

# 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),

*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

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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. Culture-independent 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 culture-independent 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 from Hongyun Honghe Tobacco (Group) Co, Ltd. (Kunming, China), and the grade of samples are L<sub>1</sub>OT. Unaged tobacco leaves and those aged for 24 months were sampled and stored at -20°C for use.

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×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'-GGTAGTCCACGCCGTAACGATG-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* DH5α) 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.

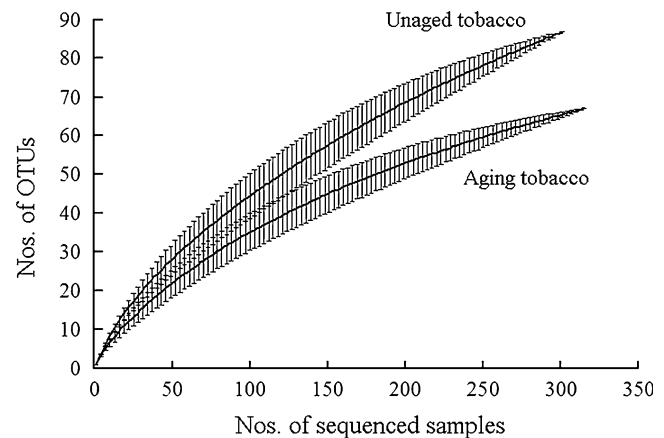
## Results

### 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*, *Methylobacterium*, *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 FCTL			Unaged FCTL			GenBank accession nos.	Identity (%)	OTU number
	No. of clones	Ratio (%)	OTUs	No. of clones	Ratio (%)	OTUs			
<i>Aerococcus</i>				1	0.35	1			
<i>Aerococcus</i> sp.				1	0.35	1	AM292070	99	09-192
<i>Cronobacter</i>				1	0.35	1			
<i>Cronobacter dublinensis</i>				1	0.35	1	GU122214	100	09-265
<i>Herbaspirillum</i>				1	0.35	1			
<i>Herbaspirillum</i> sp.				1	0.35	1	GU201556	95	09-197
<i>Roseomonas</i>				1	0.35	1			
<i>Roseomonas genomospecies</i>				1	0.35	1	AY150049	95	09-026
<i>Shigella</i>				1	0.35	1			
<i>Shigella</i> sp.				1	0.35	1	EF600631	95	09-329
<i>Novosphingobium</i>				2	0.70	2			
<i>Novosphingobium subarcticum</i>				1	0.35	1	AY151394	99	09-304
<i>Novosphingobium</i> sp.				1	0.35	1	GU086416	98	09-102
<i>Serratia</i>				3	1.05	2			
<i>Serratia</i> sp.				3	1.05	2	GU124497	97/95	09-263/09-061
<i>Xanthomonas</i>				6	2.09	1			
<i>Xanthomonas campestris</i>				6	2.09	1	GU086451	99	09-188
<i>Paenibacillus</i>	6	1.94	3						
<i>Paenibacillus hodogayensis</i>	1	0.32	1				AB179866	96	07-003
<i>Paenibacillus</i> sp.	4	1.29	1				EU741039	99	07-341
<i>Paenibacillus</i> sp.	1	0.32	1				HM233974	99	07-366
<i>Mesorhizobium</i>	2	0.65	1						
<i>Mesorhizobium</i> sp.	2	0.65	1				FJ529846	99	07-171
<i>Agrobacterium</i>	1	0.32	1						
<i>Agrobacterium</i> sp.	1	0.32	1				GU086439	98	07-314
<i>Arcobacter</i>	1	0.32	1						
<i>Arcobacter</i> sp.	1	0.32	1				DQ234101	99	07-217
<i>Bacteriovorax</i>	1	0.32	1						
<i>Bacteriovorax</i> sp.	1	0.32	1				AY294222	99	07-282
<i>Bosea</i>	1	0.32	1						
<i>Bosea</i> sp.	1	0.32	1				AF531764	99	07-246
<i>Curtobacterium</i>	1	0.32	1						
<i>Curtobacterium</i> sp.	1	0.32	1				EF411134	99	07-027
<i>Moryella</i>	1	0.32	1						
<i>Moryella indoligenes</i>	1	0.32	1				AF527773	99	07-276
<i>Sphingomonas</i>	1	0.32	1						
<i>Sphingomonas bacterium</i>	1	0.32	1				FJ932666	98	07-305
<i>Arthrobacter</i>	2	0.65	1						
<i>Arthrobacter chlorophenolicus</i>	2	0.65	1				AB495170	99	07-059
<i>Asaia</i>	2	0.65	1						
<i>Asaia krungthepensis</i>	2	0.65	1				AB292240	99	07-323
<i>Enterococcus</i>	2	0.65	1						
<i>Enterococcus</i> sp.	2	0.65	1				HQ264083	100	07-117
<i>Flavobacterium</i>	2	0.65	2						
<i>Flavobacterium</i> -like sp.	1	0.32	1				AF385549	97	07-205
<i>Flavobacterium</i> sp.	1	0.32	1				HQ329187	97	07-389

