another unnamed *Rhizobium* species also nodulated faba bean.

Diversity of *nod* genes of *V. faba* rhizobia

Nodulation gene characterization

In *nod* gene clusters, *nodD* is a regulation gene and it is related to the host specificity. The gene *nodC* is a common nodulation gene. In earlier studies, *nodD* genotype had been used to reveal the diversity of *R. leguminosarum* bv. *viciae* and specificity of symbiosis between this bacterium and its hosts (Laguerre et al. 1996, 2003; Louvrier et al. 1996; Mutch et al. 2003; Mutch and Young 2004; Zézé et al. 2001). The *nodC* gene was also recently used to study the nodulation gene diversity of soil rhizobial population (Sarita et al. 2005).

In this study, neither *nodD* nor *nodC* region was amplified from CCBAU53093-1. The *nodC* fragments were amplified from the remaining 74 isolates and the two reference strains for *R. leguminosarum*. With primers NBA12 and NBF12′, *nodD* regions (1,200–1,400 bp) were amplified from 71 isolates and from USDA 2370^T. Fragments of *nodD* (about 900 bp) were amplified from

Table 1 Root nodule isolates of *Vicia faba* from different sowing regions of China

CCBAU no.[a]	Sampling site[b]	Geographic origin[c]	rDNA type	AFLP group	BOX cluster	*Nod*-type[d]
Rhizobium leguminosarum isolates						
23121, **23125**	52, 55	Anhui	1	3	1	B–E
33192, 33193, 33196, 33203, 33204	25, 27, 28, 30	Jiangxi	1	3	1	B–E
43226, 43231, 43236, 43237, **43240**	36, 38, 40, 41, 43	Hubei	1	3	1	B–E
23126-1, 23126-2, 23130, **23131**	50, 53, 56	Anhui	1	3	2	B–E
33190, **33195**, 33199, 33198	26, 31, 32	Jiangxi	1	3	2	B–E
43233	42	Hubei	1	3	2	B–E
53072–5	24	Guangxi	1	3	2	B–E
05068	4	Hebei	1	3	3	D–D
73112, **73113**	13, 14	Gansu	1	3	3	D–D
23115, 23116	47, 48,	Anhui	1	1	4	B–E
33191	33	Jiangxi	1	1	4	C–E
43229, 43230, 43241	37, 39, 45	Hubei	1	1	4	B–E
53093-1	23	Guangxi	1	1	4	NO
53093-2	23	Guangxi	1	1	4	A–E
65310, 65311	21	Yunnan	1	1	4	B–E
11080, 11112, 11115, 11117	1, 2, 3	Liaoning	1	3	6	I–B
43235	35	Hubei	1	3	6	**E**–D
43238	44	Hubei	1	3	6	B–E
03317	7	Shanxi	1	–	7	D–D
85004	20	Soil of Tibet	1	–	7	D–D
85003	20	Soil of Tibet	2	–	7	D–D
03319, 03320, **03322**	9, 11	Shanxi	1	–	8	D–D
81102, 81103, 81104	15,16,17	Qinghai	1	–	8	D–D
81106	18	Qinghai	1	–	8	A–E
03318	9	Shanxi	1	–	9	D–D
03321	11	Shanxi	1	–	9	A–E
81107	18	Qinghai	1	–	9	A–E
83266	19	Xinjiang	1	–	9	G–C
83267, 83268	19	Xinjiang	1	–	9	F–A
03100	8	Shanxi	1	4	10	C–E
75042	12	Ningxia	1	4	10	C–E
01010, **01253**	5,6	Inner Mongolia	1	3	11	D–D
03316	11	Shanxi	1	–	11	B–E
03058	10	Shanxi	1	4	12	**N**–B
65313, 65314	21	Yunnan	1	4	12	J–B
65263, 65264	22	Yunnan	1	4	12	K–B
65315	21	Yunnan	1	4	12	**N**–B
81100	18	Qinghai	1	–	12	L–B
Rhizobium sp. isolates						
23122, 23127, **23132**	46, 49, 51	Anhui	3	2	5	B–E
23123	54	Anhui	3	2	5	M–E
33201, **33202**	29, 34	Jiangxi	3	2	5	B–E
R. leguminosarum reference strains						
bv. trifolii 162 × 68		(*Trifolium*)	1	Single	Single	**O**–G

Table 1 continued

CCBAU no.[a]	Sampling site[b]	Geographic origin[c]	rDNA type	AFLP group	BOX cluster	*Nod*-type[d]
bv. viciae USDA2370^T		(*Pisum*)	1	Single	Single	H–F

