The Genotypic Characterization of *Cronobacter* spp. Isolated in China

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Abstract

Cronobacter spp. (*Enterobacter sakazakii*) is an important pathogen contaminating powdered infant formula (PIF). To describe the genotypic diversity of *Cronobacter* isolated in China, we identified the isolates using *fusA* allele sequencing, and subtyped all of the isolates using pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and multiple-locus variable-number tandem-repeat analysis (MLVA). A total of 105 isolates were identified, which included *C. sakazakii* (58 isolates), *C. malonaticus* (30 isolates), *C. dublinensis* (11 isolates), *C. turicensis* (5 isolates), and *C. muytjensii* (1 isolate). These isolates were showed to have 85 PFGE-patterns, 71 sequence types (STs), and 55 MLVA-patterns. Comparisons among the three molecular subtyping methods revealed that the PFGE method was the most distinguishable tool in identifying clusters of *Cronobacter* spp. through DNA fingerprinting, and MLST method came second. However, ESTR-1, ESTR-2, ESTR-3, and ESTR-4 were not effective loci for subtyping *Cronobacter* spp. such that the MLVA method requires further improvement.

Introduction

Infants fed with powdered infant formula (PIF) contaminated with *Cronobacter* (*Enterobacter sakazakii*) can suffer from necrotising enterocolitis, bacteraemia, and neonatal meningitis [1–4]. Although these infections are relatively rare overall, they are of considerable significance, because infants comprise the highest-risk age group. While these infections are lethal for infants, recent studies reported possible diarrhea, wound infections, and urinary tract infections among immunocompromised people, particularly the elderly [5–8]. While the reservoir of *Cronobacter* is unknown in many cases, PIF was reported as a key source for infantile infection. Since 2004, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have addressed the issue of *E. sakazakii* in PIF: accordingly, many studies of the phenotype and genotype of *Cronobacter* were conducted. In particular, *Cronobacter* was proposed as a new genus for *E. sakazakii*, and the genus consists of ten genomospecies [9–11]. These bacteria are distributed throughout nature and are found in plants, spice, herbs, water, dry foods, and starches [12–15]. Although there were no clinical infections reported, these strains have been widely isolated from food and from the environment in China.

Several molecular techniques have been developed for subtyping *Enterobacter sakazakii*, such as ribotyping, random amplification of polymorphic DNA, pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), and multi-locus sequence typing (MLST) [16–18]. PulseNet

International, which is the international molecular subtyping network for foodborne disease surveillance, considers PFGE as a gold-standard method for bacterial molecular typing. MLVA is the second generation of molecular subtyping method. MLST data is comparable across laboratories worldwide via the central curated database(http://pubmlst.org/cronobacter). To compare the genotypes of *Cronobacter* isolates from different sources, years and regions in China, we identified 105 *Cronobacter* isolates obtained from 2006 to 2011 from 11 Chinese provinces, and using PFGE, MLVA and MLST method to type these isolates. We also investigated which molecular subtyping method is more effective in determining the genetic relationship of *Cronobacter* isolates for the purpose of surveillance and outbreak investigation.

Materials and Methods

Bacterial strains

From 2006 to 2011,105 isolates were collected from 11 provinces in China, and their sources included infant food, potable water, and rectal swabs of healthy humans. Three rectal swabs from healthy humans were collected by Jinan municipal CDC, and the participants were informed and had provided their written consent to participate in this study, which was approved by the Jinan municipal CDC Ethics committee. As controls, the isolates ATCC 51329 and ATCC 29544 were provided by the Chinese Academy of Inspection and Quarantine (CAIQ). All isolates were confirmed by biochemical test using kit API 20E (bioMerieux, Marcy l'Etoile, France) and real-time PCR using a

primer set and probe targeting the *dnaG* gene on the macromolecular synthesis operon [19]. Isolates were cultured on TSA agar (Oxoid, Basingstoke, United Kingdom), and they were cryopreservation at −80°C for long-term storage.

