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QPCR Quantification Strategy of Minor Species in a Complex Microbial Community Using Species-specific Primers

Huimin Zhang[1], Changlu Guo[1], Hongkui He[2], Kaiqiang Li[1], Yuan Sun[1], Qingwu Zhou[b] and Zhizhou Zhang[1,2]*

[1]School of Marine Science and Technology, Lab for Personalized Nutrition and Food Safety, Marine Antifouling Engineering Technology Center of Shangdong Province, Harbin Institute of Technology, Weihai - 264 209, China.
[2]The GuJing Group, Bozhou, Anhui - 236 800, China.

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People have normally known little about microbial species in any natural complex microbial community, in which minor species have been paid less attention in the context of their functional roles in maintaining the community complexity. Proper quantification of such minor species shall be a prerequisite for functional studies. In this report, TA-cloning was used to decipher the structure of 12 GuJingGong liquor-making microbial community samples; 32 minor species in the microbial community were found to already have whole genome sequences, and species-specific primers were designed and synthesized for real-time quantification polymerase chain reaction (QPCR). Among the 32 targets, 19 gave expected PCR results and then were emplyed to quantify 12 GuJingGong microbial community samples. Proper strategy for quantification of compositional structure of a complex microbial community was discussed.

Key words: Complex microbial community, Minor species, Quantification, PCR, species-specific primer.

In the past few years, multi-target pathogen detection methods have been developed[1-6] in the molecular levels. Those pathogens are normally viruses and bacteria. However, present methods are mostly established on several genes without enough reference on whole genome information, because many target pathogen or potential target pathogens don't have whole genome sequences yet. This situation makes the detection specificty a problem, since other unknown bacteria may also have one of those target gene(s).When you deal with a complex microbial community and try to quantitatively detect some species in the microbial population, things are getting complicated and even worse.

A natural microbial community may have decades or several hundreds of different microorganisms, in which most species have never been studied at all. Especially, those 20-30 dominant species in a complex microbial population may only have a partial 16s rDNA sequence and nothing else. No specific genes could be selected for quantification targets in the coming, say, 5-10 years for a specific microbial population. Besides biomedical diagnosis field, in other areas such as environment preservation/protection, fishery, natural fermentation (brewing) and human health, there are lot of microbial communities to investigate and modulate.

* To whom all correspondence should be addressed.
Tel.:/Fax: 86-631-5683176
E-mail: zhangzzbiox@hitwh.edu.cn

Recently, severe pollutions are widely spread in air, water and soil in China, and this situation is hard to be completely changed in decades. Pollutions are changing the general environment and modulate even destroy the compositional structure of a natural microbial community. In this situation, it is very necessary to develop approches to rapidly and cost-effectively quantify dominant or minor species in a microbial community.

In this study, a molecular approach based on 16s rDNA amplification/TA-cloning/sequencing was employed to decipher the compositional structure of GuJingGong-making microbial communities. GuJingGong is a famous liquor in China with a 1800-year history. Twelve samples were prepared from GuJingGong production lines and subjected to characterization of their microbial community structures. All characterized microbial species were collected in Table 1 and Table 2 and those with whole genome sequences were subjected to species-specific DNA amplification and quantification.

MATERIALS AND METHODS

TA cloning to characterize the structure of GuJingGong microbial community

Genomic DNA was extracted from 12 GuJingGong samples (S1-S4 for Daqu samples and S5-S12 for Jiaoni samples. Daqu and Jiaoni represent different stages of GuJingGong liquor production) using Solarbio D2600 kit for soil genome purification. 16s rDNA amplification was undertaken using universal primers 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'TAC GGY TAC CTT GTT ACG ACT T3'). Amplified target bands (about 1500bp) were gel-purified using Sangon SanPrep kit (Cat#: SK8132). For TA cloning, 4ul purified 16s rDNA (about 20ng) for each genome sample was ligated with 0.5ul pMD19-T vector (50ng/ul, TaKaRa) for 4 hours at room temperature, then transformed into 60ul competent DH5a cells. Cells were selected on LB plates with 100 ug/ml ampicillin and X-gal/IPTG according to standard protocols. About 130 white colonies from each of the 12 transformations were randomly picked for DNA sequencing using both 27F and 1492R. About 1299 effectively sequenced 16s rDNA fragments were subjected to Basic Local Alignment Search Tool (BLAST) analysis at the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov).

