ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 5, pp. 655–662. © Pleiades Publishing, Ltd., 2010.

EXPERIMENTAL ARTICLES

Multiple Copies of 16S rRNA Gene Affect the Restriction Patterns and DGGE Profile Revealed by Analysis of Genome Database[1]

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Received December 7, 2009

Abstract—The use of 16S rRNA gene has been a "golden" method to determine the diversity of microbial communities in environmental samples, phylogenetic relationships of prokaryotes and taxonomic position of newly isolated organisms. However due to the presence of multiple heterogeneous 16S rRNA gene copies in many strains, the interpretation of microbial ecology via 16S rRNA sequences is complicated. Purpose of present paper is to demonstrate the extent to which the multiple heterogeneous 16S rRNA gene copies affect RFLP patterns and DGGE profiles by using the genome database. In present genome database, there are 782 bacterial strains in total whose genomes have been completely sequenced and annotated. Among the total strains, 639 strains (82%) possess multiple 16S rRNA gene copies, 415 strains (53%) whose multiple copies are heterogeneous in sequences as revealed by alignment, 236 strains (30%) whose multiple copies show different restrict patterns by *CSP*6I+*Hinf*I, *Msp*I+*Rsa*I or *Hha*I as analyzed *in silico*. Polymorphisms of the multiple copies in certain strains were further characterized by G+C% and phy-logentic distances based on the sequences of V3 region, which are linked to DGGE patters. Polymorphisms of a few strains were shown as examples. Using artificial communities, it is demonstrated that the presence of multiple heterogeneous 16S rRNA gene copies potentially leads to over-estimation of the diversity of a community. It is suggested that care must be taken when interpreting 16S rRNA-based RFLP and DGGE data and profiling an environmental community.

Key words: 16S rRNA gene, DGGE, multiple copies, RFLP

DOI: 10.1134/S0026261710050103

Restriction fragment length polymorphism (RFLP) has been widely used for screening clone libraries and profiling microbial communities in environmental samples [1–4]. By this method, insert fragments are digested with restrict enzyme(s) and run on a simple agarose gel [5–8], or a complex gel [9–11]. It is assumed that clones showing the same restriction patterns belong to the same genotype or phenotype and those showing different restriction patterns belong to different genotypes. The different genotypes recognized as such are typically selected for further sequencing, or used collectively to present the profile of a community.

Denaturing gradient gel electrophoresis (DGGE), since introduced into microbial ecology by Muyzer et al. [12], has been widely used to profile microbial communities in environmental samples such as soils, sediments and bioreactor samples [13–17]. By this method, bulk DNA is extracted and amplified with GC-clamped primers. The amplified DNA fragments are purified, run on a denaturing gradient gel, and separated according to melting domains. It is usually assumed that one band on the gel represents a genotype and different bands represent different genotypes. Band patterns represent community structures in the samples.

The gene coding 16S rRNA is commonly used as a target gene when using RFLP and DGGE methods to profile microbial communities. Genes coding functional proteins are frequently used for this purpose as well. However whether the assumptions behind the RFLP and DGGE methods are true depends on the sequence homogeneity among the copies. If an organism contains multiple copies of the target gene that are heterogeneous in sequences, the assumptions becomes questionable. Consequently systematic errors may occur in the profiled communities. In fact, many bacteria have multiple copies of 16S rRNA genes and or of functional genes. *Ralstonia eutropha* JMP134, for example, has 6 copies of 16S rRNA gene and *Magnetospirillum magneticum* AMB-1 has 3 copies of *nirS* gene (coding dissimilatory nitrite reductase). Considerable sequence variation exists among the multiple copies of both genes (for details, visit http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

[1] The article is published in the original.

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Escherichia coli APEC 01, for example, has 7 copies of 16S rRNA gene and the pairwise distance (Jukes–Cantor algorithm) between the copies of 640757041 (gene object ID as in the Integrated Microbial Genomes Database) and 640753194 is as large as 0.0143. It is thus speculated that a community diversity could be over- or under-estimated by RFLP and DGGE methods because of the presence of heterogeneous multiple gene copies.

Purpose of present paper is to demonstrate what extent to which the heterogeneity of multiple 16S rRNA gene copies affects RFLP patterns and DGGE profiles by using the genome database. We limit our analysis to the 16S rRNA genes in bacterial genomes (excluding *Archaea*) that have been completely sequenced and annotated (excluding draft sequences).