– not done, *NO* not observed

[a] CCBAU: Culture Collection of China Agricultural University, Beijing, China. The isolates marked with boldface were used in sequencing analysis

[b] The name of each sampling site is presented in Supplementary Table A

[c] Chinese province

[d] The letters presented *nodD* RFLP patterns—*nodC* RFLP patterns. The *nodD* types **E**, **N** and **O** (marked with boldface) were defined from the *nodD* fragments amplified with primers NBA12 and Y6

isolates CCBAU 03058, CCBAU 65315, CCBAU 43235 and *R. leguminosarum* 162 × 68 with primers NBA12 and Y6. We combined the RFLP patterns generated from PCR-products with both primer sets because the amplification condition also reflected the genetic difference and typing rather than phylogenetic analysis was considered herein.

In RFLP analysis, the reference strains USDA 2370^T and 162 × 68 had *nodC* and *nodD* gene types different from each other and from the isolates. A total of 13 *nodD* gene types were defined among the isolates (Fig. 4). The *nodD* types B and D dominated respectively in the subtropical region (33/37) and in the temperate region (15/30). The other *nodD* types were composed of 1–4 isolates. The *nodD* types A, B, C and N were found in two or three ecological regions. The remaining types were found in one of the three regions, temperate region, subtropical region, or Yunnan.

A total of 5 *nodC* RFLP patterns were found in the isolates (Table 1 and supplementary Fig. C). The *nodC* gene types B, D and E were found in two or three ecological regions, while *nodC* types B, D, E was dominating respectively in Yunnan (5/7), temperate region (15/30) and subtropical region (36/37).

These results showed that the *nodC* gene was rather conserved and *nodD* was more diverse. A *nodC* gene type could combine with as much as five *nodD* types (in the case of *nodC* type B), but no reverse sample was detected. This situation could be explained as that the *nodD* and *nodC* had coevolved and *nodD* changed faster than *nodC*.

Previously, it was found that the *V. faba* rhizobia in European soils harbored the dominant *nodD* type *g* on the basis of its *Hae*III restriction pattern (Laguerre et al. 2003; van Berkum et al. 1995). But Mutch et al. (2003) and Mutch and Young (2004) revealed that *V. faba* rhizobia in Jordan had *nodD* types quite different from those in European soils. In Chinese field, different dominating *nod* types were found in subtropical region, temperate region and Yunnan. Thus, we might suggest the endemicity of *nod* types of *V. faba* rhizobia.

Associations among *nod* gene types, genomic backgrounds and geographic origins of *V. faba* rhizobia

Homogeneity analysis

This analysis was performed to estimate the interactions among the genomic types, *nod* types and ecological regions. The genomic types were defined same as the BOX clusters because the combined grouping results of ARDRA, AFLP and BOX-PCR were identical to the BOX clusters (Table 1). The *nod* types were designed by combining the *nodD* and *nodC* gene types, and the letters representing *nodD* gene types were used to name the *nod* types since they were exactly the same (Table 1).

In the generated two-dimension figure (Fig. 5), three groups were found based upon the distances among the genomic types, *nod* types and ecological origins. Different levels in the same variable located in the same group meant that they might have similar characteristics in certain situations. For example, four *nod* types J, K, L and N had one common genomic background (genomic type 12), and isolates of genomic types 1, 2, 5 mainly harbored one common *nod* type B and all originated from subtropical region. The distance between different variables in the same cluster represented their relative correlation. The genomic types 3, 6, 7, 8, 9, 10, 11 and *nod* types A, C, D, F, G, I were related to temperate region. Genomic types 1, 2, 4, 5 and *nod* types B, E, M were related to subtropical region. Genomic type 12 and *nod* types L, N, J and K formed a group related to Yunnan. The *nod* types C, N and genomic type 6 were distantly related to the ecological regions in each of the two groups, because all of them were found in two different ecological regions.