PFGE

Cronobacter isolates were typed as described in the PulseNet standardised PFGE *Cronobacter* subtyping protocol [17]. *Xba*I and *Spe*I were the primary and secondary restriction enzymes, respectively. DNA fragments were separated by electrophoresis (CHEF Mapper, Bio-Rad Laboratories, Hercules, California, US) through a 1% (w/v) agarose gel (Seakem Gold, Rockland, Maine, US) in 0.5× TBE buffer at 6 volts/cm using an initial switch time of 1.8 s and a final switch time of 25 s. Gels were stained in deionised water containing GelRed Nucleic Acid Gel Stain (Biotium, CA, US), and visualised under U.V. light using a GelDoc XR$^+$system (Bio-Rad laboratories, Hercules, California, US). *Xba*I-digested *Salmonella* serotype Braenderup H9812 was used as the molecular weight standard. Dendrograms were created using BioNumerics software version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) using the DICE coefficient, the unweighted pair group method with arithmetic means (UPGMA), and a band position tolerance of 1.5%.

Genomic DNA Isolation

Bacteria were cultured on TSA agar at 37°C for 18–24 h, and an isolated colony was inoculated into 5 ml tryptone soy broth (Oxoid, Basingstoke, UK). Bacteria from 1 ml of overnight culture was recovered by centrifugation at 10,000×*g* for 2 min. Total DNA was prepared using the QIA amp DNA Mini kit (Qiagen, Maryland, US) and quantified using a spectrophotometer (IMPLEN, Gemany) for MLST and MLVA method.

MLST

The seven multilocus loci of *Cronobacter* were *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA*. The primers and the detailed amplification conditions for these fragments were available at http://pubmlst.org/cronobacter/info/protocol.shtml; Baldwin *et al*. 2009. The amplified fragments were purified using a PCR purification Kit (Qiagen, Maryland, US) according to the manufacture's instructions. DNA sequencing was performed with an ABI 3730XL DNA Analyzer (Applied Biosystems, CA, US). In order to identify *Cronobacter* species, the phylogenetic and molecular evolutionary analyses of *fusA* allele were conducted by using MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 [20]. Concatenated DNA sequences for sequence types not detected in the present study were downloaded from the MLST database.

MLVA

Four loci, ESTR-1, ESTR-2, ESTR-3, ESTR-4 [16] were amplified using the primers at a concentration of 0.2 uM with EX Taq DNA polymerase (TBI, Dalian, China) in a 20 µl final reaction volume using the manufacturer's reaction buffer. Following amplification, all PCR products were electrophoresed using an Applied Biosystems 3730XL DNA Analyser (Applied Biosystems, CA, US). The band intensity of each amplicon was examined prior to analysis with Gene Mapper 4.0 software (Applied Biosystems, CA, US). All four loci were amplified three times from each isolate to ensure reproducibility. To confirm the numbers of alleles at the four loci, PCR products were processed using the BigDye kit and sequenced on an ABI 3730XL capillary DNA analyser (Applied Biosystems, CA, US).

Results

Species identification

Based on Joseph's study [18], the 105 isolates in this study were identified as follows: *C. sakazakii* (58 isolates), *C. malonaticus* (30 isolates), *C. dublinensis* (11 isolates), *C. turicensis* (5 isolates), and *C. muytjensii* (1 isolate) (Fig. 1). In addtion, the complete 16S rRNA gene was also sequenced and additional biochemical tests were performed, including the indole test, acid production from dulcitol and methyl-a-D-glucopyranoside, and malonate utilization (data not showed).

PFGE

BioNumerics software analysis showed the 105 isolates demonstrated 85 distinguishable *Xba*I-*Spe*I PFGE patterns, and the control strains (*C. sakazakii* ATCC29544, *C. muytjensii* ATCC51329) showed the two PFGE patterns different from the isolates in this study (Fig. 2). A high degree of genetic diversity was revealed using PFGE after genomic DNA digestion, and the discriminatory index was 0.9940, as it was calculated using Simpson's diversity index. Nevertheless, when the isolates digested with *Xba*I and *Spe*I respectively, 83 and 86 PFGE patterns were demonstrated. So the identification rate of PFGE with *Spe*I may be higher than with *Xba*I.