MATERIALS AND METHODS

Sequence Source

The presence of multiple 16S rRNA gene copies is viewed via the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Statistic information was obtained directly from the website. Sequences of 16S rRNA genes from complete genomes (excluding the daft genomes) were downloaded. Heterogeneity of multiple 16S rRNA gene copies within each genome is analyzed using an on-line alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

RFLP Patterns in Silico and Types by G + C% of V3 Region

For RFLP analysis, complete sequences of 16S rRNA genes were digested *in silico* (http://www.bioinformatics.org/~docreza/cgi-bin/restriction/DistinctiEnz.pl) by choosing various restriction enzymes that have been commonly used in literature. This tool provides numerical as well as graphical results. Analysis *in silico* is very sensitive, able to discriminate two bands with only one base longer or shorter, a sensitivity that is usually unachievable by real gel runs. This raises a question on how many bases are allowed for two fragments to be practically determined as different fragments. According to literature, real gel runs usually can discriminate two bands with minimal of 6 base differences if two fragments are >200 bp in size [18–20]. This practical sensitivity is adopted in present paper to make the data of RFLP patterns *in silico* comparable to those by real gel run.

To evaluate possible effect of multiple 16S rRNA gene copies on DGGE profiles, sequences of V3 region flanked by primer P2 (CCTACGGGAGGCAGCAG) and P3 (ATTACCGCGGCTGCTGG) [12] were extracted. This region has been frequently used as a marker to profile environmental communities. As the DGGE patterns can not be analyzed *in silico*, polymorphisms of the multiple copies are characterized by G + C% and phylogpnjic distance. Both characters are relevant to DGGE patterns. As for G + C%, it is assumed that different copies having the same G + C% likely have the same melting temperature and will produce an identical band, but the copies having different G + C% likely have different melting temperatures and will produce different bands. As for phylogentic distance, it has been reported [21] that two fragments with a phylogentic distance larger than 0.0005 usually show two different DGGE bands. This value is therefore adopted to characterize the multiple copies in relation to DGGE patterns. Phylogentic distance among the multiple copies (V3 region) is calculated using Phylip dnadist (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=dnadist) by choosing Jukes-Cantor algorithm. Calculation stated above applies to the artificial communities.

Artificial Communities

Five artificial communities were constructed. Community 1 is intended to represent a soil bacterial community, consisting of bacteria commonly found in soils according to the review by Janssen [22]. Community 2 consists of various species of *Pseudomonas*, a genus frequently found in soils. Community 3 is a single-species community consisting of 4 different strains of *Pseudomonas aeruginosa*. Community 4 consists of various species of *Bacillus* strains, also a genus widely found in soils. Community 5 is again a single-species community consisting of 8 different strains of *Bacillus cereus*. The multiple 16S rRNA gene copies of the selected strains were downloaded and pooled into communities. The numbers of RFLP patterns were analyzed *in silico* and heterogeneity of G + C% as well as the phylogentic distance was calculated as stated above. Details of the communities are given in Table 1.

RESULTS

An Overview of Multiple Copies of 16S rRNA Gene in the Genome Database

By August 20th, 2009, there was 782 bacterial strains whose genomes had been completely sequenced and annotated, 639 of which (81.7%) had multiple copies of 16S rRNA gene, and 143 (18.3%) had single copy (Fig. 1a). Copy numbers range from 1 to 21, depending on strains. Majority of strains have less than 8 copies (Fig. 2). Based on the alignment of complete sequences (whole length), 415 strains showed heterogeneity among the duplicated copies and only 224 strains have multiple copies but identical in sequence, accounting for respectively 53.0 and 28.6% of the total strains (782) in the database (Fig. 1b). An example of heterogeneous 16S rRNA genes within the strain of *Ralstonia eutropha* JMP134 is shown in Fig. 3. The maximal distance among the 6 copies is 0.005254.