Species-specific primer design for minor species in GuJingGong microbial community

Species-specific primers were designed using the primer-blast tool; For each genome, two or more pairs of primers were synthesized and tested for PCR specificity. The web site is www.ncbi.nlm.nih.gov/tools/primer-blast/.

QPCR

Quantitative real time PCR (qPCR) was performed with a final volume of 12ml on StepOne (ABI) machine using qPCR kit NPK62 (GREDBIO). The reaction mixture contained 6ul 2×NPK62 buffer, 0.4 ul genome sample (S1-S12, each about 10 ng/ul), 1.6 ul primer pair (2 uM each), 0.2 ul Taq DNA polymerase (5 U/ul), 3.8 ul distilled water. The kit includes 1×EvaGreen [Biotium, Cat.31000] as fluorescence reporter. PCR reaction began with a denaturation step at 94°C for 3min, followed by 65 cycles of amplification at 94°C for 20s, 58°Cfor 30s and 72°C for 30s. PCR ended with 72°C for 2min. At the end of the amplification, a melting analysis was performed to confirm the specificity of reaction products and also to examine the melting behavior of the DNA in the presence of PCR additives. All PCR reactions were repeated at least three times. PCR products were subjected to 1.2% agarose gel electrophesis.

RESULTS

GuJingGong samples included 98 different microbes

Effectively sequenced colonies in 12 samples were summarized in Table 1. Detailed information may be requested from the author. The total number for successful sequencing is 1299 (Table1 and Table 2), in which 98 different species were included and 32 of them already have whole genome sequences. Rarefaction curve analysis[7] on the colony numbers and OTUs indicated that the sampling libraries of S1-S12 were statistically big enough (data not shown).

Nineteen out of thirty-two microbes having whole genome sequences were well amplified

Species-specific PCR primers for the 32 species with whole genome sequences were designed and the 72 pairs of primer were listed in

Table 3. Only 19 pairs of primers gave decent PCR effects as in Fig1. The very likely reason was that most of the 32 species belong to the minor species. Sixteen out of 32 species only had one colony (Table 2), which meant that the a large proportion of the target genome sequences had too low concentrations. Even the final concentrations of the 12 template samples were raised 10-fold higher in the PCR set-up (to ~100ng/ul), the general PCR results were almost the same (data not shown). Another possible reason was that, among the 98 or more microbial species, most only have a partial 16s rDNA sequence so far. Their genomes are unknown yet, and these unknown genomes were mixed with 32 known genomes. So the specificity of all primers in Table 3 can not be guaranteed at all until all genomes in the microbial community, at least those of the dominant species, are decoded.

QPCR presented preliminary quantitative profiles of the 19 microbes in 12 GuJingGong samples

Rapid multiple target quantification using QPCR requires three criteria: (1) Target-specific PCR amplification must be guaranteed, (2) The same thermal cycling conditions are used for all targets, and (3) All target-specific PCR amplifications are set up in individual tubes. In this study, the first criterion is hard to achieve because whole genome sequences in the complex GuJingGong microbial community are very limited.

Since all sequenced clones were randomly picked up on TA-cloning plates in each of the 12 samples, data in Table 1 and Table 2 can largely represent the relative richness of each species. However, this relative richness was not well demonstrated in QPCR results. For example, The ratio of Bacillus subtilis : Lactobacillus plantarum : Lactobacillus fermentum : Bacillus licheniformis is about 18:22:6:5 in S2, but both the Ct values of QPCR amplification (Fig1A) and the agarose gel results (Fig1B) didn't fit the above ratio though all primer pairs had similar amplification efficiency close to 1.0 (Detailed data not shown and requestable).