MLST

The seven defined alleles of each isolate were sequenced, and each house keeping gene *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA* was found to possess 14, 5, 15, 25, 24, 14, and 27 new types of allele sequences, respectively. All the sequences were submitted to the MLST database and assigned new numbers, which were showed in Fig. 1. The 105 strains were in 71 STs, of which only 15 types had been reported by other countries. Although many new STs were found, these STs belonged to previous clonal complex (CC, single or double locus variant of dominant sequence type), such as ST183, 203, 235, 241, 242 belonged to ST4; and ST159, 161, 201, 238 belonged to ST7; and so on. The isolate *C. sakazakii* ATCC29544 showed the same ST with one isolate (BJCR0014), whereas *C. muytjensii* ATCC51329 showed different ST from all the isolates in this study. Collectively, the above results suggest *Cronobacter* isolated from China had a high diversity, particularly for the isolates identified to *C. dublinensis* and *C. turicensis*.

MLVA

Using the MLVA method developed by Mullane *et al.*, we calculated the numbers of alleles for the ESTR-1, ESTR-2, ESTR-3, and ESTR-4 loci: they were 6, 4, 12, and 2, respectively. For certain isolates, there was no PCR amplicon at a variable number of tandem repeats (VNTR) locus, which was labeled as "NP" meaning"no product", and the amplicon was absent in 11% of the isolates at the ESTR-1 locus, 17.1% at the ESTR-2 locus, 49% at the ESTR-3 locus, and 22% at the ESTR-4 locus. The genetic diversity of VNTRs for each locus was calculated using Simpson's diversity index, and the values for ESTR-1, ESTR-2, ESTR-3, and ESTR-4 loci were 71.68%, 61.16%, 89.55%, and 32.67%, respectively. Although the polymorphism indicator at the ESTR-3 locus was the best, the rate of missing amplicons was very high. For VNTRs in ESTR-1 locus, the upstream and downstream sequences were not conserved in 12% of the isolates. To distinguish these results from conserved sequences, the repeat copy number was determined by negative correlation (Fig. 2). Minimum spanning tree (MST) revealed that the 105 isolates were divided into 55 MLVA patterns, and the control strains (ATCC29544, ATCC51329) showed two MLVA patterns different from the

