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Diversity and phylogeny of bacteria on Zimbabwe tobacco leaves estimated by 16S rRNA sequence analysis

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Abstract Microorganisms play important roles in the tobacco aging process. However, microbial communities on flue-cured tobacco leaves (FCTL) remain largely unknown. In this study, the total microbial genomic DNA of unaged and aging FCTL from Zimbabwe were isolated using a culture-independent method, and the bacterial communities were investigated through analyzing two 16S rRNA gene libraries. Eighty-four and 65 operational taxonomic units were obtained from the libraries of the unaged and aging FCTL, respectively. The following genera were represented more than 4% in both libraries (aging and unaged library): Sphingomonas (4.84%, 4.18%), Stenotrophomonas (4.84%, 5.23%), Erwinia (5.81%, 4.88%), Pantoea (19.35%, 18.47%), and Pseudomonas (21.29%, 24.04%). The dominant species varied between the two libraries. Specifically, several dominant species in unaged FCTL including Pseudomonas fulva, Pseudomonas sp. (AM909658),

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W. Zhe · Y. Duan (⊠) Technology Centre of Hongyun Honghe Tobacco (Group) Co., Ltd., Kunming 650202, People's Republic of China e-mail: dyanqing@yahoo.com.cn *Klebsiella* sp. (HM584796), and *Pantoea* sp. (AY501386) were not identified in aging FCTL, while several dominant species in aging FCTL such as *Pantoea* sp. (GU566350), *Pseudomonas* sp. (EF157292), and *Buttiauxella izardii* were not found in unaged FCTL. The phylogenetic analysis showed that bacteria from unaged and aging FCTL were divided into two clades, and two unique subclades were identified in aging FCTL. Our results revealed for the first time the bacterial diversities on Zimbabwe tobacco, and provided a basis for clarifying the roles of bacteria in aging process of FCTL.

Keywords Zimbabwe tobacco · 16S rRNA clone library· Culture-independent method · Bacterial diversity· Phylogenetic analysis

Introduction

Tobacco is economically the most important nonfood crop worldwide. The flue-cured tobacco leaves (FCTL) is one of the most important type of tobacco in the world, including China. Cured but unaged tobacco leaves have a sharp, disagreeable odor and an undesirable aroma, and produce harsh, irritating smoke. In the industrial production, a further process called fermentation or aging is typically applied to improve the quality of the FCTL (Guo et al. 2004). Aging greatly improves the aroma and color, reduces irritating smoke, and improves the overall tobacco quality (Peng et al. 2009). Tobacco fermentation is a very complicated process, and it has been linked to the enzymatic actions of bacteria, fungi, and other chemical interactions within the leaves (Jensen and Parmele 1950). Tobacco fermentation can be divided into artificial and natural fermentation. Compared to natural aging, artificial fermentation can shorten the fermentation period and reduce cost (Guo et al. 2004). However, because little is known about the number and type of microorganisms appropriate for conducive aging, it has been difficult to control artificial fermentation (Yang et al. 2008). Tamayo and Cancho (1953) were the first to inoculate tobacco leaves with microorganisms to improve the aroma. In recent years, increasing reports showed these microorganisms on FCTL can accelerate the aging process and improve the quality of tobacco leaves through its growth activities (Han and Ye 1997; Yang et al. 2008; Huang et al. 2010a).

The conventional media and culture condition are not appropriate for the growth of most microorganisms in nature, and more than 85% microbes cannot be obtained in pure culture (Amann et al. 1995). The development of molecular biology techniques has provided a useful method to study these uncultured microorganisms. Cultureindependent 16S rRNA gene sequence analysis has been widely used to identify microbial diversities in soil (Williamson et al. 2003), air (Tringe et al. 2008), animal gut (Hill et al. 2005), and other environments. Recently, Huang et al. (2010b) and Zhao et al. (2007) analyzed the bacterial diversities of several FCTLs using the cultureindependent method. The analyzed tobacco leaves include several widely cultivated in China such as K326, Zhongyan 100, NC89, and Zhongyan 101. However, microbial communities in several other important tobacco leaves, such as Zimbabwe and Brazil FCTL, are not fully understood. Zimbabwe has natural conditions for the production of high-quality FCTL, and Zimbabwe tobacco leaves are the main materials for producing the high-grade cigarette. Understanding the microbial communities of Zimbabwe tobacco leaves would be very important for effectively controlling the tobacco aging process and improving the quality of FCTL. In this study, the total microbial genomic DNAs were isolated from the unaged and aging Zimbabwe FCTL, and the bacterial communities of these two samples were analyzed and compared by their 16S rRNA clone libraries. Moreover, the phylogenetic trees of bacteria in unaged and aging FCTL were constructed based on the 16S rRNA sequences.

Materials and methods

Sampling of tobacco

Unaged and aging Zimbabwe tobacco leaves were sampled

DNA extraction from the microbial community of tobacco

Sixty grams of tobacco leaves were divided into three equal parts and placed in three flasks with 250 mL sterilized 0.1 M phosphate buffer (pH 7.0) for 30 min, respectively. Later, the tobacco leaves were washed with a sonicator for 10 min, and the microorganisms were collected by centrifugation at $10,000 \times g$ for 30 min. The microbial genomic DNA was extracted according to our recently described protocol (Huang et al. 2010b). The genomic DNA isolated from three samples were mixed and used as template for 16S rRNA gene amplification.

Amplification of the bacterial 16S rRNA genes

Primers 799f (5'-GGTAGTCCACGCCGTAAACGATG-3'; position 781 through 799 according to *Escherichia coli* number) and 1492r (5'-GGTTACCTTGTTACGACTT-3'; position 1492 through 1,510 according to *E. coli* number) were selected to amplify the 16S rRNA of bacteria. These primers are specific to bacteria and have a low affinity for chloroplast DNA (Jurkevitch et al. 2000). The PCR amplification was done according to the conditions described by Sun et al. (2008), and the band size of approximately 715 bp was purified.

Construction of the 16S rRNA clone library

The purified PCR products were ligated into the pMD18-T vector (Takara, Japan) and transformed into competent cells (*E. coli* DH5 α) to construct the 16S rRNA clone library. In order to identify the bacterial diversity on unaged and aging tobacco samples, two 16S rRNA clone libraries were respectively constructed. Inserts of 16S rRNA genes from recombinant clones were re-amplified using universal primers M13 and RV. Products of PCR amplification were purified and sequenced with a 3730-nucleotide sequencer (ABI, USA).

Analysis of the 16S rRNA sequence data

Chimeric sequences were identified using the B2C2 program (Gontcharova et al. 2010). The resulting sequences were fully aligned with ClustalX (Thompson et al. 1997). On the basis of the alignment, a distance matrix was constructed by using the DNADIST program from PHYLIP (ver. 3.66, Felsenstein 1989) with the default parameters. The program DOTUR with the furthest neighbor algorithm was used to group sequences into operational taxonomical units (OTUs) or phylotypes that represented the number of 16S rRNA sequence similarity groupings (Schloss et al. 2005). A 97% cutoff value was used so that sequences with more than 97% similarity were considered the same. A

sequence was selected randomly from each OTU and compared with those available sequences in GenBank using the BLAST program to determine their approximate phylogenetic affiliation and sequence similarities (Altschul et al. 1990). The diversity of the clone libraries was investigated by rarefaction analysis, and the rarefaction curves were calculated using the RarefactWin program (Holland 2004).

Phylogenetic analysis

Nucleotide sequences were aligned initially using ClustalX (Thompson et al. 1997) and then manually adjusted. Distance matrices and phylogenetic trees were calculated according to the Kimura two-parameter model (Kimura 1980) and the neighbor-joining (Saitou and Nei 1987) algorithm using the MEGA (version 4.0) software packages (Tamura et al. 2007). One thousand bootstraps were performed to assign confidence levels to the nodes in the trees.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper were deposited in GenBank with accession numbers JF820125-JF820273.