Three pairs (p19: 5'TCG CCA AGG AAG GAA AGT3', 5'AAG TGG AGC AAG GCA GTT AG3') (P20: 5'CAC CAC TAA CTG CCT TGC TC3', 5'ACA TCT GGG GTT GGG ACA3') (P21: 5'GAA GGT GAA GGT CGG AGT3', 5'GAA GAT GGT GAT GGG ATT TC3') of house-keeping gene GAPDH primer were tested in all 12 samples, and each pair of the primers was only successfully amplified in some of the samples (data not shown), indicating that the selection of housing-keeping genes for relative quantification may be not suitable for a complex microbial community. Absolute quantification in QPCR, instead of relative quantification, shall be suitable for compositional structure quantification of a complex microbial community.

Table 1. Basic data for the twelve samples (OTU: operational taxonomic units)

No.	Randomly selected colony number for sequencing	Successfully sequenced colony number	OTUs
S1	132	123	11
S2	148	120	25
S3	127	73	13
S4	126	68	1
S5	120	111	50
S6	130	125	16
S7	129	117	20
S8	126	121	24
S9	124	92	21
S10	124	108	29
S11	134	128	45
S12	122	113	22

Table 2. TA-clong and sequencing discovered at least 98 different species in GuJingGong microbial community

No.	Name	Colony number	Genome
1	Aminobacterium colombiense	1	
2	Actinobacterium	1	
3	Aminobacterium colombiense DSM	1	√
4	Anaerobic bacterium	2	
5	Anaerobranca gottschalkii	1	
6	Anaerotruncus sp.	1	
7	Arthrobacter sp.	1	
8	Aspergillus fumigatus	4	√
9	Aspergillus niger	2	√
10	Aspergillus oryzae	5	
11	Bacillus amyloliquefaciens	3	√
12	Bacillus anthracis	1	√
13	Bacillus cereus	5	√
14	Bacillus licheniformis	5	√
15	Bacillus oleronius	1	
16	Bacillus pumilus	1	√
17	Bacillus smithii	2	
18	Bacillus sp	4	
19	Bacillus subtilis	18	√
20	Bacteroides sp.	9	
21	Bifidobacterium	1	
22	Bifidobacterium dentium	4	√
23	Brevibacterium sp	1	
24	Caloramator australicus	1	
25	Chlorobi bacterium	1	
26	Citrobacter freundii	1	
27	Clostridiaceae bacterium	1	
28	Clostridiales bacterium	2	
29	Clostridiales bacterium	6	
30	Clostridium sp	14	√
31	Dehalobacter sp	1	√
32	Desulfonosporus sp.	3	
33	Desulfosporosinus orientis	1	
34	Desulfotomaculum halophilum	1	
35	Desulfotomaculum reducens MI-1	1	√
36	Dokdonella ginsengisoli strain Gsoil	1	
37	Dyella sp.	3	
38	Enterobacter sp.	2	
39	Enterobacter aerogenes	1	√
40	Enterobacter asburiae	1	√
41	Enterococcus sp	8	
42	Erysiphe pisi	1	
43	Escherichia coli	1	√
44	Escherichia sp.	1	
45	Eubacteriaceae bacterium	3	
46	Firmicutes bacterium	5	
47	Garciella sp.	4	