It had been revealed that symbiotic genotype of rhizobia isolated from faba bean appeared to be the determinant of the success in nodule occupancy of rhizobial genotypes independent of the associated genomic background (Laguerre et al. 2003; Louvrier et al. 1996; Mutch and Young 2004). However, our results in Fig. 5 indicated that the *nod* types were not randomly associated with the geno-

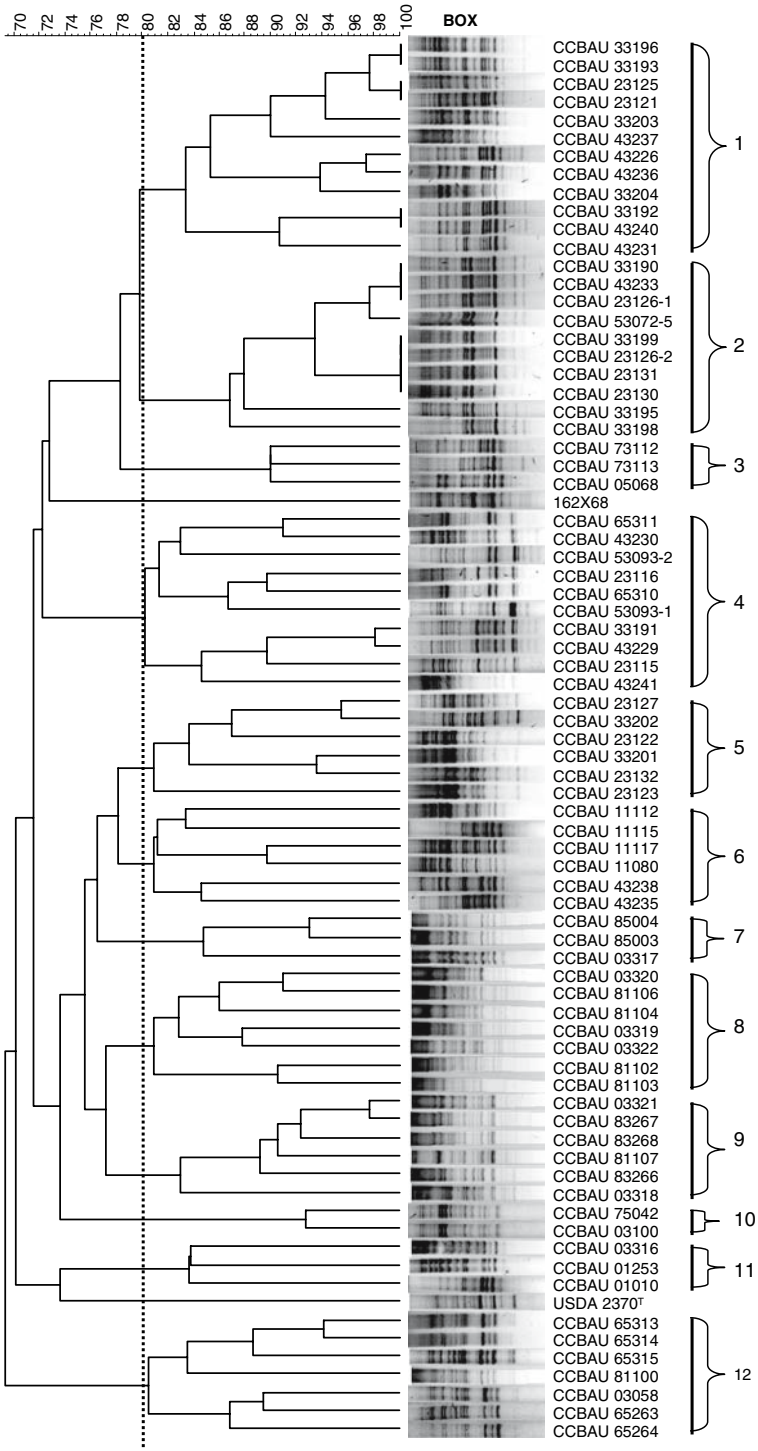

Fig. 2 Genomic diversity of *Vicia faba* rhizobia revealed by BOX-PCR. The dendrogram was constructed by the UPGMA method in the GelCompar program

mic types and the associations between *nod* type and genomic types displayed endemicity, despite few noises. For example, the *nod* type B was mainly harbored by subtropical isolates in five genomic types and by only a temperate isolate, while *nod* type D was only found in five genomic types in temperate region. According to the plasmid/chromosome evolutionary patterns proposed by Souza et al. (1997), an epidemic symbiotic gene transfer pattern among different genomic backgrounds within an ecological region and relatively limited transfer pattern between different regions could be deduced from these results. Moreover, as shown in Table 1, it was also revealed that one genomic type could harbor different *nod* types at different field sites, such as the cases of genomic types 4, 5, 6, 8, 9, 11 and 12. This phenomenon might be related to the survival strategy of *V. faba* rhizobia because different *nod* genotypes could

