ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 6, pp. 888–895. © Pleiades Publishing, Ltd., 2014.

EXPERIMENTAL ARTICLES

Analysis of Endophytic Bacterial Community Composition by 16S rDNA Clone Library in *Achnatherum inebrians*[1]

Ying Wu Shi, Xuebing Zhang, and Kai Lou[2]

Institute of Microbiology, Xinjiang Academy of Agricultural Sciences, Urumqi 830091

Received December 2, 2013

Abstract—The endophytic bacterial diversity of drunken horse grass (DHG) (*Achnatherum inebrians*) growing in native habitat station in Xinjiang Province, China was analyzed by 16S rDNA cloning, amplified ribosomal DNA restriction analysis (ARDRA), and sequence homology comparison. To effectively exclude the interference of chloroplast DNA and mitochondrial DNA of DHG, a pair of bacterial PCR primers was selected to specifically amplify bacterial 16S rDNA sequences directly from DHG root tissues. Among 249 positive clones in the 16S rDNA library of endophytes, 57 OTUs were identified based on the similarity of the ARDRA banding profiles. Sequence analysis revealed diverse phyla of bacteria in the 16S rDNA library, which consisted of alpha, beta, gamma, delta, and epsilon subclasses of the *Proteobacteria*, *Flavobacteria*, *Actinobacteria*, *Sphingobacteria*, and uncultured bacteria. The dominant group was *Proteobacteria*, whie the most numerous genus was *Bacillus*. More than 8.00% of the total clones showed a high similarity to uncultured bacteria, suggesting that nonculturable bacteria make a significant part of DHG endophytic bacterial community. To our knowledge, this is the first report that endophytic bacteria associated with DHG are revealed by the culture-independent approach. The results suggest that the diversity of endophytic bacteria is abundant in DHG.

Keywords: endophytic bacteria, *Achnatherum inebriants*, bacterial diversity, 16S rDNA library

DOI: 10.1134/S0026261714060174

Endophytic bacteria are defined as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plants and that are not observed to harm the host plants [1]. The asymptomatic internal colonization of healthy plant tissue by endophytic bacteria is a widespread and well-documented phenomenon. Most information on endophytic bacterial diversity has been obtained by using culture-dependent approaches. However, due to the unknown conditions for growth requirements of many bacteria and the presence of cells which are in a viable but noncultivable state, the portion of microbial diversity which has been obtained by conventional cultivation techniques is less than 1% of the bacterial species present. Culture-dependent biodiversity studies of the endophytic community are somewhat limited. The use of fingerprinting techniques and clone analysis can provide additional information for analysing the community composition of endophytic bacteria, such as PCR amplification of 16S rDNAs, amplified ribosomal DNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). It have been successfully used for bacterial community analysis in a great variety of environments, including soil ecosystems [2], marine [3], rhizosphere [4], food [5], and human intestine [6] to overcome the limitations of culture-dependent approaches.

Drunken horse grass (*Achnatherum inebrians*) is an important perennial Chinese bunchgrass which usually grows on roadsides, in gullies, on shady slopes, and in the harsh conditions of the alpine or subalpine grasslands of the Qinghai-Tibetan, Tianshan and Qilian mountains in Gansu, Xinjiang, Qinghai, Tibet and Inner Mongolia. It could, therefore, have potential use as a sustainable soil-water conservation plant. *Neotyphodium gansuense* obtained using culture-dependent approaches is a fungal endophyte symbiotic with the Among planctomycetes that is native to Gansu, China. *N. gansuense* provide *A. inebrians* with a strong competitive ability due to an increased host tolerance to drought [7], salt [8], cold [9], pathogenic fungi [10], and pests [11, 12]. Although endophyte infections have been detected and the *Neotyphodium—A. inebrians* association investigated widely (Li et al., 2004a), very little is known about the endophytic bacterial diversity in *A. inebrians*.

The goal of this paper was to explore the feasibility of identifying *A. nebrians* endophytic bacteria and, thus, to obtain a better understanding of bacterial community structure and diversity using culture-independent methods, such as amplified ribosomal DNA

[1] The article is published in the original.

[2] Corresponding author; e-mail: loukai02@mail.tsinghua.edu.cn

restriction analysis (ARDRA) and sequencing of 16S rDNA clones.

MATERIALS AND METHODS

Sampling of plants. Plant samples (*Achnatherum inebriansc*) were obtained from their native habitat from a mountain near Urumuqi City in Xinjiang Province, China (46°30′24.1″ N, 106°27′43.3″ E) in May and July 2008 and immediately transported to the laboratory. The physical and chemical characteristics of the *Achnatherum inebrians* field soil were as follows: pH 7.68, 36% clay, 1.86% organic matter, and 0.144% total nitrogen.