Table 1. Data sources of the artificial communities

Artificial communities	Organisms	Copy number of 16S rRNA gene	Scaffold ID
1	*Agrobacterium tumefaciens* C58 (Cereon)	4	NC_003062; NC_003063
	Arthrobacter chlorophenolicus A6	5	NC_011886
	Bacillus anthracis Ames	11	NC_003997
	Bacillus cereus ATCC 10987	12	NC_003909
	Flavobacterium johnsoniae UW101	6	NC_009441
	Nocardia farcinica IFM 10152	3	NC_006361
	Pseudomonas aeruginosa PAO1	4	NC_002516
	Pseudomonas putida GB-1	7	NC_010322
	Streptomyces avermitilis MA-4680	6	NC_003155
	Streptomyces coelicolor A3(2)	6	NC_003888
2	*Pseudomonas aeruginosa* LESB58	4	NC_011770
	Pseudomonas entomophila L48	7	NC_008027
	Pseudomonas fluorescens Pf-5	5	NC_004129
	Pseudomonas mendocina ymp	4	NC_009439
	Pseudomonas putida F1	6	NC_009512
	Pseudomonas stutzeri A1501	4	NC_009434
	Pseudomonas syringae pv. *phaseolicola* 1448A	5	NC_005773
3	*Pseudomonas aeruginosa* LESB58	4	NC_011770
	Pseudomonas aeruginosa PA7	4	NC_009656
	Pseudomonas aeruginosa PAO1	4	NC_002516
	Pseudomonas aeruginosa UCBPP-PA14	4	NC_008463
4	*Bacillus amyloliquefaciens* FZB42	10	NC_009725
	B. anthracis Ames	11	NC_003997
	B. cereus AH187	14	NC_011658
	B. clausii KSM-K16	7	NC_006582
	B. halodurans C-125	8	NC_002570
	B. licheniformis ATCC 14580 (Goettingen)	7	NC_006322
	B. pumilus SAFR-032	7	NC_009848
	B. subtilis 168	10	NC_000964
	B. thuringiensis Al Hakam	14	NC_008600
	B. weihenstephanensis KBAB4	14	NC_010184
5	*Bacillus cereus* AH187	14	NC_011658
	B. cereus AH820	12	NC_011773
	B. cereus ATCC 10987	12	NC_003909
	B. cereus ATCC 14579	13	NC_004722
	B. cereus B4264	14	NC_011725
	B. cereus cytotoxis NVH 391-98	13	NC_009674
	B. cereus E33L	13	NC_006274
	B. cereus G9842	13	NC_011772

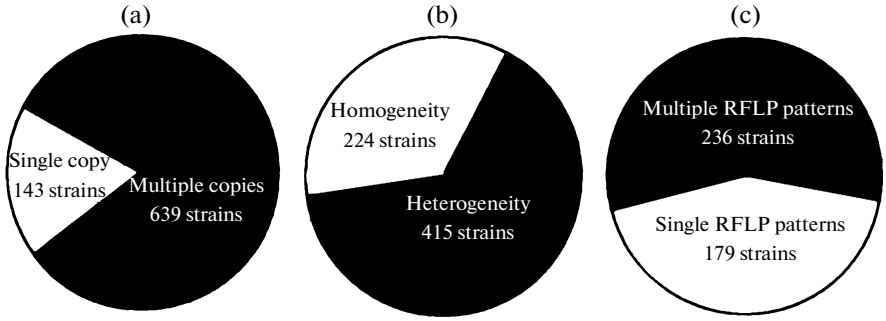

Fig. 1. Statistics showing the number of genomes having multiple copies of 16S rRNA gene (a), heterogeneous sequence of the multiple copies by alignment (b), and multiple RFLP patterns (see text) (c). All analysis was based on complete 16S rRNA sequences.

RFLP Patterns in Silico of the Multiple Copies

Using the restrict enzymes commonly reported in literature, RFLP patters of each strain in the database were analyzed *in silico*. It was found that 236 strains showed multiple RFLP patters by either *CSP*6I + *Hinf*I, *Msp*I + *Rsa*I or *Hha*I (Fig. 1c), accounting for 30% of total number of strains available in present database, whereas 179 strains showed a single RFLP pattern despite the presence of multiple copies. Because of the heterogeneous sequence of multicopy 16S rRNA genes, a single strain may exhibit 2 or 3 different RFLP patterns. *Pseudomonas stutzeri* A1501 is an example among many others. This strain has 4 copies of 16S rRNA genes, which shows 2 types of RFLP patterns by digestions of *CSP*6I + *Hinf*I, *Hha*I, and *Msp*I + *Rsa*I (Fig. 4). In contrast, different species within a genus may show the same RFLP patterns despite the presence of multicopy 16S rRNA genes. *Brucella* is an example among several other genera. *B. melitensis* bv Abortus 2308, *B. abortus* bv 1 9-941 and *B. ovis* ATCC 25840 are three species all possessing three copies of 16S rRNA genes but showed the same *CSP*6I + *Hinf*I patterns not only within strain but also within the species (Fig. 5). Digestion with *Hha*I and *Msp*I + *Rsa*I also produced homogeneous patterns (data not shown).