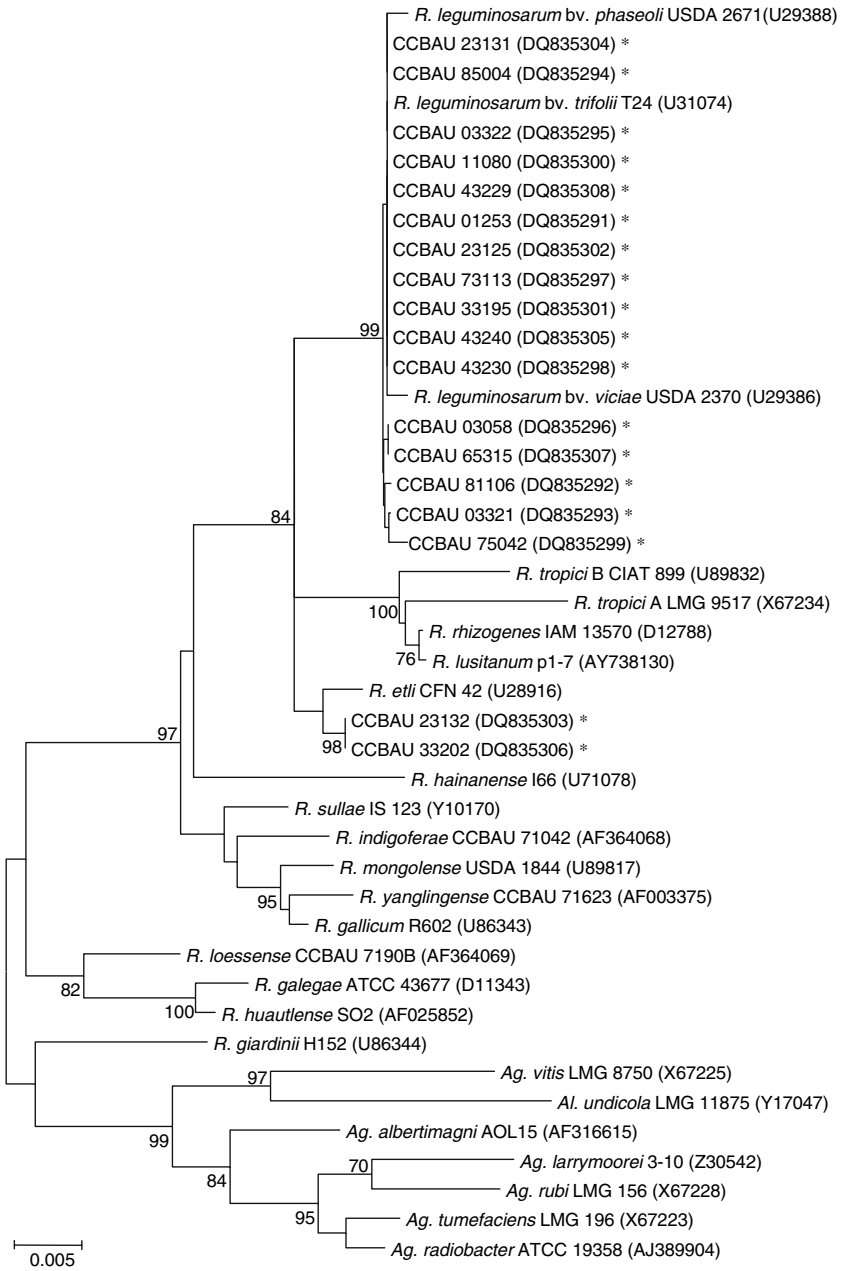

Fig. 3 Phylogenetic tree of 16S rDNA of *V. faba* rhizobia isolated from Chinese fields (marked with *asterisk*). This tree was constructed from sequences of the 16S rRNA genes using the Neighbor-Joining method in MEGA3.1. Bootstrap probability values above 70% are indicated at the branch points. The *bar* represents a 0.5% nucleotide divergence

help a genomic type to be competitive in nodulation with different cultivars of *V. faba* on different field sites.