Surface sterilization of *Achnatherum inebrians*. The plant samples (root, stem, leaf, seed) were first washed three times with tap water to remove attached soil. Subsequently, the roots were immersed in 70% ethanol for 3 min, washed with fresh sodium hypochlorite solution for 5 min, rinsed three times with 70% ethanol for 30 s, and finally washed five times with sterile distilled water. To confirm that the disinfection process was successful, aliquots of the sterile distilled water used in the final rinse were detected by cultivation and molecular method to determine the results of surface disinfection. The cultivation was performed by setting 100 mL of the final rinse on Luria-Bertani (LB) medium plates, and the plates were examined for bacterial growth after incubation at 30°C for 3 days.

DNA extraction and PCR amplification. About 1.2 g of the surface-sterilized DHG plants was frozen with liquid nitrogen and ground to a fine powder. The hot CTAB procedure [13] was used with some modifications. The powder was suspended in preheated 11 mL CTAB extraction buffer and mixed by inverting the tube several times, followed by incubation in a 60°C water bath for 60 min. DNA was then extracted twice with chloroform-isoamyl alcohol (24 : 1 v/v), followed by precipitation with 0.6 volumes of isopropanol for 2 h at −20°C. DNA was centrifuged at 12000 *g* for 10 min at 4°C, washed with 70% ethanol, and then air-dried. Finally, the DNA was resuspended in 30 μL of sterile water.

The pair of primers 799f and 1492r [14] was selected to amplify the DNA of *Achnatherum inebrians* endophytic bacteria. The 50 μL PCR reaction mixture contained 100 ng of DNA extract, 1× *Taq* reaction buffer, 20 pmol of each primer, 200 μM each dNTP, and 1.5 U of *Taq* DNA polymerase (Sangong). After initial denaturation at 95°C for 4 min, each thermal cycling was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 60 s, and elongation at 72°C for 1.5 min. At the end of 30 cycles, the final extension step was at 72°C for 8 min.

Clone library construction and ARDRA screening. The purified PCR products were ligated into the pMD19-T vector (Takara Co., China). *Escherichia coli* DH5α competent cells (Tiangen Co., China) were transformed with the ligation products and spread onto Luria-Bertani agar plates with ampicillin (100 μg mL^{-1}) for standard blue and white screening. Colonies randomly picked were screened directly for inserts by performing colony PCR with primers for the vector (primers M13F and M13R).

Randomly selected colonies were screened directly for inserts by performing colony PCR with primers Primer M13F (−47) 5'-CgCCAgggTTTTCCCAgTCACgAC-3' and Primer M13R (−48) 5'-AgCggATAACAATTTCACACAggA-3') for the vector (Takara Co., China). Further screening of PCR fragments was done via ARDRA to determine unique pattern profles, representatives clones of which were selected for subsequent sequence analysis. Digestion of the PCR product was done using the restriction enzymes *Hae*III. The fragments were separated on a 2.5% agarose gel running in 1× TAE buffer at 100 V for approximately 1 h. According to ARDRA patterns, clones were grouped into OTUs. Diversity of the clone library was investigated by rarefaction analysis.

Sequencing and phylogenetic analysis. Partial sequences of cloned 16S rRNA genes were sequenced using an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co., Ltd). The presence of possible chimeric sequences was investigated by using the CHIMERA_CHECK program of the Ribosomal Database Project II (RDP II) [15]. All sequences obtained were compared with sequences in the GenBank database by using the BLASTN. Phylogenetic trees were deposited in the GenBank under accession numbers GU942752–GU942808 [16].

Diversity estimations. Diversity indexes including the coverage rate (Coverage value, C), the Shannon diversity index, Simpson's index, McIntosh's index, and the Berger-Parker index were all calculated using the BIO-DAP software package (http://nhsbig.inhs.uiuc.edu) [17], a biodiversity analysis package. Diversity indices were calculated on the basis of the number of uniquely classified clones in clone library. C value of the theory that the microbial species including 16S rRNA gene clone library (OTU) in the total microbial species in the sample proportion. $C = 1 - n1/N$, N represents 16S rRNA clone library storage capacity, the number of n1 represent only a OTU in a 16S rRNA clone library [18]. Calculation of Shannon's diversity index and Simpson index by Biodap (Biodiversity Data Analysis Package) analysis software [17].