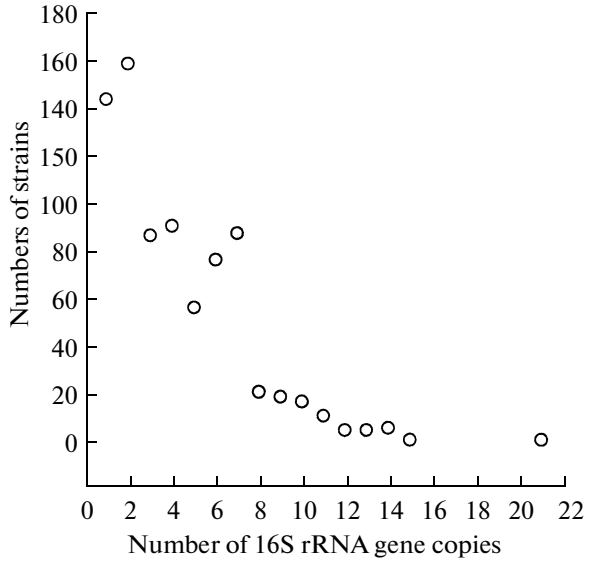

Fig. 2. Number of 16S rRNA gene copies against number of bacterial genomes in database (by August 20, 2009).

Characterization of Multiple Copies by G + C% and Phylogentic Distance

As characterized by G + C%, the V3 region of multiple copies of 16S rRNA gene within a strain also showed heterogeneity. *Clostridium botulinum* A ATCC 3502, for example, has 9 copies of 16S rRNA genes. The V3 region of 6 copies contains 56.21% of G + C content, 1 copy contains 56.80% and 2 copies contain 57.40% of G + C content (three G + C% tyres). In contrast, different species within a genus may show the same G + C% despite the presence of multiple copies. *Escherichia* is an example among several other genera. *E. coli* UTI89 and *E. fergusonii* ATCC 35469 are the two species all having 7 copies of 16S rRNA genes but having 54.6% G + C content in V3 region (only one G + C% type). As characterized by phylogentic distance, *Clostridium botulinum* A ATCC 3502 showed 6 types among the 9 copies. The largest distance is 0.0054 between the copy 640571672 and 640571662.

RFLP Patterns of Artificial Bacterial Communities Analyzed in Silico

Community 1 consisted of 10 bacteria belonging to 7 genera, all of which are commonly found in soils. Numbers of RFLP patterns *in silico* were 13, 12 and 14 when digested with *CSP*6I + *Hinf*I, *Hha*I and *Msp*I + *Rsa*I respectively (Table 2), significantly higher than the actual number of phenotypes. Community 2 consisted of 7 species within the genus of *Pseudomonas*, which are also common soil bacteria. Digestion with the same set of enzymes produced 10, 7 and 8 RFLP patterns respectively. Community 3 consisted of 4

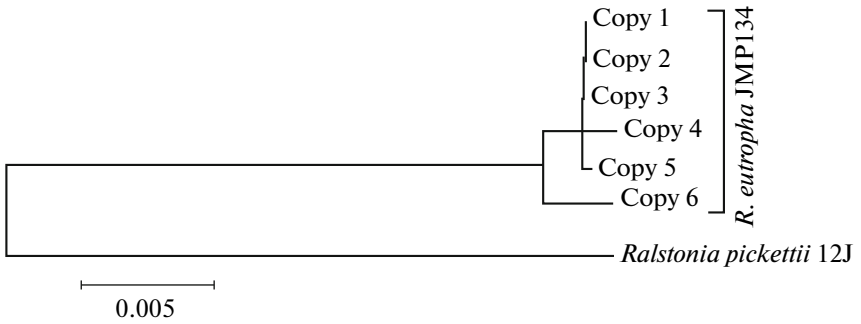

Fig. 3. Heterogeneity of multiple copies of 16S rRNA gene (1528 bp) within the strain of *Ralstonia eutropha* JMP134. Sequence from *Ralstonia pickettii* 12J (access number NC_010678) is used as an outgroup.