Results

Statistical analysis of the clone library

According to the Phylipwx program, the 303 16S rRNA clones from the unaged FCTL were grouped into 87 OTUs, and the 316 clones from the aging FCTL sample were grouped into 66 OTUs. Through the BLAST analysis, three OTUs containing 16 clones in unaged FCTL and one OTU containing 6 clones in aging FCTL belonged to the tobacco chloroplast. The calculated rarefaction curve (Fig. 1) showed that the two samples are close to their respective OTU saturation curve, indicating that the clone libraries already included most of the dominant bacteria species on tobacco samples.

Analyses of bacterial communities and dominant species on unaged and aging FCTL

As shown in Table 1, the two samples contained a large diversity of bacteria, which were divided mainly into Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Twenty-two genera including *Pseudomonas, Bacillus, Rhizobium, Sphingomonas, Achromobacter, Methylobacteriun, Stenotrophomonas*, and *Buttiauxella* were detected in



Fig. 1 Rarefaction curve of bacterial 16S rRNA clone library of the unaged and aging Zimbabwe FCTL

both the unaged and aging FCTL; these genera contained 49 OTUs (81.88% clone ratio) and 37 OTUs (81.61% clone ratio) in unaged and aging FCTL, respectively. Eight genera including *Roseomonas, Novosphingobium*, and *Xanthomonas* were only detected in the unaged FCTL and these genera contained 10 OTUs and 5.57% of the total clone library. In contrast, 16 genera including *Agrobacterium, Curtobacterium, Arthrobacter, Flavobacterium*, and *Actinomyces* were only detected in aging FCTL, which consisted of 19 OTUs and 11.94% of the total clone library.

In both samples, the genera with greater than 4% of the clone libraries were Sphingomonas, Stenotrophomonas, Erwinia, Pantoea, and Pseudomonas, and the most dominant genera were Pantoea and Pseudomonas, representing 18.47-24.04% of the clone libraries. The main OTUs in the genus Pantoea included Pantoea vagans and Pantoea sp. (GU566350), and those in the genus Pseudomonas included Pseudomonas putida, Pseudomonas fulva, Pseudomonas sp. (AM909658), and Pseudomonas sp. (EF157292). The OTUs of Pseudomonas decreased significantly from 11 to 3 after aging treatment. Moreover, the percentages of Buttiauxella and Klebsiella differed significantly between the two samples: Buttiauxella was represented by 0.35% in unaged FCTL and by 7.10% in aging FCTL; Klebsiella was represented by 6.62% in unaged FCTL and by 0.65% in aging FCTL.

The dominant species (i.e., those present in greater than 3% of the libraries) were also variable between the two samples (Table 2). *P. vagans* and *Pseudomonas* sp. (AM909658) were both present by more than 10% in unaged FCTL, while *Pantoea* sp. (GU566350) and *Pseudomonas* sp. (EF157292) were represented by more than 10% in aging FCTL. *P. vagans* decreased from 14.63% to 6.77% after aging treatment, while *P. putida* increased from 2.79% to 9.03%. *Erwinia* sp. (EU336938) and *Stenotrophomonas maltophilia* were equally dominant in these two

Table 1 Bacterial communities in aging and unaged Zimbabwe FCTL

Name	Aging	Aging FCTL			I FCTL	1	GenBank accession nos.	Identity	OTU number
	No. of clones	Ratio (%)	OTUs	No. of clones	Ratio (%)	OTUs		(%)	
Aerococcus				1	0.35	1			
Aerococcus sp.				1	0.35	1	AM292070	99	09-192
Cronobacter				1	0.35	1			
Cronobacter dublinensis				1	0.35	1	GU122214	100	09-265
Herbaspirillum				1	0.35	1			
Herbaspirillum sp.				1	0.35	1	GU201556	95	09-197
Roseomonas				1	0.35	1			
Roseomonas genomospecies				1	0.35	1	AY150049	95	09-026
Shigella				1	0.35	1			
Shigella sp.				1	0.35	1	EF600631	95	09-329
Novosphingobium				2	0.70	2			
Novosphingobium subarcticum				1	0.35	1	AY151394	99	09-304
Novosphingobium sp.				1	0.35	1	GU086416	98	09-102
Serratia				3	1.05	2			
Serratia sp.				3	1.05	2	GU124497	97/95	09-263/09-061
Xanthomonas				6	2.09	1			
Xanthomonas campestris				6	2.09	1	GU086451	99	09-188
Paenibacillus	6	1.94	3						
Paenibacillus hodogavensis	1	0.32	1				AB179866	96	07-003
Paenibacillus sp.	4	1.29	1				EU741039	99	07-341
Paenibacillus sp.	1	0.32	1				HM233974	99	07-366
Mesorhizohium	2	0.65	1				11111200971		0,200
Mesorhizobium sp	2	0.65	1				F1529846	99	07-171
Agrahacterium	1	0.32	1				10020010	,,	07 171
Agrobacterium sp	1	0.32	1				GU086439	98	07-314
Arcobacter	1	0.32	1				00000129	20	07.511
Arcobacter sp	1	0.32	1				DO234101	99	07-217
Bacteriovorax	1	0.32	1				5251101	,,	0, 21,
Bacteriovorax sp	1	0.32	1				AY294222	99	07-282
Bosea	1	0.32	1					,,	07 202
Bosea sp	1	0.32	1				AF531764	99	07-246
Curtobacterium	1	0.32	1				11 331701	,,	07 210
Curtobacterium sp	1	0.32	1				FF411134	99	07-027
Morvella	1	0.32	1					,,	01 021
Morvella indoligenes	1	0.32	1				AF527773	99	07-276
Sphingomonas	1	0.32	1				111 521115	,,	07 270
Sphingomonas hacterium	1	0.32	1				F1932666	98	07-305
Arthrohacter	2	0.52	1				13732000	70	07-505
Arthrobacter chlorophonolicus	2	0.65	1				AB405170	00	07.059
Annrobacter chiorophenolicus	2	0.65	1				AD495170	<u>,,,</u>	07-039
Asaia brungthanansis	2	0.65	1				A D 202240	00	07 222
лыши кrunginepensis Futerococcus	∠ 2	0.05	1				AD272240	37	07-323
Enterococcus sp	2	0.05	1				HO264083	100	07 117
Envelococcus sp.	2	0.65	1 2				11Q204003	100	0/-11/
Flavohactarium liko en	∠ 1	0.05	∠ 1				A F385540	07	07 205
Flavobacterium-like sp.	1	0.32	1				AT 303347	プ/ 07	07-203
riavobacterium sp.	1	0.32	1				HQ32918/	91	07-389

Table 1 (continued)