RESULTS

DNA extraction and PCR amplification. Total DNA was extracted from DHG plants using the CTAB approach. After electrophoresis, two bands of PCR products were displayed on the agarose gel. One band between 700 and 800 bp could be the bacterial 16S rDNA fragments, and the other band between 800 and 900 bp could be the *A. inebrians* mitochondrial 18S rDNA fragments. The purified PCR prod-

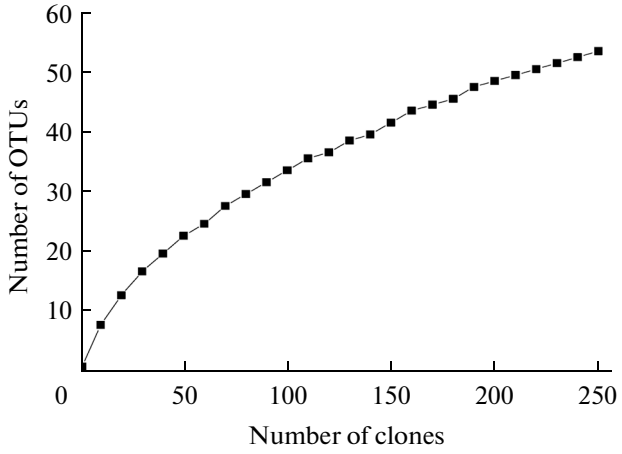

Fig. 1. Rarefaction curve of endophytic bacterial 16S rDNA clone library of *Achnatherum inebrians*.

ucts between 700 and 800 bp were used for constructing a 16S rDNA clone library of DHG endophytic bacteria.

Endophytic bacterial communities analysesIn order to identify the diversity of endophytic bacteria from DHG plants, the 16S rDNA clone library was constructed. Two hundred and forty-nine clones containing the inserts of correct size were verified by colony PCR. All of the positive clones were screened by ARDRA. An OTU was defined as a group of clones that had identical banding patterns obtained from the independent digestions. According to ARDRA patterns, 249 colones were grouped into 57 OTUs. The result of statistical analysis showed that coverage, Shannon diversity index, Evenness index, Simpson index of the clone library were 93.17%, 2.96, 0.91, 0.11, respectively, and the calculated rarefaction curve is shown in Fig. 1.

According to ARDRA results, representative clones of all OTUs were selected for 16S rDNA sequence analysis. In order to confirm the ARDRA validity in this study, 2–3 clones of five randomly selected OTUs were sequenced, which showed that sequences of different clones from the same OTU were uniform. Then one clone selected randomly from each OTU of the rest was sequenced. Using the CHIMERA_CHECK program, two sequences representing two OTUs were identified as chimeric sequences and discarded, so this clone library included 57 OTUs comprising 249 clones.

The phylogenetic analysis of the 249 clones revealed that the majority of clones were affiliated with *Proteobacteria* (57.00%). Other clones belonged to *Flavobacteria* (12.00%), *Actinobacteridae* (7.00%), *Sphingobacteria* (5.00%). 8.00% of the sequences showed high similarity with uncultured bacterial sequences. Details of all OTUs in the clone library are listed in table.

Six predominant OTUs including more than ten clones were found in the clone library (table). Three out of the six predominant OTUs belonged to the *Betaproteobacteria*. Among the three other one represented the phylum *Firmicutes* showing the highest similarity with *Bacillus subtilis* (29 clones), while the other two belonged to *Alphaproteobacteria*.

Phylogenetic analysis of clones. The phylogenetic relationship of clones in comparison to the type strains of related species is demonstrated for three selected bacterial groups (Fig. 2). The sequences related to *Proteobacteria* made up the largest fraction of the clone library, which included alpha, beta, gamma, and delta subgroups. The *Betaproteobacteria* comprising 18 OTUs were the most dominant subgroups of *Proteobacteria* (Fig. 2a). Among them, four predominant groups were related to *Methylophilus* sp., *Janthinobacterium* sp., *Acidovorax* sp. and *Massilia* sp. The sequences related to *Alphaproteobacteria*, the second most abundant fraction, comprised 5 OTUs (Fig. 2a). In the *Alphaproteobacteria*, OTUC1 including 19 clones was identified as *Ochrobactrum* spp., which was the second dominant genus in the clone library. The other dominant OTUs in this subclass was clustered with *Devosia* sp. The *Gammaproteobacteria* were the third abundant subclass (Fig. 2a). Most dominant OTU in the subclass had the highest similarity to *Acinetobacter* sp. The phylogenetic diversity of *Delta-* and *Epsilonproteobacteria* was much less, only 4 OTUs belonged to *Deltaproteobacteria* (Fig. 2a). Except for *Proteobacteria*, the remaining OTUs belonged to Low GC gram positive bacteria division (Fig. 2b), High GC gram positive bacteria division (Fig. 2b), and *Bacteroidetes* (Fig. 2b).