**Table 1** (continued)

Name	Aging FCTL			Unaged FCTL			GenBank accession nos.	Identity (%)	OTU number
	No. of clones	Ratio (%)	OTUs	No. of clones	Ratio (%)	OTUs			
<i>Actinomyces</i>	8	2.58	1						
<i>Actinomyces</i> sp.	8	2.58	1				EU341158	99	07-089
<i>Pedobacter</i>	2	0.65	1						
<i>Pedobacter hartonius</i>	2	0.65	1				AM491371	99	07-148
<i>Rhodococcus</i>	4	1.29	1	1	0.35	1			
<i>Rhodococcus</i> sp.	4	1.29	1	1	0.35	1	FJ973466	99//97	07-041//09-085
<i>Microbacterium</i>	1	0.32	1	1	0.35	1			
<i>Microbacterium profundi</i>				1	0.35	1	EF623999	96	09-296
<i>Microbacterium</i> sp.	1	0.32	1				AB480762	99	07-291
<i>Saccharibacillus</i>	4	1.29	1						
<i>Saccharibacillus kuerlensis</i>	4	1.29	1				EU046270	98	07-290
<i>Enterobacter</i>	1	0.32	1	5	1.74	2			
<i>Enterobacter cloacae</i>				4	1.39	1	HM030748	95	09-186
<i>Enterobacter hormaechei</i>				1	0.35	1	EU047556	95	09-242
<i>Enterobacter</i> sp.	1	0.32	1				FJ950692	96	07-308
<i>Bacillus</i>	2	0.65	2	1	0.35	1			
<i>Bacillus</i> sp.				1	0.35	1	GU366039	99	09-016
<i>Bacillus simplex</i>	1	0.32	1				GU086427	98	07-159
<i>Bacillus subtilis</i>	1	0.32	1				FJ984449	99	07-150
<i>Rhodobacter</i>	2	0.65	1	1	0.35	1			
<i>Rhodobacter</i> sp.	2	0.65	1	1	0.35	1	EU703498	96//96	09-021//07-141
<i>Buttiauxella</i>	22	7.10	1	1	0.35	1			
<i>B. izardii</i>	22	7.10	1				NR025331	99	07-345
<i>Buttiauxella warmboldiae</i>				1	0.35	1	NR028893	98	09-097
<i>Phyllobacterium</i>	2	0.65	1	2	0.70	1			
<i>Phyllobacterium</i> sp.	2	0.65	1	2	0.70	1	FJ178785	99//99	09-266//07-002
<i>Klebsiella</i>	2	0.65	1	19	6.62	1			
<i>Klebsiella</i> sp.				19	6.62	1	HM584796	99	09-057
<i>Klebsiella</i> sp.	2	0.65	1				AY942948	96	07-195
<i>Aurantimonas</i>	3	0.97	1	3	1.05	2			
<i>Rhizobiales bacterium</i> <i>Tianshan 513-1</i>	3	0.97	1	2	0.70	1	FJ005011	96//96	09-093//07-352
<i>Aurantimonas ureilytica</i>				1	0.35	1	DQ883810	99	09-222
<i>Rhizobium</i>	3	0.97	1	5	1.74	2			
<i>Rhizobium</i> sp.				3	1.05	1	HM224406	99	09-340
<i>Rhizobium huautlense</i>				2	0.70	1	EU694389	99	09-002
<i>Rhizobium soli</i>	3	0.97	1				EF363715	99	07-372
<i>Sphingobacterium</i>	4	1.29	2	3	1.05	2			
<i>Sphingobacterium composti</i>				1	0.35	1	EF122436	97	09-327
<i>Sphingobacterium</i> sp.	2	0.65	1				DQ984207	99	07-339
<i>Sphingobacterium</i> sp.	2	0.65	1				FM958444	95	07-367
<i>Sphingobacterium</i> sp.				2	0.70	1	EF426437	99	09-341
<i>Achromobacter</i>	4	1.29	1	3	1.05	1			
<i>Achromobacter</i> sp.				3	1.05	1	HQ200411	98	09-030
<i>Achromobacter</i> sp.	4	1.29	1				HQ327263	99	07-386
<i>Massilia</i>	4	1.29	1	5	1.74	2			
<i>Massilia</i> 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			
<i>Massilia</i> sp.	4	1.29	1	4	1.39	1	GQ853362	98//98	09-049//07-085
<i>Methylobacterium</i>	4	1.29	2	5	1.74	3			
<i>Methylobacterium variabile</i>	3	0.97	1				AB302931	97	07-096