No. of Ratio OTUS No. of Ratio No. of	Name	Aging FCTL			Unaged FCTL			GenBank accession nos.	Identity	OTU number	
Actionanyces sp.82.581IUUUActionanyces sp.82.681UUVall1589907-089Padobacter20.651UAM4913719907-148Parbactectic hartonias20.651UNA4913719907-148Badococcus41.2910.351FIP346699/9707-041/09-085Macrobacterinin10.3210.351FIP33909609-396Microbacterinin sp.10.321UAB4807629907-391Saccharibactifiks karelraxi41.291UEU0452569509-146Enternbacter closcae-41.391HM0307489509-146Enternbacter closcae41.391HM0307489907-150Enternbacter closcae10.351EU0475569509-142Enternbacter lownaechei10.351EU047569907-150Bacillus spingle10.321FIP34499907-150Bacillus spingle10.32110.351EU047569907-150Bacillus spingle10.32110.351EU047569907-150Bacillus spingle10.32110.351E		No. of clones	Ratio (%)	OTUs	No. of clones	Ratio (%)	OTUs		(%)		
Actimonyces sp.82.581II </td <td>Actinomyces</td> <td>8</td> <td>2.58</td> <td>1</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>	Actinomyces	8	2.58	1							
Pedobacter harronins20.651.Ma49137190.7-148Pedobacter harronins20.65110.351F19734669/070.7-148Rhodeocccus sp.41.2910.351F19734669/070.7-148Rhodeocccus sp.41.2910.351EF6239999609-296Microbacterium sp.10.3210.351EF6239999609-296Microbacterium sp.10.321UVEF6239999609-296Scacharibacillis kuerlensis41.291U3<1EU0462709807-290Enterobacter obrane41.291U3<1EU045769809-186Enterobacter barca40.32140.351EU045769609-242Enterobacter barca40.321U0.351EU045769609-186Enterobacter barca20.321U0.351EU045769609-16Bacillus sp.20.3210.351EU045769609-16Bacillus sp.20.3210.351EU7349896/0609-21/07141Bacillus sp.20.55110.351EU7349896/0609-01/071451Bacillus sp.20.62110.351 <th< td=""><td>Actinomyces sp.</td><td>8</td><td>2.58</td><td>1</td><td></td><td></td><td></td><td>EU341158</td><td>99</td><td>07-089</td></th<>	Actinomyces sp.	8	2.58	1				EU341158	99	07-089	
Pacholactor harinanias20.651	Pedobacter	2	0.65	1							
Rhodeneccus sp.41.29110.351FUT346699/9707-041/09-085Rhodeneccus sp.41.2910.351F17346699/9707-041/09-085Microbacterium profindi10.351F1623999609-296Microbacterium sp.10.321-NNNNSaccharibacilla kuerlensis41.291-EU0462709607-291Saccharibacilla kuerlensis41.291-EU0462709607-201Enterobacter loance41.391HM0307489509-186Enterobacter hormaceled41.391HM0307489509-186Enterobacter hormaceled41.391EU0475569609-2142Enterobacter hormaceled10.321-EU04864279807-159Bacillus sp.10.351EU047349896/9609-021/07-141Bacillus subfils10.3210.351EU047349896/9609-021/07-141Batilus subfils10.351EU047349896/9609-021/07-1411Batilus subfils27.1010.351EU047349896/9609-021/07-141Batilus subfils27.1010.351EU047349896/9609-021/07-141Batilus su	Pedobacter hartonius	2	0.65	1				AM491371	99	07-148	
Rhodococcus sp.41.29110.351F197346699//9707-041//09-085Microbacterium prifundi10.32110.351EF623999609-296Microbacterium sp.10.3211EF623999907-291Saccharibacillus kuelensis41.2911EU0462708807-290Saccharibacillus kuelensis41.29151.421EU0462709809-186Enterobacter kormacchei10.32151.4EU0475569509-186Enterobacter kormacchei10.32151EU0475569509-242Enterobacter kormacchei10.3211EU047569009-016Bacillus simplex10.3211GU351GU3660399009-016Bacillus simplex10.3211GU364179807-159Bacillus simplex10.3211GU364179807-159Rodobacter sp.20.65110.351EU031489607-159Rodobacter sp.20.65110.351EU031499909-016Rodobacter sp.20.65110.351EU031499909-016Rodobacter sp.20.65110.351EU0314999 <td>Rhodococcus</td> <td>4</td> <td>1.29</td> <td>1</td> <td>1</td> <td>0.35</td> <td>1</td> <td></td> <td></td> <td></td>	Rhodococcus	4	1.29	1	1	0.35	1				
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Enterobacter sp. 1 0.32 1 U <thu< th=""> U <thu< th=""></thu<></thu<>	Enterobacter cloacae				4	1.39	1	HM030748	95	09-186	
Enerobacter sp. 1 0.32 1	Enterobacter hormaechei				1	0.35	1	EU047556	95	09-242	
Bacillus sp.20.65210.351IBacillus sp.10.321IGU360399909-016Bacillus simplex10.321GU360399807-159Bacillus simplex10.321FJ9844499907-150Rhodobacter sp.20.65110.351IRhidobacter sp.20.65110.351EU70349896/9609-021//07-141Butiauxella227.10110.351EU7034989607-345Butiauxella warmboldiae27.10110.351NR0253319907-345Phyllobacterium20.65120.701F1787859/9909-266//07-002Klebsiella20.651196.6211110.55Klebsiella sp.1196.6211110.5511Aurantinonas30.97131.052111096.6211Rhizobiales bacterium30.97131.05211110.351110050119009-266//07-032Klebsiella sp.10.551131.05211111111111111	Enterobacter sp.	1	0.32	1				FJ950692	96	07-308	
Bacillus sp. 1 0.32 1 CU366039 99 09-016 Bacillus sinplex 1 0.32 1 50.20 GU086427 98 07-159 Bacillus subtilis 1 0.32 1 5784449 99 07-150 Bacillus subtilis 2 0.65 1 1 0.35 1 EUT070498 96/96 09-021/07-141 Buttiaucella 22 7.10 1 1 0.35 1 EUT070498 96/96 09-021/07-141 Buttiaucella 22 7.10 1 1 0.35 1 EUT070498 96/96 09-021/07-141 Buttiaucella 22 7.00 1 1 0.35 1 NR025331 98 09-097 Phyllobacterium 2 0.65 1 2 0.70 1 FJ178785 99/99 09-266/07-002 Klebsiella sp. 2 0.65 1 2 0.70 1 HMS4796 99 09-037 <td>Bacillus</td> <td>2</td> <td>0.65</td> <td>2</td> <td>1</td> <td>0.35</td> <td>1</td> <td></td> <td></td> <td></td>	Bacillus	2	0.65	2	1	0.35	1				
Bacillus subilis10.321 \cdot GU0864279807-159Bacillus subilis10.321 \cdot FJ9844499907-150Rhodobacter20.65110.351EU70349896/9609-021/07-141Rhodobacter sp.20.65110.351EU70349896/9609-021/07-141Buttauxella warmboldiae227.10110.351NR0288939809-097Phyllobacterium20.65120.701FJ17878599/9909-266/07-002Klebsiella sp.20.65120.701FJ17878599/9909-266/07-002Klebsiella sp.20.65120.701FJ17878599/9909-266/07-002Klebsiella sp.196.621HM5847969909-057Klebsiella sp.1931.0521FJ00501196/9609-093/07-352Murantimonas30.97120.701FJ00501196/9609-093/07-352Rhizobium sp10.351EV244069909-340Rhizobium sp10.351EV24369909-327Sphingobacterium sp.20.651-EF3637159909-337Sphingobacterium sp.20.651-EF4243799	Bacillus sp.				1	0.35	1	GU366039	99	09-016	
Bacillus subtilis10.321FJ9844499907-150Rhodobacter20.65110.351FU70349896/9609-021/07-141Rhodobacter sp.20.65110.351EU70349896/9609-021/07-141Butiauxella227.10110.351EU7034989907-345Butiauxella varmboldiae10.351NR0288939809-097Phyllobacterium sp.20.65120.701FJ17878599/9909-266/07-002Klebsiella sp.20.65120.701FJ17878599/9909-266/07-002Klebsiella sp.20.65120.701FJ050119909-057Aurantimonas30.97131.052110.35Aurantimonas treilytica10.351DQ8838109909-222Rhizobium sp.30.97151.742Rhizobium soli30.97151HM2244069909-340Rhizobium sp.20.651EU6943899909-022Rhizobium soli30.97151EU6943899909-034Rhizobium soli30.97151EU6943699909-340Sphingobacterium sp.20.651E	Bacillus simplex	1	0.32	1				GU086427	98	07-159	
Rhodobacter 2 0.65 1 1 0.35 1 EUT03498 96/96 09-021/07-141 Rhodobacter sp. 2 0.65 1 1 0.35 1 EUT03498 96/96 09-021/07-141 Buttiauxella 22 7.10 1 0.35 1 EUT03498 99 07-345 Buttiauxella warnboldiae 2 0.65 1 2 0.70 1 NR028893 98 09-097 Phyllobacterium 2 0.65 1 2 0.70 1 F178785 9//99 09-266/07-002 Klebsiella 2 0.65 1 2 0.70 1 HM584796 99 09-057 Klebsiella sp. 2 0.65 1 3 1.05 2 3 3 0.71 2 3 1.05 2 3 3 0.71 2 3 1.05 1 PM224406 9 0-340 3 0-021/07-352 3 <	Bacillus subtilis	1	0.32	1				FJ984449	99	07-150	