DISCUSSION

Endophytic bacteria ubiquitously inhabit most plant species, however there have been few reports of endophytic bacteria in DHG. To our knowledge, this is the first report that endophytic bacteria associated with DHG are revealed by the culture-independent approach. The results from this study suggest that the diversity of endophytic bacteria is abundant within *Achnatherum inebrians*. The coverage result of the clone library suggested that the unique sequence types sampled from this library approached the total number of unique sequences within the library. In addition, the rarefaction curve also tended to plateau, indicating that this library was large enough to reflect the endophytic bacterial diversity of DHG roots.

The most dominant group in our clone library was related to *Proteobacteria*, which was consistent with other studies [14]. A total of eight genera of *Bacillus*, *Pseudomonas*, *Actinomyces*, *Corynebacterium*, *Acinetobacter*, *Sphingomonas*, *Paenibacillus*, *Phyllobacterium* were isolated from roots, stems, leaves and seeds of health *A. inebrians* by grinding separation method, and the frequency of *Bacillus* species was highest in

Distribution of 16S rRNA detected from endophytes in *Achnatherum inebrians*

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Alphaproteobacteria	5	40	0.16		
	C1	19	0.08	*Ochrobactrum anthropi* (AY917134)	90
	C5	2	0.01	*Phenylobacterium lituiforme* (NR029117)	96
	C19	5	0.02	*Brevundimonas* sp. (FJ197848)	99
	C122	12	0.05	*Devosia insulae* (EF012357)	97
	C152	2	0.01	*Rhizobium galegae* (Z79620)	100
Betaproteobacteria	18	70	0.29		
	C12	11	0.04	*Janthinobacterium agaricidamnosum* (AY167838)	99
	C13	9	0.04	*Acidovorax delafieldii* (AB269774)	100
	C58	2	0.01	*Massilia* sp. (FM955855)	99
	C59	3	0.01	*Massilia brevitalea* (EF546777)	93
	C61	3	0.01	*Massilia aurea* (AM231588)	99
	C62	1	0.01	*Oxalicibacterium faecigallinarum* (AB469788)	90
	C123	1	0.01	*Beta proteobacterium* (GU213399)	97
	C139	2	0.01	*Pelomonas saccharophila* (AM501432)	98
	C149	3	0.01	*Polaromonas rhizosphaerae* (EF127651)	99
	C156	3	0.01	*Massilia niabensis* (EU808006)	99
	C157	1	0.01	*Janthinobacterium* sp. (D84590.2)	98
	C166	4	0.02	*Oxalobacteraceae bacterium* (DQ337591)	90
	C170	1	0.01	*Herbaspirillum rubrisubalbicans* (FJ605419)	94
	C174	12	0.05	*Methylophilus freyburgensis* (AJ517772)	99
	C191	1	0.01	*Janthinobacterium lividum* (EU275366)	90
	C207	2	0.01	*Variovorax paradoxus* (GQ920616)	100
	C254	1	0.01	*Methylibium fulvum* (AB245357)	98
	C309	10	0.04	*Massilia timonae* (AJ871463.1)	98
Gammaproteobacteria	5	20	0.08		
	C6	8	0.03	*Acinetobacter johnsonii* (GQ169068)	100
	C25	4	0.02	*Lysobacter brunescens* (GQ859167)	97
	C57	2	0.01	*Xanthomonas albilineans* (FP565176)	97
	C77	4	0.02	*Perlucidibaca piscinae* (DQ664237)	98
	C226	2	0.01	*Pseudomonas thivervalensis* (EU721607)	100
Deltaproteobacteria	4	5	0.02		
	C7	1	0.01	*Geobacter bremensis* (NR_026076.1)	97
	C146	2	0.01	*Sorangium cellulosum* (GU299045.1)	95
	C160	1	0.01	*Myxobacterium* (AB246770)	95
	C271	1	0.01	*Geobacter bemidjiensis* (CP001124)	98
Epsilonproteobacteria	2	8	0.03		
	C16	5	0.02	*Epsilonproteobacterium* (GQ354902)	94
	C17	3	0.01	*Epsilonproteobacterium* (AB174846)	100
Flavobacteria	5	17	0.12		
	C3	8	0.09	*Algoriphagus* (AB197852)	93
	C108	1	0.03	*Phycicoccus jejuensis* (DQ345443)	93
	C138	1	0.01	*Flavobacterium segetis* (AY581115)	99
	C155	5	0.02	*Flavisolibacter ginsengiterrae* (AB267476)	93
	C159	2	0.01	*Algoriphagus locisalis* (AY835923)	93

Table. (Contd.)