48	Iron-reducing bacterium	10	
49	Kurthia sp	1	
50	Lachnospiraceae bacterium	1	
51	Lactobacillus brevis	3	√
52	Lactobacillus casei	2	√
53	Lactobacillus fermentum	6	√
54	Lactobacillus gasseri	1	√
55	Lactobacillus plantarum	22	√
56	Lactobacillus pontis	2	
57	Lactobacillus rossiae	2	
58	Lactobacillus sanfranciscensis	1	√
59	Lactobacillus sp	7	
60	Leuconostoccitreum	1	√
61	Lichtheimia corymbifera	1	
62	Lichtheimia ramosa	1	
63	Moorella sp.	4	
64	Oceanobacillus sp	2	
65	Pantoea ananatis	1	√
66	Pantoea vagans	1	√
67	Pantoea sp	24	
68	Pediococcus acidilactici	1	
69	Pelotomaculum sp.	3	
70	Pseudomonas hibiscicola	3	
71	Pseudomonas putida	1	√
72	Rhizobium sp	1	
73	Rhizopus delemar	15	
74	Rhizopus oryzae	14	
75	Rikenellaceae bacterium	1	
76	Rumen bacterium	5	
77	Ruminobacillus xylanolyticum	1	
78	Salmonella bongori	1	√
79	Staphylococcus kloosii	3	
80	Staphylococcus saprophyticus	4	√
81	Staphylococcus sp	18	
82	Staphylococcus succinus	1	
83	Stenotrophomonas maltophilia	10	√
84	Streptococcus uberis	1	√
85	Syntrophomonas bryantii	2	
86	Syntrophomonas sapovorans	3	
87	Syntrophomonas sp.	1	
88	Syntrophomonas wolfei	11	√
89	Talaromyces thermophilus	1	
90	Tepidanaerobacter acetatoxydans	9	√
91	Thermoactinomyces sanguini	124	
92	Thermomyces lanuginosus	8	
93	Thermoactinomycetaceae bacterium	3	
94	Tissierella creatinini	2	
95	Uncultured bacterium	718	
96	Virgibacillus sp	99	
97	Weissella cibaria	3	
98	Xanthobacter sp.	1	
	Total	1299	

Table 3. Primers (bolded means sucessful PCR amplification) for 32 microbe species that have whole genome sequences

Species No.	Primer name	Primer Sequences(5'-3')	Amplicon (bp) Size(bp)
3	**Gzhm-1F**	**AAGTTCCGCCAAACGCTTTC**	156
	Gzhm-1R	**TTCGGTTTCGGTCGTGGAAT**	
12	Gzhm-2F	ACACAGTATATCGTGTTGTGGA	192
	Gzhm-2R	ACCCACATGTGATTAAAACTGTCC	
	Gzhm-2-1F	GCAAAGCTAAATGAAACTGAGCA	549
	Gzhm-2-1R	TGATGTGGCTTGTGCGTTTG	
	Gzhm-2-2F	CGCGGAGCTAACCCATGTAT	282
	Gzhm-2-2R	AGAGATAAAGCATCGCACCTT	
14	**Gzhm-3F**	**TGGTACAGATGAGCACGGAC**	284
	Gzhm-3R	**CATCAACGAGCTGGGTTTCG**	
19	**Gzhm-4F**	**CCGTTCCGCTTGCTATTAACG**	192
	Gzhm-4R	**TTCTTTGGACGGAGCGTGTA**	
	Gzhm-4-1F	**TGCGAAGGTCTGCTGCTTTA**	994
	Gzhm-4-1R	TGCTCACCCTCAAACGAGAC	
	Gzhm-4-2F	ATTTGCGAAGGTCTGCTGCT	363
	Gzhm-4-2R	GCGCGTCAGTAGAGCCAATA	
16	Gzhm-5F	AGCCCATTCTCTTCAAGGTGA	126
	Gzhm-5R	AGCTCTTCACCTGTCAGTTCG	
	Gzhm-5-1F	GTGATACGCTCACAATTACGGC	841
	Gzhm-5-1R	GCACCCTCCAGTTCACGAAT	
	Gzhm-5-2F	**CGGTATTCCAGTCGTCTTAATGT**	315
	Gzhm-5-2R	**AGACCCCAGTGGATACCGAA**	
22	Gzhm-6F	GAGTACGGCTACGATTGGGG	241
	Gzhm-6R	GCACGGAGTCACCGGTATAG	
	Gzhm-6-1F	GCTCTTCCTCGCTGTATCCC	206
	Gzhm-6-1R	CCCGTTATACGAAGACGCGA	
	Gzhm-6-2F	TCGGTGTGATCGTAGGCTGT	903
	Gzhm-6-2R	CACCACGTTACTTCACACGC	
30	Gzhm-7F	GTGTCAGGCTTTGGAAGCAC	247
	Gzhm-7R	CGCAGGTGTGTATGAGGGAA	
	Gzhm-7-1F	TCCAGATAATGCAGCCCTTCG	493
	Gzhm-7-1R	GAAAGGGACCGGGATACGTG	
	Gzhm-7-2F	ACCATATCCTGGGAAGGCAC	101
	Gzhm-7-2R	GTAGAAGCCCAGGCAGAGTG	
31	Gzhm-8F	GCGGGAACACCTTGGAAAAC	155
	Gzhm-8R	GCCTGCGTGGAAAATGTGTT	
	Gzhm-8-1F	GAATCTGCCTCTGTCCAGGT	424
	Gzhm-8-1R	TGATCGGAACCCCAAACACC	
	Gzhm-8-2F	AGTGGTCCCCTATTACGGCT	911
	Gzhm-8-2R	CAGGTTGCTTCCCAAAAGGC	
43	**Gzhm-9F**	**CGTTTCCAGGTGCTTTCCCT**	105
	Gzhm-9R	**GACCTTCACGTCCAGCCAAA**	
51	**Gzhm-10F**	**CGTGCGCCCAAACGAAATTA**	129
	Gzhm-10R	**AAGTCGCGTAGGCTGTCTTC**	
52	**Gzhm-11F**	**GGACAATGGCTGACGAGACT**	221