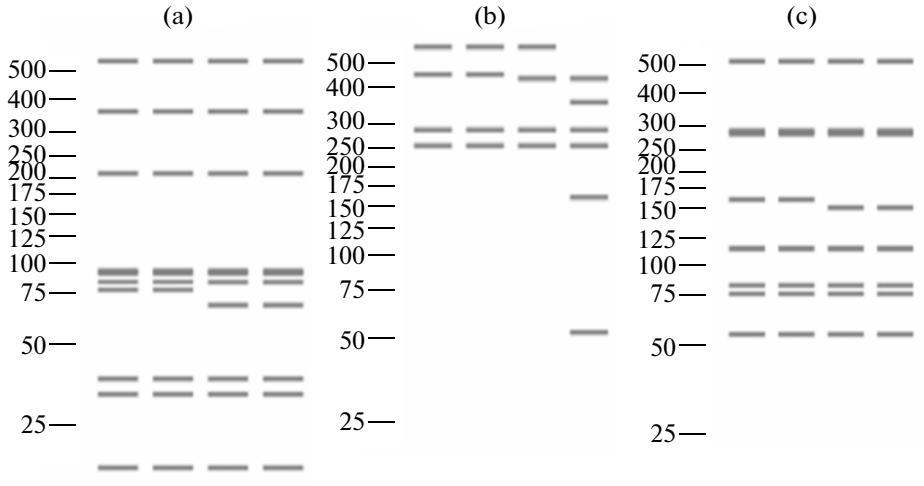

Fig. 4. RFLP patterns *in silico* of 4 copies of 16S rRNA gene from *Pseudomonas stutzeri* A1501 digested by *CSP*6I+*Hinf*I (a), *Hha*I (b) and *Msp*I+*Rsa*I (c). Analysis was based on complete 16S rRNA sequences.

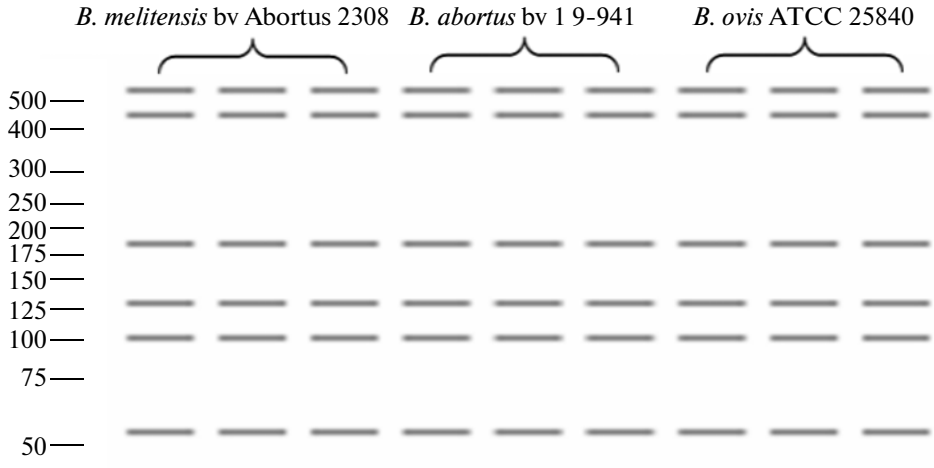

Fig. 5. RFLP patterns *in silico* of 3 copies of 16S rRNA gene from *Brucella melitensis* bv Abortus 2308, *Brucella abortus* bv 1 9-941 and *Brucella ovis* ATCC 25840 digested by *CSP*6I+*Hinf*I. Patterns digested by *Hha*I and *Msp*I+*Rsa*I are also identical among the species (data not shown). Analyses were based on complete 16S rRNA sequences.

Table 2. Number of polymorphisms of artificial communities as characterized by restriction digestion *in silico*, phylogentic distance and G + C%

Artificial communities[a]	By *CSP*6² + *Hinf*² digestion	By *Hha*² digestion	By *Msp*² + *Rsa*² digestion	By phylogentic distance	By G + C%
1 (10)	13	12	14	9	8
2 (7)	10	7	8	6	4
3 (4)	3	3	3	1	1
4 (10)	16	8	17	17	6
5 (8)	5	4	5	7	4

Note: [a] Values in parentheses indicate the number of strains in the artificial community.