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Sphingobacteria	5	12	0.05		
	C34	6	0.02	*Cyclobacteriaceae bacterium* (FJ798610)	92
	C55	2	0.01	*Niastella koreensis* (DQ244077)	97
	C69	1	0.01	*Terrimonas ferrugineum* (AM230484)	97
	C132	2	0.01	*Algoriphagus hitonicola* (EF488486)	99
	C133	1	0.01	*Sphingobacteriales bacterium* (AB540003)	97
Bacilli	3	31	0.12		
	C11	29	0.11	*Bacillus subtilis* (GQ980239)	98
	C204	1	0.01	*Bacillus niacini* (EU221338)	99
	C315	1	0.01	*Bacillus licheniformis* (GU323372)	99
Actinobacteridae	4	17	0.07		
	C144	1	0.01	*Actinoplanes nipponensis* (AB047498)	94
	C161	4	0.02	*Arthrobacter sulfonivorans* (FM955888)	100
	C268	5	0.02	*Rhodococcus* sp. (DQ337546)	99
	C286	5	0.02	*Actinobacterium* (GU167988)	99
Uncultured bacteria	5	19	0.08		
	C30	3	0.01	Uncultured marine bacterium clone (FJ826007)	99
	C88	2	0.01	Uncultured actinobacterium clone (EF220368)	99
	C182	7	0.03	Uncultured prokaryote clone (GU208351)	97
	C224	1	0.01	Uncultured bacterium clone (AF422685)	93
	C225	6	0.02	Uncultured Haliangiaceae bacterium clone (EU665118)	96

eight genera, accounting for 58.4% of the total number of endophytic bacteria [19], which was inconsistent with our studies using the 16S rDNA library technique. However, the proportion of subgroups of *Proteobacteria* was different in endophyte clone libraries of various plants. In some studies on the endophytes by culture-independent approaches, *Deltaproteobacteria* were rarely reported. It is indicated that the diversity of endophytic bacteria was abundant in DHG roots, and there might be some extent of specificity between the endophytes and their host plants.

The most dominant group in our clone library was related to *Bacillus subtilis* including 29 clones, which was consistent with our previous study [19]. By the culture-dependent method, *B. subtilis* was isolated as endophytes from cotton roots and stems [20]. *B. subtilis* plays important roles in agricultural production as a plant growth-promoting bacterium, which could suppress disease development by secretion of antibiotics, production of extracellular enzymes such as protease and chitinase [21], and potential root colonization [22].

In this clone library, some sequences had high identity with *Rhizobium galegae*, *Acidovorax delafieldii*, *Variovorax paradoxus*, *Acinetobacter johnsonii*, *Pseudomonas thivervalensis*, and *Arthrobacter sulfonivorans*. To our knowledge, these strains have not been observed previously as an endophytic bacterium. In addition, sequences of some clones showed low identity with the cultured bacterial genera but high identity with the uncultured bacteria, revealing the presence of some uncultured bacteria in the DHG endophytic bacterial community. The OTUC122 was related to *Devosia insulae* with 97% identity of 16S rDNA partial sequence. The result indicates that this bacterium is likely to be a potential novel species.

Nevertheless, studies on endophytic bacterial diversity by 16S rDNA cloning and sequencing have some limitations. The biases in genomic DNA extrac-