<i>Methylobacterium</i> sp.	1	0.32	1				AB252203	97	07-026
<i>Methylobacterium</i> sp.				4	1.39	2	FJ157964	99/99	09-082/09-040
<i>Methylobacterium</i> sp.				1	0.35	1	AY741717	95	09-308
<i>Acinetobacter</i>	5	1.61	2	10	3.48	1			
<i>Acinetobacter junii</i>	1	0.32	1				HM030746	99	07-320
<i>Acinetobacter lwoffii</i>	4	1.29	1				FJ999939	99	07-178
<i>Acinetobacter</i> sp.				10	3.48	1	HM137026	99	09-036
<i>Sphingomonas</i>	15	4.84	6	12	4.18	6			
<i>Sphingomonas faeni</i>	1	0.32	1	4	1.39	1	FR682703	99//99	09-035//07-073
<i>Sphingomonas</i> sp.				2	0.70	1	FJ192627	99	09-110
<i>Sphingomonas</i> sp.				2	0.70	1	FJ716236	98	09-001
<i>Sphingomonas</i> sp.	1	0.32	1				AF184221	97	07-164
<i>Sphingomonas</i> sp.	5	1.61	1				EU131002	99	07-266
<i>Sphingomonas</i> sp.				1	0.35	1	FJ828932	96	09-60
<i>Sphingomonas</i> sp.				1	0.35	1	HQ331138	99	09-212
<i>Sphingomonas</i> sp.	3	0.97	1				GQ339888	98	07-408
<i>Sphingomonas</i> sp.	5	1.61	2				AB495350	100/97	07-021/07-306
<i>Sphingomonas</i> sp.				2	0.70	1	AF335468	97	09-125
<i>Chryseobacterium</i>	8	2.58	4	1	0.35	1			
<i>Chryseobacterium</i> sp.				1	0.35	1	DQ337588	97	09-039
<i>Chryseobacterium</i> sp.	1	0.32	1				GU086430	99	07-051
<i>Chryseobacterium</i> sp.	5	1.61	1				DQ337588	98	07-338
<i>Chryseobacterium massiliae</i>	1	0.32	1				AF531766	97	07-315
<i>Chryseobacterium daeguense</i> strain	1	0.32	1				EF076759	99	07-334
<i>Escherichia</i>	8	2.58	1	6	2.09	2			
<i>E. coli</i>	8	2.58	1	6	2.09	2	DQ118017	99/96// 99	09-236/09-318// 07-235
<i>Stenotrophomonas</i>	15	4.84	1	15	5.23	2			
<i>Stenotrophomonas maltophilia</i>	15	4.84	1	13	4.53	1	GU385870	99//99	09-019//07-046
<i>Stenotrophomonas</i> sp.				2	0.70	1	FN666196	95	09-020
<i>Erwinia</i>	18	5.81	1	14	4.88	2			
<i>Erwinia tasmaniensis</i>				1	0.35	1	AB480775	96	09-111
<i>Erwinia</i> sp.	18	5.81	1	13	4.53	1	EU336938	99//99	09-106//07-328
<i>Pantoea</i>	60	19.35	2	53	18.47	3			
<i>Pantoea agglomerans</i>				1	0.35	1	HM130697	96	09-037
<i>P. vagans</i> C9-1	21	6.77	1	42	14.63	1	CP002206	100//99	09-034//07-056
<i>Pantoea</i> sp.				10	3.48	1	AY501386	99	09-059
<i>Pantoea</i> sp.	39	12.58	1				GU566350	99	07-193
<i>Pseudomonas</i>	66	21.29	3	69	24.04	11			
<i>P. fulva</i>				16	5.57	1	AY741159	99	09-344
<i>P. putida</i>	28	9.03	2	8	2.79	1	HQ164547	97//99/ 97	09-173//07-322/ 07-231
<i>Pseudomonas</i> sp.				1	0.35	1	HM030395	96	09-083
<i>Pseudomonas</i> 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	Ratio (%)	OTUs			
<i>Pseudomonas</i> sp.				6	2.09	3	EF471903	96/95/98	09-155/09-161/ 09-126
<i>Pseudomonas</i> sp.				31	10.80	1	AM909658	99	09-343
<i>Pseudomonas</i> sp.	38	12.26	1				EF157292	99	07-173
<i>Pseudomonas</i> sp.				1	0.35	1	DQ991143	95	09-006
<i>Pseudomonas</i> sp.				3	1.05	1	HQ264094	99	09-015
<i>Pseudomonas</i> 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