	Gzhm-11R	**AAGCCGGTCGTTCATTGGTT**	
53	**Gzhm-12F**	**TGGCAACTTGGCTACTCACC**	148
	Gzhm-12R	**CACACCTCGACTAGAAGGGC**	
54	Gzhm-13F	AATGATCAAAACGGTGCGGAAT	128
	Gzhm-13R	CCTTGAGCATCTTTGCCATCAG	
	Gzhm-13-1F	AGCAATAACCCAAGTGGCGA	489
	Gzhm-13-1R	GATCTAGGCGGAGAAGTGGC	
	Gzhm-13-2F	CCAGGACAAACGGCCAAAAG	107
	Gzhm-13-2R	GGCAACATGTGGGAAACACC	
55	**Gzhm-14F**	**CGCAAGTGGCTTGCTTAGTC**	224
	Gzhm-14R	**CCACTGTCCGTACCAGCAAT**	
58	Gzhm-15F	TTGATCCCGCCACTTTGACA	280
	Gzhm-15R	GGGGTTCATATCATGACACTCTT	
	Gzhm-15-1F	**ATACCCGGCAATGCTCTACG**	642
	Gzhm-15-1R	**CCCAAGTAAAGGGCCACGAT**	
	Gzhm-15-2F	TGCTAAAATTAACCACCACATTGA	690
	Gzhm-15-2R	TCATCGACTGGTTTGGGGTT	
60	**Gzhm-16F**	**ATTGGGCCGTCTGAGTGAAG**	269
	Gzhm-16R	**GCACTTCGCGGCAAACTTAT**	
71	**Gzhm-17F**	**GCGTGCTTGGAGGTAGTCTT**	177
	Gzhm-17R	**GAGACGGTCAAGAAAGGGCA**	
80	Gzhm-18F	TGTAATGCCCTCGGGAGAGA	281
	Gzhm-18R	GTAGCGACATCGAACAACGC	
	Gzhm-18-1F	TCCCAAACCCACAAACACCTT	835
	Gzhm-18-1R	TTAGCTGGTGCTTCTGCCAC	
	Gzhm-18-2F	**CTACCCGATAAGCAGAAGCGT**	727
	Gzhm-18-2R	**GGCCGTAATATCTCATCCGGT**	
83	Gzhm-19F	GCGCAAAGCGTTGATCCTTC	277
	Gzhm-19R	CATGAGGGCATCCGTAGCAA	
	Gzhm-19-1F	GATGTTCACAGCCAACCAGCC	638
	Gzhm-19-1R	GCGCTGTACAACAAGTACCAC	
	Gzhm-19-2F	CGCAAAGCGTTGATCCTTCTC	651
	Gzhm-19-2R	GTACTGCAACGCGTAGTGGA	
88	**Gzhm-20F**	**GTGGTTGCCAAAAGACGACC**	101
	Gzhm-20R	**GGCCAGGACTCTCCTTTCAC**	
90	Gzhm-21F	AGGTGGACTCGGATCGGTTA	266
	Gzhm-21R	GTTTACCTCCGTGCTGGACA	
	Gzhm-21-1F	GTCTTTGGGCGGTTTTGGAC	331
	Gzhm-21-1R	CGAGTGCCCTTTTGTGTTGG	
	Gzhm-21-2F	GCGCTACTTGCAGGCATAAC	725
	Gzhm-21-2R	ATTGCCGGAGAATAGCCGAG	
11	**Gzhm-22F**	**AACGGTTCAGGTCTTGCAGT**	142
	Gzhm-22R	**GGCGCACCTTTTACGGTTTG**	
13	**Gzhm-23F**	**GGTTAAGGGTAAGAAAAGGAGCA**	208
	Gzhm-23R	**GAAGGGGAACAACCGCGTTA**	
	Gzhm-23-1F	GGTTAAGGGTAAGAAAAGGAGCAG	873
	Gzhm-23-1R	TTAGCCCCTTTGCCGCTATT	
	Gzhm-23-2F	TCCGGTGGAACGTTTAGGTG	590
	Gzhm-23-2R	ACAGGTGAACCCCACTGATT	
35	Gzhm-24F	GCCCCATAGTCAAGAAAAACGG	297