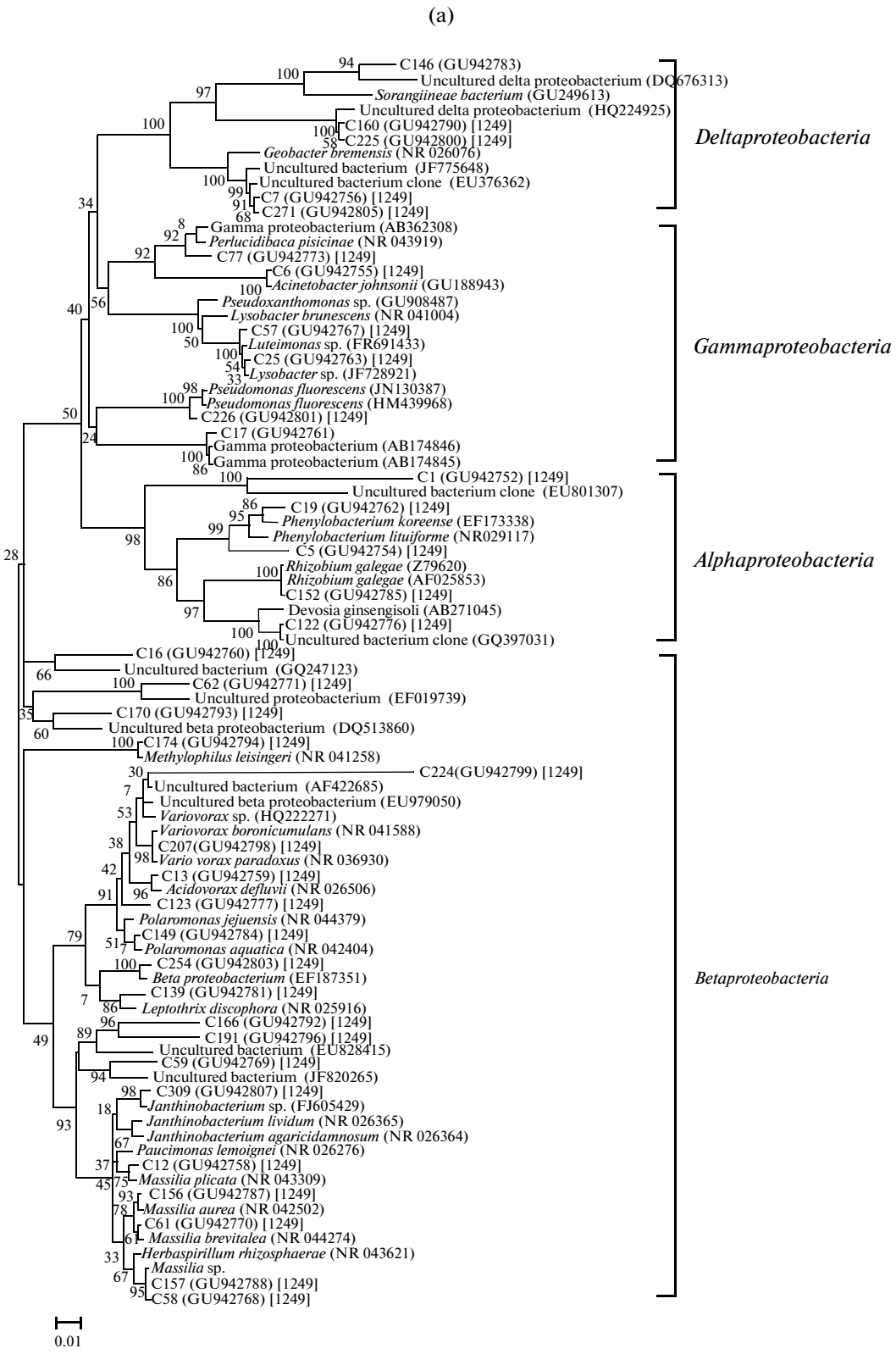

Fig. 2. 16S rRNA based dendrogram showing the phylogenetic relationship of clones from *Achnatherum inebrians*. Phylogenies were inferred using neighbor-joining analysis and trees were generated using MEGA 5 software. Numbers in parentheses represent the sequence accession numbers in GenBank. Numbers in square brackets indicate the clone number out of the total clones. Numbers at branch points indicate bootstrap values. The scale bar represents a 1 and 2% estimated difference in nucleotide sequence. (a) α-Proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria bacteria; (b) High G+C Gram positive bacteria, Bacteroidetes and Low G+C Gram positive bacteria.