Rhodobacter sp. 2 0.65 1 1 0.35 1 EU703498 96/96 09-021/07-141 Buttiauxella 22 7.10 1 1 0.35 1 EU703498 99 07-345 B. tandii 22 7.10 1 0.35 1 NR025331 99 09-097 Buttiauxella warnboldiae 2 0.65 1 2 0.70 1 NR028893 98 09-097 Phyllobacterium sp. 2 0.65 1 2 0.70 1 FJ178785 99/99 09-266/07-002 Klebsiella sp. 2 0.65 1 19 6.62 1 HM584796 99 09-057 Klebsiella sp. 2 0.65 1 3 1.05 2 7 1 3 0.95 2 Rhizobiales bacterium 3 0.97 1 3 1.05 1 DQ883810 99 09-03/07-352 Rhizobium sp. 2 0.70 1 5 1.74 2 2 0.70 1 EU64	Rhodobacter	2	0.65	1	1	0.35	1				
Buttiauxella227.101110.351B. izardii227.1011 1.035 1NR0253319907-345Buttiauxella warnboldiae10.351NR0288939809-097Phyllobacterium20.65120.701FJ17878599/9909-266//07-002Klebsiella20.65120.701FJ17878599/9909-266//07-002Klebsiella sp.20.65120.701FJ17878599/9909-266//07-002Klebsiella sp.20.6511NY9429489607-195Aurantimonas30.97131.052Rhizobiales bacterium30.97120.701FJ00501196/9609-093//07-352Rhizobium sp10.351DQ8838109909-222Rhizobium sp31.051HM2244069909-340Rhizobium spl30.97151.742Sphingobacterium sp.20.651DQ8838109909-032Rhizobium soli30.97151.742Sphingobacterium sp.20.651DQ982079907-327Sphingobacterium sp.20.651	Rhodobacter sp.	2	0.65	1	1	0.35	1	EU703498	96//96	09-021//07-141	
B. izardii 22 7.10 1 NR025331 99 07-345 Buttiauxella warmboldiae 1 0.35 1 NR028893 98 09-097 Phyllobacterium sp. 2 0.65 1 2 0.70 1 FJ178785 99//99 09-266//07-002 Klebsiella 2 0.65 1 19 6.62 1 FJ178785 99//99 09-057 Klebsiella sp. 2 0.65 1 19 6.62 1 HM584796 99 09-057 Alrantimonas 3 0.97 1 3 1.05 2 Z 70.6 1 FJ005011 96//96 09-037/07-352 Rhizobiales bacterium 3 0.97 1 2 0.70 1 FJ005011 96//96 09-037/07-352 Rhizobianes ureilytica 3 0.97 1 5 1.74 2 2 7.0 1 EV694389 99 09-327 Rhizobium spi 3 0.97 1 5 1.74 2 EF363715 99 07-332<	Buttiauxella	22	7.10	1	1	0.35	1				
Butitackella warmboldiae10.351NR0288939809-097Phyllobacterium20.65120.701FJ17878599//9909-266//07-002Klebsiella20.65120.701FJ17878599//9909-266//07-002Klebsiella sp.20.651196.621Klebsiella sp.20.651-AY9429489607-195Aurantimonas30.97131.052Rhizobiales bacterium30.97151.742Rhizobium sp10.351DQ8838109909-0340Rhizobium sp31.051HM2244069909-340Rhizobium soli30.97151.742Sphingobacterium sp.20.651-EF3637159907-372Sphingobacterium sp.20.651-EF3637159907-372Sphingobacterium sp.20.651-EF4264379909-331Sphingobacterium sp.20.651Sphingobacterium sp.20.651Sphingobacterium sp.20.651Sphingobacterium sp.20.651 </td <td>B. izardii</td> <td>22</td> <td>7.10</td> <td>1</td> <td></td> <td></td> <td></td> <td>NR025331</td> <td>99</td> <td>07-345</td>	B. izardii	22	7.10	1				NR025331	99	07-345	
Phyllobacterium20.65120.701F117878599//9909-266//07-002 $Phyllobacterium$ sp.20.65120.701F117878599//9909-266//07-002 $Klebsiella$ sp.20.651196.621HMS847969909-057 $Klebsiella$ sp.20.651 \cdot AY9429489607-195 $Aurantimonas$ 30.97131.052 \cdot $Rhizobiales bacterium30.97120.701FJ00501196//9609-093//07-352Itanshan 513-1\cdot10.351DQ8838109909-222Aurantimonas ureilytica\cdot151.742Rhizobium sp.\cdot\cdot30.97151.742Rhizobium sp.\cdot\cdot30.97151.742Rhizobium soli30.97151.742\cdot\cdotSphingobacterium composti\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdotSphingobacterium sp.20.651\cdot$	Buttiauxella warmboldiae				1	0.35	1	NR028893	98	09-097	
Phyllobacterium sp.20.65120.701FJ17878599//9909-266//07-002Klebsiella20.651196.621FJ17878599//9909-266//07-002Klebsiella sp.20.651196.621HM5847969909-057Klebsiella sp.20.651196.621Atya429489607-195Aurantimonas30.97131.052ZZZZZZAurantimonas ureilytica10.351DQ8838109909-092/22Rhizobium sp.30.97151.742Rhizobium sp.20.701FU0501196//9699-0340Rhizobium soli30.97151.742Sphingobacterium composti20.701EU6943899909-0327Sphingobacterium sp.20.651EDQ9842079907-339Sphingobacterium sp.20.651EEPM9584449507-367Sphingobacterium sp.20.651EEPM224369909-030Achromobacter sp.41.29131.051HQ2004119809-030Achromobacter sp.41.29151.742Massilia sp.41.29151.742Ma	Phyllobacterium	2	0.65	1	2	0.70	1				
Lebsiella20.651196.621Klebsiella sp.20.651196.621HM5847969909-057Klebsiella sp.20.651 $AY942948$ 9607-195Aurantimonas30.97131.052 $AY942948$ 9609-093//07-352Rhizobiales bacterium30.97120.701FJ00501196//9609-093//07-352Marantimonas ureilytica10.351DQ8838109909-222Rhizobium sp.30.97151.742Rhizobium sp.30.971EE9637159909-340Rhizobium soli30.971EF3637159907-372Sphingobacterium composti10.351EF1224369709-327Sphingobacterium sp.20.651EM984449507-367Sphingobacterium sp.20.651EM984449507-367Sphingobacterium sp.20.651EM984449507-367Sphingobacterium sp.20.651EM984449507-367Sphingobacterium sp.20.651EM224069909-030Achromobacter sp.31.051HQ2004119809-030Achromobacter sp.41.29151.742Massilia sp.41.291 <td>Phvllobacterium sp.</td> <td>2</td> <td>0.65</td> <td>1</td> <td>2</td> <td>0.70</td> <td>1</td> <td>FJ178785</td> <td>99//99</td> <td>09-266//07-002</td>	Phvllobacterium sp.	2	0.65	1	2	0.70	1	FJ178785	99//99	09-266//07-002	
Klebsiella sp.19 6.62 1HM5847969909-057Klebsiella sp.2 0.65 1 $AY942948$ 96 $07-195$ Aurantimonas3 0.97 13 1.05 2Rhizobiales bacterium3 0.97 12 0.70 1FJ005011 $96/96$ $09-093/07-352$ Aurantimonas weilytica1 0.35 1DQ88381099 $09-222$ Aurantimonas weilytica1 5 1.74 2 Z Rhizobium sp.3 0.97 1 5 1.74 2Rhizobium sp.2 3 1.05 1HM22440699 $09-340$ Rhizobium nuautlense2 0.70 1EU69438999 $09-002$ Rhizobium soli3 0.97 1 Z 3 1.05 2 Sphingobacterium composti1 1.29 2 3 1.05 2 Sphingobacterium sp.2 0.65 1 Y $DQ984207$ 99 $07-339$ Sphingobacterium sp.2 0.65 1 Y $DQ984207$ 99 $07-367$ Sphingobacterium sp.2 0.65 1 Y $DQ984207$ 99 $07-367$ Sphingobacterium sp.2 0.65 1 Y $DQ984207$ 99 $07-367$ Sphingobacterium sp.2 0.65 1 Y $DQ17478$ 95 $07-367$ Sphingobacterium sp.2 0.65 1 </td <td>Klebsiella</td> <td>2</td> <td>0.65</td> <td>1</td> <td>19</td> <td>6.62</td> <td>1</td> <td></td> <td></td> <td></td>	Klebsiella	2	0.65	1	19	6.62	1				
Kitching p20.651ATY 429489607-195Aurantimonas30.97131.052 X X Rhizobiales bacterium30.97120.701FJ00501196/96 9 -093/07-352Tanshan 513-110.351DQ8838109909-222Aurantimonas ureilytica10.351DQ8838109909-222Rhizobium30.97151.742 Z Rhizobium sp.20.701EU6943899909-002Rhizobium huautlense20.701EU6943899909-002Rhizobium soli30.971 $EF363715$ 9907-372Sphingobacterium composti10.351EF1224369709-327Sphingobacterium sp.20.651DQ9842079909-341Achromobacter sp.20.651EF4264379909-341Achromobacter sp.20.651HQ2004119809-030Achromobacter sp.41.29131.051HQ2004119809-030Achromobacter sp.41.29151.742 Z <td>Klebsiella sp.</td> <td></td> <td></td> <td></td> <td>19</td> <td>6.62</td> <td>1</td> <td>HM584796</td> <td>99</td> <td>09-057</td>	Klebsiella sp.				19	6.62	1	HM584796	99	09-057	