	Gzhm-24R	TCTTGAGCAACCCTCCAAACT	
	Gzhm-24-1F	TGCCCCATAGTCAAGAAAAACG	749
	Gzhm-24-1R	TCCCTAAACCAACACCACCG	
	Gzhm-24-2F	TATCAACTGAGAAGGGGGAGG	193
	Gzhm-24-2R	CGTGAACATCAGCGTAGGGA	
39	Gzhm-25F	CAGCAGCGACGTTCCGTTAT	118
	Gzhm-25R	ATAGTGCGGGATTGGCAGAG	
	Gzhm-25-1F	GCACGTAGGGATGGGGATAAA	130
	Gzhm-25-1R	TGTCCCCGATTTTTCGTGCT	
	Gzhm-25-2F	**CCCTGCAATAAGGAAATGCCG**	972
	Gzhm-25-2R	**ATACGAAGTTGGTGCCCTCG**	
40	**Gzhm-26F**	**ATTTTCGCACGTTTTCGCGT**	189
	Gzhm-26R	**CCCACGATTTAGCCCGCATA**	
	Gzhm-26-1F	ATTCCCGCGTATTTTCGCAC	717
	Gzhm-26-1R	GTTTGCGTGACTGGCTTTGT	
	Gzhm-26-2F	GCGCTCAAAATTGTCGGGATG	296
	Gzhm-26-2R	GCAATCATCGACCCGTCCTT	
65	Gzhm-27F	GATAGTCCACGCCAAGCCAG	230
	Gzhm-27R	TGGCTTTTCGTTTAAGCAGGC	
	Gzhm-27-1F	CTTGACGCAGACGGACAATC	234
	Gzhm-27-1R	CGAGGTCCCACCGAATTTCA	
	Gzhm-27-2F	CGAGAAGTGATAGTCCACGC	428
	Gzhm-27-2R	CACCGTTCGCAATGGGTTAG	
66	Gzhm-28F	GCAGGGTGCGGTTTACAGAT	165
	Gzhm-28R	CGCTGAATCTGCCACTTTCC	
	Gzhm-28-1F	GCACTTTCCAGATGTTGCCATT	300
	Gzhm-28-1R	CGGTGAGTGAAACCAAGCTG	
	Gzhm-28-2F	**CATTGACGTGGCTAACGGCT**	693
	Gzhm-28-2R	**CATTAAGGGGGCGCAAAGTG**	
78	Gzhm-29F	CGTGAGCAGAATGCGGAATG	284
	Gzhm-29R	TCGCATGGTTGTTACCTCGT	
	Gzhm-29-1F	AAACTGATGATGACCCACCGA	959
	Gzhm-29-1R	ATCGCAACCATCGGTCAACT	
	Gzhm-29-2F	TCAGAAAGTGTTAGTCCGGCA	356
	Gzhm-29-2R	AAGCACTGGAAACCCACCTC	
84	Gzhm-30F	GCCAACGGCACTCTTCACTA	242
	Gzhm-30R	ACTAAGGCTCCCGCCAATTC	
	Gzhm-30-1F	TTTGGTCCAGTGTACGTGCT	629
	Gzhm-30-1R	CCAAGCCTCTGGTGCTACTC	
	Gzhm-30-2F	GCAGAACAAAGCAAGCGGAA	288
	Gzhm-30-2R	TTCGGTCCGCATAATCCGAG	
8	**Gzhm-31F**	**TGGAAGTAGCACAGCCACAG**	227
	Gzhm-31R	**GATACCCTCGGCACGCAATA**	
9	Gzhm-32F	CATACTTGGTACGCGCAAGC	279
	Gzhm-32R	TCCCGTCGGATAACCCTTCT	
	Gzhm-32-1F	CACACGACCATCGCTCTCTG	991
	Gzhm-32-1R	AACCTCTGGGTCTAGCTCGT	
	Gzhm-32-2F	CTGGGAAAGGCAGCTCAAAC	404