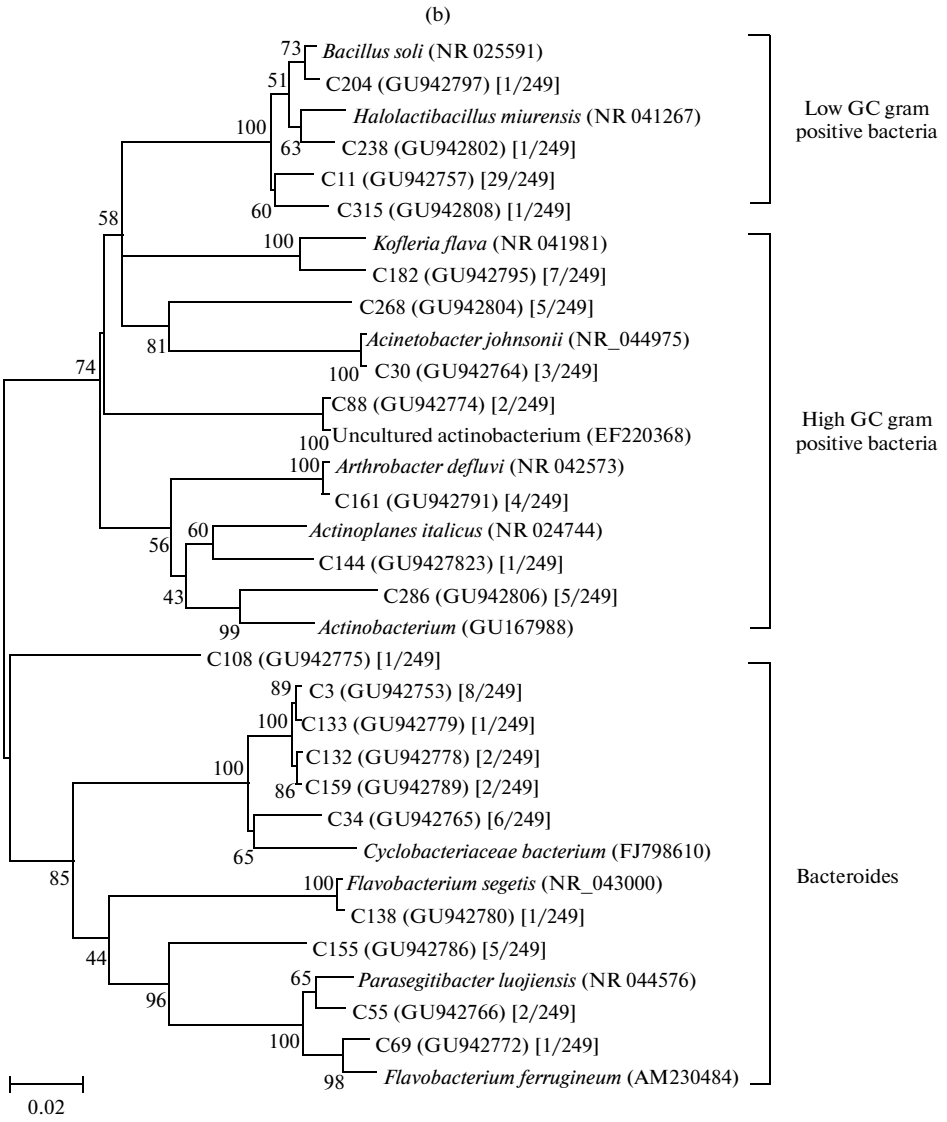

Fig. 2. Contd.

tion, PCR amplification, and cellular rDNA copy number were well known limitations for all molecular approaches relying on PCR amplification of rRNA genes from genomic DNA [23–25]. In addition, the cloning sequences usually could not provide any information on the function of the individual community members. Therefore, combining the above approach with quantitative techniques, such as FISH or RT-PCR of functional genes, may explore the dominant populations more effectively. Although the approach of library construction may distort the structure of a bacterial community to some extent, it is a practicable method to provide better knowledge about the endophytic bacterial diversity and a preview for obtaining the endophytic cultures. It was also found that the DHG endophytic community was very complex and a lot of uncultured DHG endophytes could be interesting subjects for further exploration.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (project no. 31060018).

REFERENCES

1. Kloepper, J.W. and Beauchamp, C.J., A review of issues related to measuring colonization of plant roots by bacteria, *Can. J. Microbiol.*, 1992, vol. 38, pp. 1219–1232.
2. Bartram, A.K., Jiang, X., Lynch, M.D., Masella, A.P., Nicol, G.W., Dushoff, J., and Neufeld, J.D., Exploring links between pH and bacterial community composition in soils from the Craibstone Experimental Farm,

FEMS Microbiol. Ecol., 2013. doi: 10.1111/1574-6941.12231

3. Aravindraja, C. Viszwapriya, D., and Karutha Pandian, S., Ultradeep 16S rRNA sequencing analysis of geographically similar but diverse unexplored marine samples reveal varied bacterial community composition, *PLoS One*, 2013, 8:e76724.

4. Singh, A.K., Rai, G.K., Singh, M., and Dubey, S.K., Bacterial community structure in the rhizosphere of a Cry1Ac Bt-Brinjal crop and comparison to its non-transgenic counterpart in the tropical soil, *Microb. Ecol.*, 2013, vol. 66, pp. 927–939.