Aurantimonas 3 0.97 1 3 1.05 2 Rhizobiales bacterium 3 0.97 1 2 0.70 1 FJ005011 96//96 09-093//07-352 Rhizobiales bacterium 3 0.97 1 2 0.70 1 FJ005011 96//96 09-093//07-352 Rhizobium 3 0.97 1 5 1.74 2 2 Rhizobium sp. 3 0.97 1 5 1.74 2 2 Rhizobium sp. 2 0.70 1 EU694389 99 09-340 Rhizobium soli 3 0.97 1 5 1.74 2 Sphingobacterium soli 3 0.97 1 EE363715 99 07-372 Sphingobacterium composti 1 0.35 1 EF122436 97 09-327 Sphingobacterium sp. 2 0.65 1 DQ984207 99 07-339 Sphingobacterium sp. 2 0.65 1 FM958444 95 07-367 Sphingobacterium	Klebsiella sp	2	0.65	1	.,	0.02		AY942948	96	07-195	
Rhizobiales bacterium 3 0.97 1 2 0.70 1 FJ005011 96//96 09-093//07-352 Rhizobiales bacterium 3 0.97 1 2 0.70 1 FJ005011 96//96 09-093//07-352 Aurantimonas ureilytica 1 0.35 1 DQ883810 99 09-222 Rhizobium 3 0.97 1 5 1.74 2 Rhizobium sp. 3 0.97 1 5 1.74 2 Rhizobium sp. 2 0.70 1 EU694389 99 09-002 Rhizobium soli 3 0.97 1 5 1.74 2 Sphingobacterium soli 3 0.97 1 EU694389 99 09-002 Sphingobacterium sp. 2 0.65 1 EF122436 97 09-327 Sphingobacterium sp. 2 0.65 1 DQ984207 99 07-339 Sphingobacterium sp. 2 0.65 1 EF426437 99 09-341 Achromobacter 4	Aurantimonas	3	0.97	1	3	1.05	2		20	07 190	
Aurantimonas ureilytica1 0.35 1DQ8838109909-222Rhizobium3 0.97 15 1.74 2Rhizobium sp.3 0.97 15 1.74 2Rhizobium huautlense2 0.70 1EU6943899909-002Rhizobium soli3 0.97 1 $= 1.29$ 2 0.70 1EU6943899909-002Rhizobium soli3 0.97 1 $= 1.29$ 2 0.70 1EU6943899909-002Sphingobacterium composti3 0.97 1 $= 1.29$ 2 3 1.05 2 2 2 3 1.05 2 Sphingobacterium sp.2 0.65 1 $= 1.29$ 2 0.7372 2 0.7372 Sphingobacterium sp.2 0.65 1 $= 1.29$ 2 0.35 1EF122436 97 $09-327$ Sphingobacterium sp.2 0.65 1 $= 1.29$ 1.055 1EF426437 99 $09-341$ Achromobacter sp.4 1.29 1 3 1.05 1HQ200411 98 $09-030$ Achromobacter sp.4 1.29 1 5 1.74 2 $= 1.035$ 1 1.035 1 1.035 1.00177478 95 $09-334$	Rhizobiales bacterium Tianshan 513-1	3	0.97	1	2	0.70	1	FJ005011	96//96	09-093//07-352	
Rhizobium3 0.97 15 1.74 2Rhizobium sp.3 1.05 1HM22440699 $09-340$ Rhizobium huautlense2 0.70 1EU69438999 $09-002$ Rhizobium soli3 0.97 1 $EI63715$ 99 $07-372$ Sphingobacterium4 1.29 23 1.05 2 $EF363715$ 99 $07-372$ Sphingobacterium composti4 1.29 23 1.05 2 $EF122436$ 97 $09-327$ Sphingobacterium sp.2 0.65 1 $EF122436$ 99 $07-339$ Sphingobacterium sp.2 0.65 1 $EF426437$ 99 $09-341$ Achromobacter4 1.29 13 1.05 1HQ20041198 $09-030$ Achromobacter sp.4 1.29 15 1.74 2 </td <td>Aurantimonas ureilytica</td> <td></td> <td></td> <td></td> <td>1</td> <td>0.35</td> <td>1</td> <td>DQ883810</td> <td>99</td> <td>09-222</td>	Aurantimonas ureilytica				1	0.35	1	DQ883810	99	09-222	
Rhizobium sp.3 1.05 1HM22440699 $09-340$ Rhizobium huautlense2 0.70 1EU69438999 $09-002$ Rhizobium soli3 0.97 1EF36371599 $07-372$ Sphingobacterium4 1.29 23 1.05 2 2 2 Sphingobacterium composti1 0.35 1EF12243697 $09-327$ Sphingobacterium sp.2 0.65 1 2 $DQ984207$ 99 $07-339$ Sphingobacterium sp.2 0.65 1 2 0.70 1EF42643799 $09-341$ Achromobacter4 1.29 13 1.05 1HQ20041198 $09-030$ Achromobacter sp.4 1.29 15 1.74 2 2 2 0.35 1 $DQ177478$ 95 $09-334$	Rhizobium	3	0.97	1	5	1.74	2				
Rhizobium huautlense2 0.70 1EU69438999 $09-002$ Rhizobium soli3 0.97 1 $EF363715$ 99 $07-372$ Sphingobacterium4 1.29 23 1.05 2Sphingobacterium composti1 0.35 1 $EF122436$ 97 $09-327$ Sphingobacterium sp.2 0.65 1 U $DQ984207$ 99 $07-339$ Sphingobacterium sp.2 0.65 1 U $EF426437$ 99 $09-341$ Achromobacter4 1.29 13 1.05 1 $HQ200411$ 98 $09-030$ Achromobacter sp.4 1.29 15 1.74 2 U U U U Massilia4 1.29 15 1.74 2 U U U U Massilia sp.4 1.29 15 1.74 2 U	Rhizobium sp.				3	1.05	1	HM224406	99	09-340	
Rhizobium soli3 0.97 1EF36371599 $07-372$ Sphingobacterium composti4 1.29 23 1.05 2Sphingobacterium sp.2 0.65 1EF12243697 $09-327$ Sphingobacterium sp.2 0.65 1DQ98420799 $07-339$ Sphingobacterium sp.2 0.65 1EF42643799 $09-341$ Achromobacter4 1.29 13 1.05 1EF42643799 $09-030$ Achromobacter sp.4 1.29 13 1.05 1HQ20041198 $09-030$ Achromobacter sp.4 1.29 15 1.74 2I1 0.35 1DO17747895 $09-334$	Rhizobium huautlense				2	0.70	1	EU694389	99	09-002	
Sphingobacterium 4 1.29 2 3 1.05 2 Sphingobacterium composti 1 0.35 1 EF122436 97 09-327 Sphingobacterium sp. 2 0.65 1 DQ984207 99 07-339 Sphingobacterium sp. 2 0.65 1 EF122436 97 09-327 Sphingobacterium sp. 2 0.65 1 FM958444 95 07-367 Sphingobacterium sp. 2 0.65 1 EF426437 99 09-341 Achromobacter 4 1.29 1 3 1.05 1 HQ200411 98 09-030 Achromobacter sp. 4 1.29 1 5 1.74 2 1 0.35 1 PO177478 95 09-334	Rhizobium soli	3	0.97	1				EF363715	99	07-372	
Sphingobacterium composti1 0.35 1EF12243697 $09-327$ Sphingobacterium sp.2 0.65 1DQ98420799 $07-339$ Sphingobacterium sp.2 0.65 1FM95844495 $07-367$ Sphingobacterium sp.2 0.65 1EF42643799 $09-341$ Achromobacter4 1.29 13 1.05 1 $HQ200411$ 98 $09-030$ Achromobacter sp.4 1.29 15 1.74 2 $HQ327263$ 99 $07-386$ Massilia4 1.29 15 1.74 2 I I I I I I I I Massilia sp. I <td>Sphingobacterium</td> <td>4</td> <td>1.29</td> <td>2</td> <td>3</td> <td>1.05</td> <td>2</td> <td></td> <td></td> <td></td>	Sphingobacterium	4	1.29	2	3	1.05	2				
Sphingobacterium sp.20.651DQ9842079907-339Sphingobacterium sp.20.651FM9584449507-367Sphingobacterium sp.20.701EF4264379909-341Achromobacter41.29131.051 $HQ200411$ 9809-030Achromobacter sp.41.29151.742 $HQ327263$ 9907-386Massilia41.29151.742 $HQ327263$ 9509-334	Sphingobacterium composti				1	0.35	1	EF122436	97	09-327	
Sphingobacterium sp. 2 0.65 1 FM958444 95 07-367 Sphingobacterium sp. 2 0.70 1 EF426437 99 09-341 Achromobacter 4 1.29 1 3 1.05 1 HQ200411 98 09-030 Achromobacter sp. 4 1.29 1 5 1.74 2 Use of the second se	Sphingobacterium sp.	2	0.65	1				DQ984207	99	07-339	
Sphingobacterium sp. 2 0.70 1 EF426437 99 09-341 Achromobacter 4 1.29 1 3 1.05 1 <td>Sphingobacterium sp.</td> <td>2</td> <td>0.65</td> <td>1</td> <td></td> <td></td> <td></td> <td>FM958444</td> <td>95</td> <td>07-367</td>	Sphingobacterium sp.	2	0.65	1				FM958444	95	07-367	
Achromobacter 4 1.29 1 3 1.05 1 Achromobacter sp. 3 1.05 1 HQ200411 98 09-030 Achromobacter sp. 4 1.29 1 HQ327263 99 07-386 Massilia 4 1.29 1 5 1.74 2 2 Massilia sp. 1 0.35 1 DO177478 95 09-334	Sphingobacterium sp.				2	0.70	1	EF426437	99	09-341	
Achromobacter sp. 3 1.05 1 HQ200411 98 09-030 Achromobacter sp. 4 1.29 1 HQ327263 99 07-386 Massilia 4 1.29 1 5 1.74 2 2 Massilia sp. 1 0.35 1 DO177478 95 09-334	Achromobacter	4	1.29	1	3	1.05	1				
Achromobacter sp. 4 1.29 1 HQ327263 99 07-386 Massilia 4 1.29 1 5 1.74 2 Massilia sp. 1 0.35 1 DO177478 95 09-334	Achromobacter sp.				3	1.05	1	HQ200411	98	09-030	
Massilia 4 1.29 1 5 1.74 2 Massilia sp. 1 0.35 1 DO177478 95 09-334	Achromobacter sp.	4	1.29	1				HQ327263	99	07-386	
Massilia sp. 1 0.35 1 DO177478 95 09-334	Massilia	4	1.29	1	5	1.74	2	~			
- 130 i DQ1/110 /0 0/001	Massilia sp.				1	0.35	1	DQ177478	95	09-334	