In the homogeneity analysis, the ecological regions could be replaced by ecotypes of faba bean, as mentioned in introduction. Then the non-random correlation between the ecological regions and the genomic or *nod* types also represented the same correlation between faba bean ecotypes and the rhizobia genomic or *nod* genotypes. From Table 1, it was clear that different sampling sites in the same ecological region could harbor rhizobia with similar genomic background and *nod* type, such as BOX cluster 1 (with *nod* type B) included isolates originated from 11 sites in three provinces, BOX cluster 2 (with *nod* type B) from eight sites in four provinces, BOX cluster 3 (with *nod* type D) from three sites in two distant provinces. Moreover, the same site could harbor rhizobia in different genomic and *nod* types, as the cases of sites 9 and 11 in Shanxi and site 21 in Yunnan. Considering that the sampling sites covered a vast territory with many different soil types and other environmental characters, it could be estimated that the faba bean ecotype might be the determinant for the distribution of rhizobial genomic and *nod* types. However, further experiments were needed to clearly confirm this association or coevolution.

Conclusively, the present study revealed a novel microsymbiont (*Rhizobium* sp.) of *V. faba* in addition to *R. legu-*

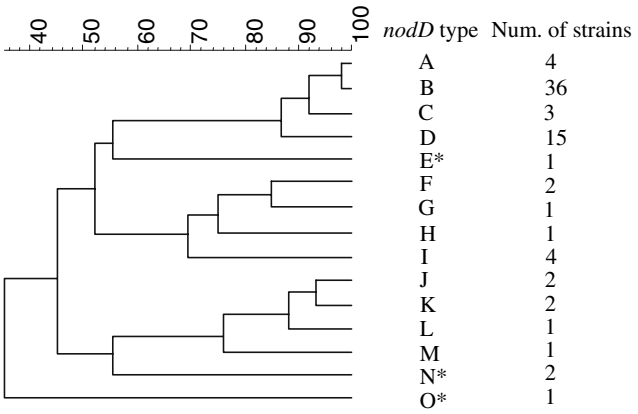

Fig. 4 Simplified dendrogram showing the *nodD* diversity in *V. faba* rhizobia defined by RFLP of amplified *nodD* fragments. The dendrogram was generated with UPGMA method from the RFLP patterns in the GelCompar program. *Asterisk* RFLP patterns of about 900 bp *nodD* fragments amplified from isolates CCBAU 03058 (type N), CCBAU 65315 (type N), CCBAU 43235 (type E) and 162 × 68 (type O) with primers NBA12 and Y6 were included together with other patterns obtained with RFLP analysis of PCR products with primers NBA12 and NBF12′, because the amplification condition also reflected the genetic difference

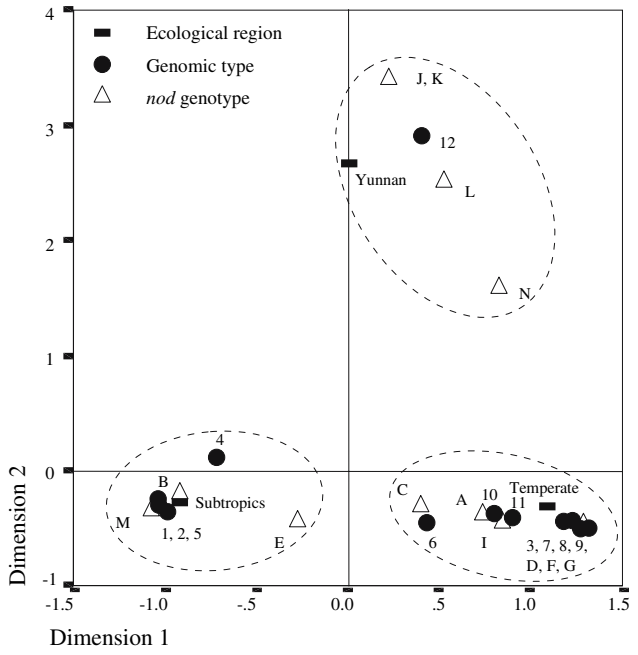

Fig. 5 Homogeneity analysis of ecological regions, genomic groups and nodulation genotypes. The Dimensions 1 and 2 were the linear combination of the three variables and may have no real meaning. Eigen value for Dimension 1 and 2 was 0.938 and 0.666, respectively, indicating both the dimensions were closely related to all the three variables. The discrimination measures were 0.942, 0,953 and 0.931, respectively for ecological region, genomic type, and *nod* type in Dimension 1; and were 0.759, 0.921 and 0.949, respectively for the three variables in Dimension 2. These discrimination measures indicated that the two dimensions could distinguish all the variables with high confidence

minosarum. Furthermore, the population of *V. faba* rhizobia displayed endemic distributions in Chinese fields. The non-random association *V. faba* ecotypes (or ecological origins), rhizobial chromosomal backgrounds and nodulation genotypes should be considered in screening rhizobial inoculants in agricultural production.

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