5. Coton, M., Joffraud, J.J., Mekhtiche, L., Leroi, F., and Coton, E., Biodiversity and dynamics of the bacterial community of packaged king scallop (*Pecten maximus*) meat during cold storage, *Food Microbiol.*, 2013, vol. 35, pp. 99–107.

6. Heilig, H.G., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D., and de Vos, W.M., Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA, *Appl. Environ. Microbiol.*, 2002, vol. 68, pp. 114–123.

7. Li, F., Effects of endophyte infection on drought resistance to drunken horse grass (*Achnatherum inebrians*), *Master Thesis*, Lanzhou Univ., China, 2007.

8. Gou, X.Y., Effect of *Neotyphodium endophyte* on salt tolerance to drunken horse grass (*Achnatherum inebrians*), *Master Thesis*, Lanzhou Univ., China, 2007.

9. Chen, N., Genetic diversity of drunken horse grass (*Achnatherum inebrians*) and effects of its endophyte infection on cold tolerance, *Master Thesis*, Lanzhou Univ., China, 2007.

10. Li, C.J., Nan, Z.B., and Li, F., Biological and physiological characteristics of *Neotyphodium gansuense* symbiotic with *Achnatherum inebrians, Microbiol. Res.*, 2008, vol. 163, pp. 431–440.

11. Li, C.J., Zhang, X.X., Li, F., Nan, Z.B., and Schardl, C.L., Disease and pests resistance of endophyte infected and non-infected drunken horse grass, in *Proc. 6th Int. Symp. Fungal Endophytes of Grasses*, Popau, A. and Thom, E.R., Eds., Dunedin, New Zealand, 2007, pp. 111–114.

12. Zhang, X.X. and Li, C.J., Effects on bird cherry-oat aphid resistance to drunken horse grass by *Neotyphodium endophyte* infection, in *Multi Functional Grasslands in a Changing World, vol. 2*, Guangzhou: Guangdong People's Publishing House, 2008, p. 833.

13. Xie, Z.W., Ge, S., and Hong, D.Y., Preparation of DNA from silica gel dried mini-amount of leaves of *Oryza rufipogon* for RAPD study and total DNA bank construction, *Acta Botanica Sinica,* 1999, vol. 41, pp. 802–807.

14. Li, Y.H., Zhu, J.N., Zhai, Z.H., and Zhang, Q., Endophytic bacterial diversity in roots of *Phragmites australis* in constructed, Beijing Cuihu Wetland (China), *FEMS Microbiol. Lett.*, 2010, vol. 309, pp. 84–93.

15. Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, G.M., Jr., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M., and Woese, C.R., A new version of the RDP (Ribosomal Database Project), *Nucl. Acids Res.*, 1999, vol. 27, pp. 171–173.

16. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 2011, vol. 28, pp. 2731–2739.

17. Thomas, G.M., BIO-DAP. A biodiversity analysis package, 2000, downloadable from: http://nhsbig.inhs.uiuc. edu/ www/populations.html

18. Good, I.L., The population frequencies of species and the estimation of population parameters, *Biometrika*, 1953, vol. 40, pp. 237–264.

19. Zhang, X.B., Shi, Y.W., Wang, X.X., Zhang, W., and Lou, K., Isolation, identification and insecticidal activity of endophyte from *Achnatherum inebrians, Acta Microbiologica Sinica*, 2010, vol. 50, pp. 530–536.

20. McInroy, J.A. and Kloepper, J.W., Survey of indigenous bacterial endophytes from cotton and sweet corn, *Plant and Soil*, 1995, vol. 173, pp. 337–342.

21. Seo, W.T., Lim, W.J., Kim, E.J., Yun, H.D., Lee, Y.H., and Cho, K.M., Endophytic bacterial diversity in the young radish and their antimicrobial activity against pathogens, *J. Korean Soc. Appl. Biol. Chem.*, 2010, vol. 5, pp. 493–503.

22. Li, S.Q., Zhang, N., Zhang, Z.H., Luo, J., Shen, B., Zhang, R.F., and Shen, Q.R., Antagonist *Bacillus subtilis* HJ5 controls *Verticillium* wilt of cotton by root colonization and biofilm formation, *Biol. Fertil. Soils*, 2013, vol. 49. pp. 295–303.

23. Farrelly, V., Rainey, F.A., and Stackebrandt, E., Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species, *Appl. Environ. Microbiol.*, 1995, vol. 61. pp. 2798–2801.

24. More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C., and Madsen, E.L., Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 1572–1580.

25. Suzuki, M.T. and Giovannoni, S.J., Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 625–630.