Table 1 (continued)

Name	Aging FCTL			Unaged FCTL			GenBank accession nos.	Identity	OTU number	
	No. of clones	Ratio (%)	OTUs	No. of clones	Ratio (%)	OTUs		(%)		
Massilia sp.	4	1.29	1	4	1.39	1	GQ853362	98//98	09-049//07-085	
Methylobacterium	4	1.29	2	5	1.74	3				
Methylobacterium variabile	3	0.97	1				AB302931	97	07-096	
Methylobacterium sp.	1	0.32	1				AB252203	97	07-026	
Methylobacterium sp.				4	1.39	2	FJ157964	99/99	09-082/09-040	
Methylobacterium sp.				1	0.35	1	AY741717	95	09-308	
Acinetobacter	5	1.61	2	10	3.48	1				
Acinetobacter junii	1	0.32	1				HM030746	99	07-320	
Acinetobacter lwoffii	4	1.29	1				FJ999939	99	07-178	
Acinetobacter sp.				10	3.48	1	HM137026	99	09-036	
Sphingomonas	15	4.84	6	12	4.18	6				
Sphingomonas faeni	1	0.32	1	4	1.39	1	FR682703	99//99	09-035//07-073	
Sphingomonas sp.				2	0.70	1	FJ192627	99	09-110	
Sphingomonas sp.				2	0.70	1	FJ716236	98	09-001	
Sphingomonas sp.	1	0.32	1				AF184221	97	07-164	
Sphingomonas sp.	5	1.61	1				EU131002	99	07-266	
Sphingomonas sp.				1	0.35	1	FJ828932	96	09-60	
Sphingomonas sp.				1	0.35	1	HQ331138	99	09-212	
Sphingomonas sp.	3	0.97	1				GQ339888	98	07-408	
Sphingomonas sp.	5	1.61	2				AB495350	100/97	07-021/07-306	
Sphingomonas sp.				2	0.70	1	AF335468	97	09-125	
Chryseobacterium	8	2.58	4	1	0.35	1				
Chryseobacterium sp.				1	0.35	1	DQ337588	97	09-039	
Chryseobacterium sp.	1	0.32	1				GU086430	99	07-051	
Chryseobacterium sp.	5	1.61	1				DQ337588	98	07-338	
Chryseobacterium massiliae	1	0.32	1				AF531766	97	07-315	
Chryseobacterium daeguense strain	1	0.32	1				EF076759	99	07-334	
Escherichia	8	2.58	1	6	2.09	2				
E. coli	8	2.58	1	6	2.09	2	DQ118017	99/96// 99	09-236/09-318// 07-235	
Stenotrophomonas	15	4.84	1	15	5.23	2				
Stenotrophomonas maltophilia	15	4.84	1	13	4.53	1	GU385870	99//99	09-019//07-046	
Stenotrophomonas sp.				2	0.70	1	FN666196	95	09-020	
Erwinia	18	5.81	1	14	4.88	2				
Erwinia tasmaniensis				1	0.35	1	AB480775	96	09-111	
Erwinia sp.	18	5.81	1	13	4.53	1	EU336938	99//99	09-106//07-328	
Pantoea	60	19.35	2	53	18.47	3				
Pantoea agglomerans				1	0.35	1	HM130697	96	09-037	
P. vagans C9-1	21	6.77	1	42	14.63	1	CP002206	100//99	09-034//07-056	
Pantoea sp.				10	3.48	1	AY501386	99	09-059	
Pantoea sp.	39	12.58	1				GU566350	99	07-193	
Pseudomonas	66	21.29	3	69	24.04	11				
P. fulva				16	5.57	1	AY741159	99	09-344	
P. putida	28	9.03	2	8	2.79	1	HQ164547	97//99/ 97	09-173//07-322/ 07-231	
Pseudomonas sp.				1	0.35	1	HM030395	96	09-083	
Pseudomonas sp.				2	0.70	1	EF554919	95	09-347	