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

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

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 (gamma-proteobacteria) 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 *Achromobacter*, *Herbaspirillum*, and *Massilia*. Subclade 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

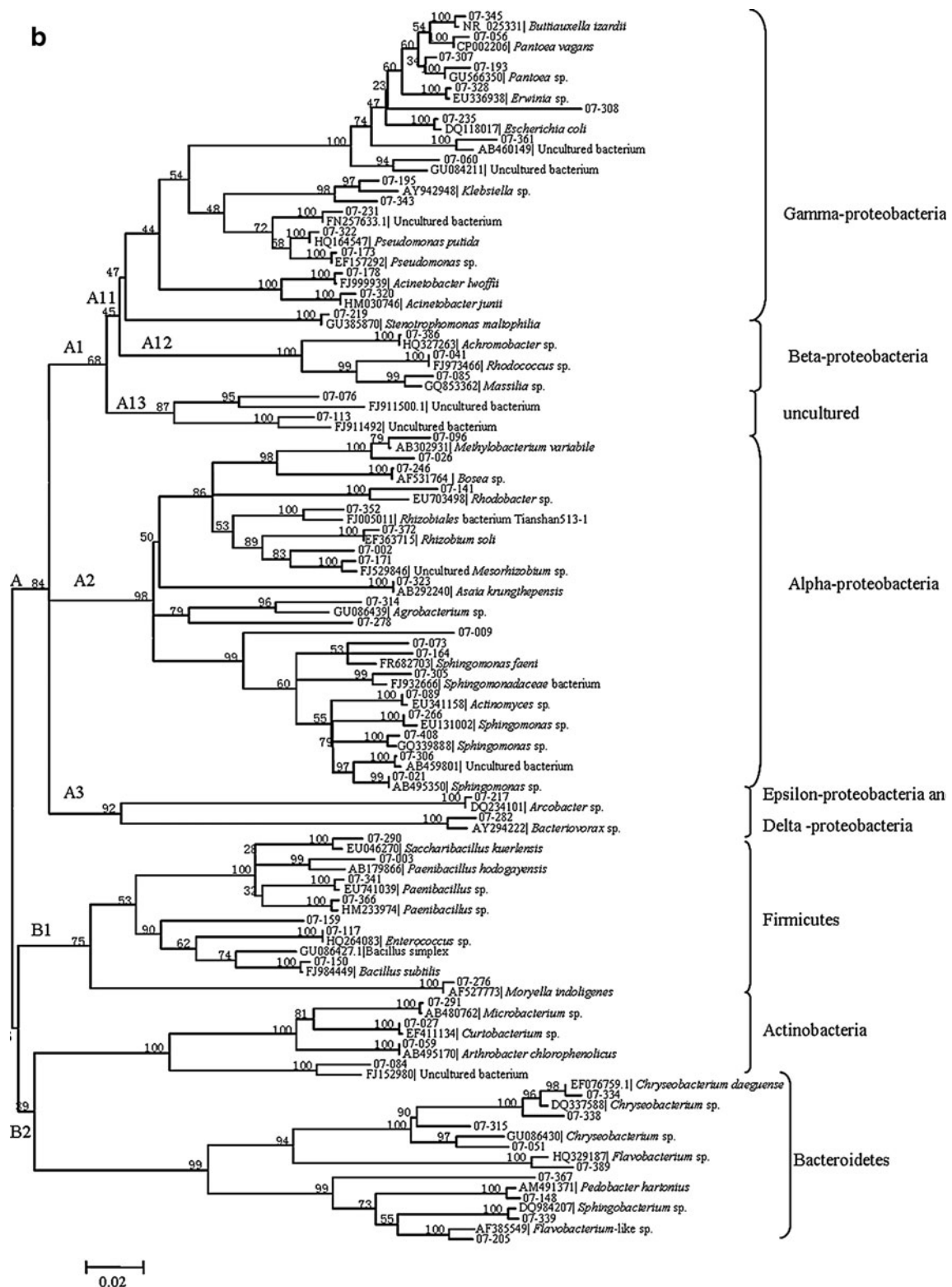
included two clusters Actinobacteria and Bacteroidetes, containing 4 OTUs (1.74% clones) in three genera *Microbacterium*, *Chryseobacterium*, and *Sphingobacteria*. More bacteria OTUs were identified in aging sample: subclade B1 contained 8 OTUs (4.84% clones) in five genera *Saccharibacillus*, *Paenibacillus*, *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*, *Flavobacterium*, *Pedobacter*, *Sphingobacterium*, 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** (continued)

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 beta-proteobacteria 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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