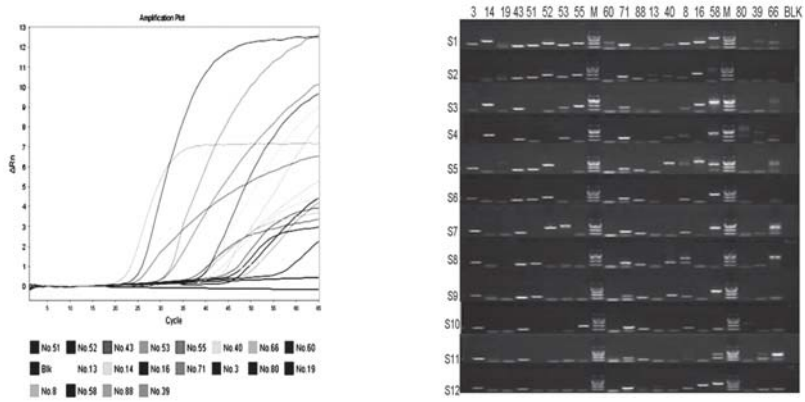

Fig. 1. QPCR results of 19 pairs of primers. (A) QPCR amplification plots with 19 pairs of primers and one blank control for S8 sample showed some species had large Ct values (Detailed information can be requested); (B) Agarose gel (1.2%) electrophesis for species-specific QPCR amplification of 19 target genome sequences in 12 GuJingGong samples (S1-S12). M: MW marker DL2000 (100,250,500,750,1000,2000bp); BLK: blank control without primers

DISCUSSION

There are at least 2 aspects important for accurate quantification for microbes in a complex microbial communiy. First, the copy number of target gene is normally unknown or unnoticed. If different target genes are chosen as QPCR amplicons while the copy numbers of those genes in their own genomes are unclear, systematic error will occur; second, the complexity of a microbial community makes it possible that an appropriate housekeeping gene may be hard to choose as a marker for relative quantification in QPCR. Different housekeeping genes have different detection stability in different species, even in the same bacterium[8-10]. Especially, most microbial communities have a large proportion of species that only have partial 16s rDNA sequences and no any other genetic information available, let alone the whole genome sequences[11-13]. So, technologically, it is still hard by now to accurately quantify the number of different microbes in any natural complex microbial community using the relative quantification approachs. However, absoloute quantification is a solution for species-specific DNA amplification of a complex microbial community. Though such DNA quantification is not necessarily equal to cell quantification (because the copy numbers of target DNA fragments in those known genomes are normally not determined), it is still a good strategy, though in need of detailed proof tests in the near future, to provide a practical way for basic composition quantification of a complex microbial community.

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