strains within the species of *Pseudomonas aeruginosa*, but shows only 3 RFLP patterns *in silico* by either set of enzymes. Community 4 was a mixture of 10 species within the genus of *Bacillus*, which are also easily isolatable soil bacteria. Numbers of RFLP patterns *in silico* were 16, 8 and 17 when digested with the same set of enzymes respectively, which are significantly different from the actual number of phenotypes. Community 5 was a single-species community consisting of 8 different strains of *Bacillus cereus*. Digestion with the same set of enzymes produced 5, 4 and 5 RFLP patterns respectively, significantly lower than the actual number of strains. Obviously these differences are resulted from heterogeneity of multiple copies. When digested with *CSP*6I + *Hinf*I, *Streptomyces coelicolor* A3(2) (in community 1), for example, produced 3 distinct bands (corresponding to gene object ID 640698046, 640698077 and 640698105), contributing to the increased number of RFLP patters in the community.

Artificial Communities Characterized by G + C% and Phylogentic Distance

As characterized by G + C% on V3 region, the number of types for each artificial community does not match the actual number of strains in the community (Table 2). Community 1, for example, contains 10 strains but shows 8 G + C% types. Community 4 also contains 10 strains but shows 6 G + C% types only. Similar results were obtained when characterized by phylogentic distance (Table 2). In community 4, for example again, number of types characterized by phylogentic distance was 17, significantly higher than the actual number of strains in the community. Again, these differences were obviously resulted from the presence of heterogeneous multiple copies of 16S rRNA gene.

DISCUSSION

The genes coding 16S rRNA has been widely used for determination phylogenetic relationships of prokaryotes, taxonomic position of newly isolated organisms, and diversity of microbial communities in environmental samples. An assumption behind these applications is that each phenotype or genotype is represented by a unique sequence of the 16S rRNA gene. This assumption is undoubtedly true if each organism contains a single copy of the 16S rRNA gene, or multiple copies but identical in sequences. This assumption probably stands for the majority of prokaryotes in the nature. However, sequence heterogeneity of multiple 16S rRNA copies has been frequently reported for certain strains [23–25]. The rapid expanding genome database provides increasing number of examples of this kind. The effect of heterogeneous multiple 16S rRNA copies on the analysis of community diversity must be re-evaluated.

Data presented in this paper clearly showed that the presence of heterogeneous multiple copies of 16S rRNA gene affect RFLP and DGGE patterns on both individual strain and community levels, suggesting that the diversity of a community can be over-estimated. In fact, a pure strain showing multiple DGGE bands is not uncommon. *E. coli*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*, for example, were reported to show 5, 3 and 3 DGGE bands respectively [26]. Similarly *Paenibacillus polymyxa* [27], several species of *Lactobacillus*, *Staphylococcus* and *Kocuria* [28–30] were also reported to show multiple DGGE bands by using sequence of V1 or V3 regions. Understanding the effects of multiple copies of 16S rRNA genes on DGGE patterns will help meaningful interpretation of DGGE data, and sometimes RFLP data as well.

Present paper showed that 30% of total strains available in present genome database show multiple RFLP patterns and possibly DGGE patterns as well (Fig. 1), which affects diversity estimation of artificial communities (Table 2). To what extend to which a true

environmental community is affected by heterogeneous multiple copies of 16S rRNA genes is, however, difficult to evaluate for two reasons that: (1) Even the whole genome database does not represent any true environmental community, as priority for genome projects has been given to the strains of economic or clinical importance. (2) Most genome projects work on pure cultures but the uncultured bacteria are usually the major population in an actual environmental sample. Nonetheless the interpretation of microbial ecology via 16S rRNA sequences is complicated as a result of presence of multiple heterogeneous 16S rRNA gene copies in many strains. Furthermore variation of 16S rRNA gene sequences is not sufficient for determination of phylogenetic relationships between certain species, as *Brucella* shown in Fig. 5, which further complicates the use of 16S rRNA genes as a sole marker, a problem that has been extensively discussed in literature.

CONCLUSIONS

Nearly 30% of total strains available in present genome database show multiple RFLP patterns and possibly DGGE patterns as well. Presence of multiple heterogeneous 16S rRNA gene copies in many strains may potentially lead to over-estimation of the diversity of an environmental community. Care must be taken when interpreting 16S rRNA-based RFLP and DGGE data.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (nos. 40471072 and 40871117) and the Knowledge Innovation Program of the Chinese Academy of Sciences (no. KZCX2-YW-407), and the National Key Basic Research and Development Program of China (no. 2005CB121108).

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