Table 1 (continued)

Name	Aging FCTL			Unaged FCTL			GenBank accession nos.	Identity	OTU number	
	No. of clones	Ratio (%)	OTUs	No. of clones	No. of Ratio OTUs clones (%)			(%)		
Pseudomonas sp.				6	2.09	3	EF471903	96/95/98	09-155/09-161/ 09-126	
Pseudomonas sp.				31	10.80	1	AM909658	99	09-343	
Pseudomonas sp.	38	12.26	1				EF157292	99	07-173	
Pseudomonas sp.				1	0.35	1	DQ991143	95	09-006	
Pseudomonas sp.				3	1.05	1	HQ264094	99	09-015	
Pseudomonas sp.				1	0.35	1	EU703498	97	09-342	
Uncultured bacterium	20	6.45	9	36	12.54	25				
Uncultured bacterium	1	0.32	1				AB460149	97	07-361	
Uncultured bacterium				4	1.39	2	EU029106	97/99	09-014/09-009	
Uncultured bacterium				1	0.35	1	GQ156837	93	09-176	
Uncultured bacterium				3	1.05	1	FJ911449	99	09-090	
Uncultured bacterium	1	0.32	1				GQ128233	92	07-278	
Uncultured bacterium				1	0.35	1	FJ984481	92	09-234	
Uncultured bacterium				1	0.35	1	HM459665	97	09-145	
Uncultured bacterium				3	1.05	3	GU272314	99/92/95	09-339/09-177/09- 032	
Uncultured bacterium				1	0.35	1	FJ612202	93	09-185	
Uncultured bacterium	1	0.32	1	2	0.70	1	FJ911492	96//97	07-113//09-003	
Uncultured bacterium	1	0.32	1	2	0.70	2	FJ911500	95/95// 93	09-348/09-091// 07-076	
Uncultured bacterium				1	0.35	1	HM920027	98	09-127	
Uncultured bacterium	6	1.94	1				HM920067	97	07-343	
Uncultured bacterium				1	0.35	1	HM920042	95	09-124	
Uncultured bacterium				1	0.35	1	FJ432396	96	09-100	
Uncultured bacterium				1	0.35	1	GU293192	93	09-072	
Uncultured bacterium	1	0.32	1				GU722212	91	07-009	
Uncultured bacterium				1	0.35	1	EU828415	95	09-285	
Uncultured bacterium				1	0.35	1	GU272236	94	09-332	
Uncultured bacterium	1	0.32	1				FJ152980	96	07-084	
Uncultured bacterium	2	0.65	1				GU084211	97	07-060	
Uncultured bacterium				1	0.35	1	AB460054	97	09-073	
Uncultured bacterium				1	0.35	1	AB460149	95	09-043	
Uncultured bacterium				1	0.35	1	HM099647	93	09-159	
Uncultured bacterium				5	1.74	1	HQ610781	99	09-029	
Uncultured bacterium				2	0.70	1	GU084217	98	09-146	
Uncultured bacterium				2	0.70	1	FJ911497	98	09-307	
Uncultured bacterium	6	1.94	1				HQ610797	98	07-307	

samples. Moreover, several dominant species such as *P. fulva, Pseudomonas* sp. (AM909658), *Klebsiella* sp. (HM584796), *Acinetobacter* sp. (HM137026), and *Pantoea* sp. (AY501386) were only detected in unaged FCTL, while several dominant species such as *Pantoea* sp. (GU566350), *Pseudomonas* sp. (EF157292), and *Buttiauxella izardii* were only detected in aging FCTL.

Phylogenetic analysis of the bacterial community on tobacco leaves

Phylogenetic trees (Fig. 2) were constructed based on the 16S rRNA sequences of the 84 OTUs and 65 OTUs from unaged and aging FCTL, respectively. Two trees of bacteria from unaged (Fig. 2a) and aging FCTL (Fig. 2b) shared

The closest bacterial type strain	GenBank accession nos.	Identity (%)	Aging FCTL		Unaged FCTL		
			No. of clones	Ratio (%)	No. of clones	Ratio (%)	
P. putida	HQ164547	98/97	28	9.03	8	2.79	
P. vagans	CP002206	99/100	21	6.77	42	14.63	
Erwinia sp.	EU336938	99/99	18	5.81	13	4.53	
S. maltophilia	GU385870	99/99	15	4.84	13	4.53	
Pantoea sp.	GU566350	99	39	12.58	0	0	
Pseudomonas sp.	EF157292	99	38	12.26	0	0	
B. izardii	NR025331	99	22	7.10	0	0	
P. fulva	AY741159	99	0	0	16	5.57	
Pseudomonas sp.	AM909658	99	0	0	31	10.80	
Klebsiella sp.	HM584796	99	0	0	19	6.62	
Acinetobacter sp.	HM137026	99	0	0	10	3.48	
Pantoea sp.	AY501386	99	0	0	10	3.48	

Table 2 Analysis of dominant bacteria (clone ratio greater than 3%) in aging and unaged Zimbabwe FCTL

similar shape, and those bacteria were clustered into two clades (A and B). In unaged FCTL, clade A contained 77 OTUs (97.21% clones) and were divided into two subclades (A1 and A2), and subclade A1 consisted of two clusters (A11 and A12, Fig. 2a). Cluster A11 (gammaproteobacteria) contained 51 OTUs (81.88% clones), which included 13 genera, and the dominant genera were *Stenotrophomonas, Pantoea*, and *Pseudomonas*. Cluster A12 (beta-proteobacteria) contained five OTUs (3.48% clones) and included A2 (alpha-proteobacter) contained 21 OTUs (11.85% clones) and included *Aurantimonas, Methylobacterium, Rhizobium*, and *Rhodobacter*.

In the aging FCTL, clade A contained 44 OTUs (occupies 88.39% clones) and were divided into three subclades (A1, A2, and A3), subclade A1 included three clusters (A11, A12, and A13; Fig. 2b). Cluster A11 (gamma-proteobacteria) contained 17 OTUs (68.39% clones), which included 11 genera and the dominant ones were Buttiauxella, Pantoea, and Pseudomonas. Cluster A12 (beta-proteobacteria) contained three OTUs (3.87% clones) and included Achromobacter and Massilia. Subclade A2 (alpha-proteobacter) includes 20 OTUs (14.84% clones) and included Aurantimonas, Methylobacterium, Rhizobium, and Rhodobacter. Subclade A3 contained two OTUs, epsilon-proteobacteria and delta-proteobacteria with one clone each. Interestingly, subclade A3 and cluster A13 were unique in aging FCTL, and the member of cluster A13 belongs to uncultured bacteria.

Similarly, clade B could be further divided into two subclades (B1 and B2). In the unaged sample, subclade B1 contained 3 OTUs (1.05% clones) in genera *Aerococcus, Bacillus*, and one uncultured bacterium which has 93% similarity to *Paenibacillus* sp. (HM099647); subclade B2

included two clusters Actinobacteria and Bacteroidetes, containing 4 OTUs (1.74% clones) in three genera *Microbacterium*, *Chryseobacterium*, and *Sphingobacteria*. More bacteria OTUs were identified in aging sample: subclade B1 contained 8 OTUs (4.84% clones) in five genera *Saccharibacillus, Paenibacillus, Bacillus, Enterococcus*, and *Moryella*; subclade B2 also included two clusters Actinobacteria and Bacteroidetes, containing 13 OTUs (6.77% clones) in genera *Microbacteri, Curtobacterium, Arthrobacter, Chryseobacterium*, and an uncultured bacterium.

Discussion

In this study, we systematically analyzed the bacterial communities in the aging and unaged Zimbabwe FCTL using culture-independent method. Compared to our previous report (Huang et al. 2010b), the bacterial communities in Zimbabwe FCTL were different from those in tobacco K326 (grade C3F): 84 and 65 OTUs were detected from the unaged and aging Zimbabwe FCTL, respectively, while 50 and 42 OTUs were obtained from the unaged and aging FCTL (K326). Although the OTU numbers of bacteria in these two tobacco samples showed similar trends, their bacterial species and dominant genera were quite different. Bacillus spp. and Pseudomonas spp. were the two dominant genera in FCTL (K326), while the most dominant genera were Pantoea and Pseudomonas in Zimbabwe FCTL. These differences of bacterial communities might be related to the tobacco varieties, and further analysis could help understand effect of bacterial diversities in the aging process of FCTL.



Fig. 2 The phylogenetic tree of bacteria in Zimbabwe FCTL based on 16S rRNA sequences. a Unaged FCTL, b aging FCTL. OTU sequences to the most closely related sequences obtained from GenBank. Bootstrap values are 1,000 replicates





From the phylogenetic analysis (Fig. 2), the OTUs and the clone ratios of bacteria in clade A decreased, while these in clade B increased significantly after aging process. Tobacco leaves lose water gradually during aging process, which could impact the bacterial communities in FCTL, and the physiological activities of these bacteria would in turn contribute to bioconversion of macromolecular compounds (e.g., starch, cellulose, and protein) and improving the quality of tobacco leaves. Different from FCTL of K326 tobacco leaves where beta-proteobacteria were only detected in the unaged FCTL, these bacteria were found in both aging and unaged Zimbabwe FCTL. In addition, the clone ratio of beta-proteobacteria increased through the aging, except for *Achromobacter* detected in tobacco K326. Other genera such as *Massilia* and *Herbaspirillum* were only found in Zimbabwe FCTL. At present, the effects of these betaproteobacteria in the tobacco aging process were unknown.

Alpha-proteobacteria increased during aging of Zimbabwe FCTL, similar to those of tobacco K326 (Huang et al. 2010b), except that more alpha-proteobacteria bacteria were detected in Zimbabwe FCTL, such as *Roseomonas, Novosphingobium, Aurantimonas, Agrobacterium*, and *Bosea.* Moreover, in Zimbabwe FCTL, gamma-proteobacteria had an absolute advantage over other bacterium groups in both the number of OTU and clone. Different from the increase of OTU in alpha-proteobacteria, the number of OTU for gamma-proteobacteria decreased from 51 to 17 after aging treatment, and the dominant bacteria were *B. izardii, Pseudomonas* sp., *Pantoea* sp., *S. maltophilia, Erwinia* sp., *P. vagans*, and *P. putida.*

In Zimbabwe FCTL, Pseudomonas and Pantoea were the most dominant genera with significant changes during the aging process (Table 2). The clone ratio of P. vagans decreased by 7.86% after aging treatment, while P. putida increased by 6.27%. Similarly, P. fulva, Pseudomonas sp. (AM909658) and Klebsiella sp were the dominant species in unaged FCTL, while Pantoea sp. (GU56350), B. izardii and Pseudomonas sp. (EF157292) were more prevalent in aging FCTL. P. putida and its close relatives are capable of degrading nicotine in tobacco leaves (Newton et al. 1977; Civilini et al. 1997; Li et al. 2010). The prevalence of other species such as Klebsiella sp., Acinetobacter sp., and B. izardii also showed obvious change during the aging process. An increasing number of reports (Ruan et al. 2005; Chen et al. 2008; Li et al. 2010) have showed Pseudomonas could speed the aging process and improve the quality of tobacco leaves, while the role of other dominant bacteria in the aging process of tobacco remain unknown.

Recently, the microbial community structures on leaves of three varieties Zhongyan 100, NC89, and Zhongyan 101 were studied by denaturing gradient gel electrophoresis (DGGE) technique (Zhao et al. 2007). Five dominant 16S rDNA DGGE bands were sequenced, and they were found most similar to two cultured microbial species *Bacteriovorax* sp. EPC3, *Bacillus megaterium*, and three uncultured microbial species, respectively. The microbial community structure and dynamics on Italian Toscano cigar were also investigated by culture-based and culture-independent approaches (Di Giacomo et al. 2007); they found that Staphylococcaceae (*Jeotgalicoccus* and *Staphylococcus*) and Lactobacillales (*Aerococcus, Lactobacillus*, and *Weissella*), *Bacillus* spp., and Actinomycetales (*Corynebacterium* and *Yania*) were the most commonly detected bacteria at the early, middle, and late periods of fermentation, respectively. More recently, Huang et al. (2010b) reported that the dominant microbial population was *Pseudomonas* and *Bacillus* in tobacco K326 by 16S rRNA RFLP technology. These results showed that the dominant microbial population might be closely related to the tobacco varieties.

In this study, a large number of bacterial OTUs were identified on unaged and aging FCTL from Zimbabwe through direct sequencing. Although some microflora might be missed due to the limitation of the method, our results revealed for the first time the bacterial diversities on unaged and aging Zimbabwe FCTL. This study identified many uncultured bacteria in unaged and aging FCTL. Overall, more uncultured bacteria species were found in the aging Zimbabwe FCTL than in those of K326 FCTL (Huang et al. 2010b). These uncultured bacteria likely contribute to flue-cured tobacco aging process.

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