	fusA	apD	glnS	gltB	gyrB	infB	pps	ST
SDCR0003	1	5	3	3	5	5	4	4
TJCR0001	1	5	3	1	5	5	4	242
HBCR0002	1	1	1	1	1	1	1	1
BJCR0012	1	5	3	1	5	1	4	241
BJCR0011	1	1	1	3	1	1	1	240
BJCR0010	1	1	1	3	1	1	1	240
HECR0004	1	5	3	22	5	5	4	183
HECR0002	1	1	1	13	22	1	1	181
SDCR0041	1	10	68	5	86	87	100	178
SDCR0035	1	5	3	3	5	5	4	4
SDCR0009	1	15	66	80	14	79	25	146
SCCR0025	1	1	1	1	1	1	1	1
SCCR0016	1	5	3	3	5	5	4	4
SCCR0015	1	5	3	3	5	5	4	4
SCCR0014	1	5	3	3	18	5	4	203
SCCR0012	1	5	3	3	5	5	4	4
SCCR0011	1	1	1	1	1	1	1	1
SCCR0004	1	5	3	3	5	5	4	4
SCCR0003	1	1	1	1	1	1	1	1
GXCR0002	1	55	89	5	5	36	131	208
SDCR0013	67	15	49	9	14	19	15	148
SCCR0013	17	18	10	12	18	24	18	12
SDCR0022	17	48	10	69	71	5	81	42
GXCR0004	11	3	13	18	11	17	13	21
GXCR0005	37	3	15	100	102	101	133	209
SCCR0019	36	3	74	97	86	20	107	184
SCCR0021	36	3	74	97	86	20	107	184
HECR0005	36	3	74	97	86	20	107	184
SDCR0004	15	3	28	22	5	15	19	144
SDCR0045	15	3	28	22	5	38	19	40
HECR0006	15	3	28	5	3	38	19	185
GXCR0006	15	44	3	5	5	38	59	37
HECR0008	3	3	3	5	3	3	3	3
HECR0009	3	3	3	5	10	3	3	187
SCCR0022	8	3	3	3	18	46	127	198
SCCR0024	8	15	15	3	22	5	16	206
SDCR0006	8	3	65	77	74	77	93	145
SDCR0015	8	16	13	83	15	15	10	150
SDCR0019	8	34	68	84	78	83	19	151
SDCR0044	8	3	72	94	88	3	102	154
SDCR0047	8	69	13	95	89	56	103	155
SDCR0048	8	69	13	95	89	56	103	155
SDCR0049	8	69	13	95	89	56	103	155
HECR0010	8	16	13	40	15	15	10	64
HECR0012	8	16	13	40	15	15	10	64
BJCR0014	8	11	7	5	8	15	10	8
ATCC29544	8	11	7	5	8	15	10	8
SCCR0010	10	11	7	3	91	46	26	202
HECR0015	10	71	68	18	94	92	121	188
BJCR0013	10	71	68	18	94	92	121	188
SDCR0014	12	3	16	5	16	20	14	17
SDCR0024	12	3	16	5	16	20	14	17
SCCR0023	14	69	13	95	89	46	103	205
HECR0003	14	15	15	3	5	5	16	182
SDCR0025	68	3	49	88	81	14	96	152
SDCR0037	68	3	49	88	81	56	96	143
HECR0013	18	20	16	10	3	20	27	23
LNCR0001	18	20	16	10	3	20	27	23
LNCR0002	18	5	45	3	5	5	4	235
SCCR0017	13	61	25	7	101	14	130	204
TJCR0002	7	64	67	75	17	41	97	247
TJCR0003	7	64	67	75	17	41	97	247
SXCR0001	7	10	6	7	9	14	9	7
SXCR0002	7	10	6	7	9	115	9	234
HNCR0001	7	10	6	7	9	14	9	7
HBCR0001	7	10	6	7	9	14	9	7
GDCR0002	7	10	6	7	9	14	4	238
GDCR0001	7	10	6	7	9	14	9	7
BJCR0008	7	10	6	7	9	16	9	201
BJCR0007	7	10	6	7	9	14	9	7
BJCR0006	7	10	6	7	9	14	9	7
BJCR0005	7	10	6	7	9	14	9	7
HECR0011	7	12	8	8	10	16	43	60
SCCR0001	7	10	6	99	9	14	9	201
SCCR0002	7	10	6	99	9	14	9	201
SCCR0005	7	10	6	7	9	14	9	7
SCCR0006	7	10	6	7	9	14	9	7
SCCR0007	7	10	6	7	9	14	9	7
SCCR0008	7	10	6	7	9	14	9	7
SCCR0009	7	10	6	99	9	14	9	201
SCCR0018	7	10	6	99	9	14	9	201
SCCR0020	7	10	6	99	9	14	9	201
SDCR0011	7	61	67	81	77	81	95	169
SDCR0016	7	10	6	7	9	14	9	7
SDCR0026	7	10	6	7	9	16	9	159
SDCR0027	7	64	67	75	17	84	97	172
SDCR0046	7	10	6	7	5	14	19	161
HECR0001	7	12	8	1	1	16	43	180
HECR0007	7	64	79	75	93	22	120	186
SDCR0010	65	60	22	81	76	80	94	147
SDCR0021	65	60	22	86	76	80	94	171
SDCR0039	39	68	42	93	60	49	99	153
LNCR0003	39	47	101	121	118	49	153	236
LNCR0004	39	47	101	121	118	49	4	237
GXCR0001	24	4	30	31	4	4	6	207
ATCC51329	24	4	36	31	4	4	6	81
SDCR0005	63	58	75	76	73	76	108	167
GXCR0003	43	39	45	49	49	52	57	77
SDCR0012	66	37	76	82	92	82	111	163
SDCR0020	23	62	68	85	79	83	112	170
BJCR0009	23	85	69	123	119	16	57	239
SDCR0008	20	59	70	79	75	78	110	168
SDCR0034	20	70	71	90	83	5	118	176
SDCR0023	20	63	69	87	80	43	113	164
SDCR0028	20	65	77	89	82	84	114	173
SDCR0031	20	67	71	91	84	43	116	166
SDCR0033	20	67	76	92	85	41	98	175

Species groupings (right-hand brackets): *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*
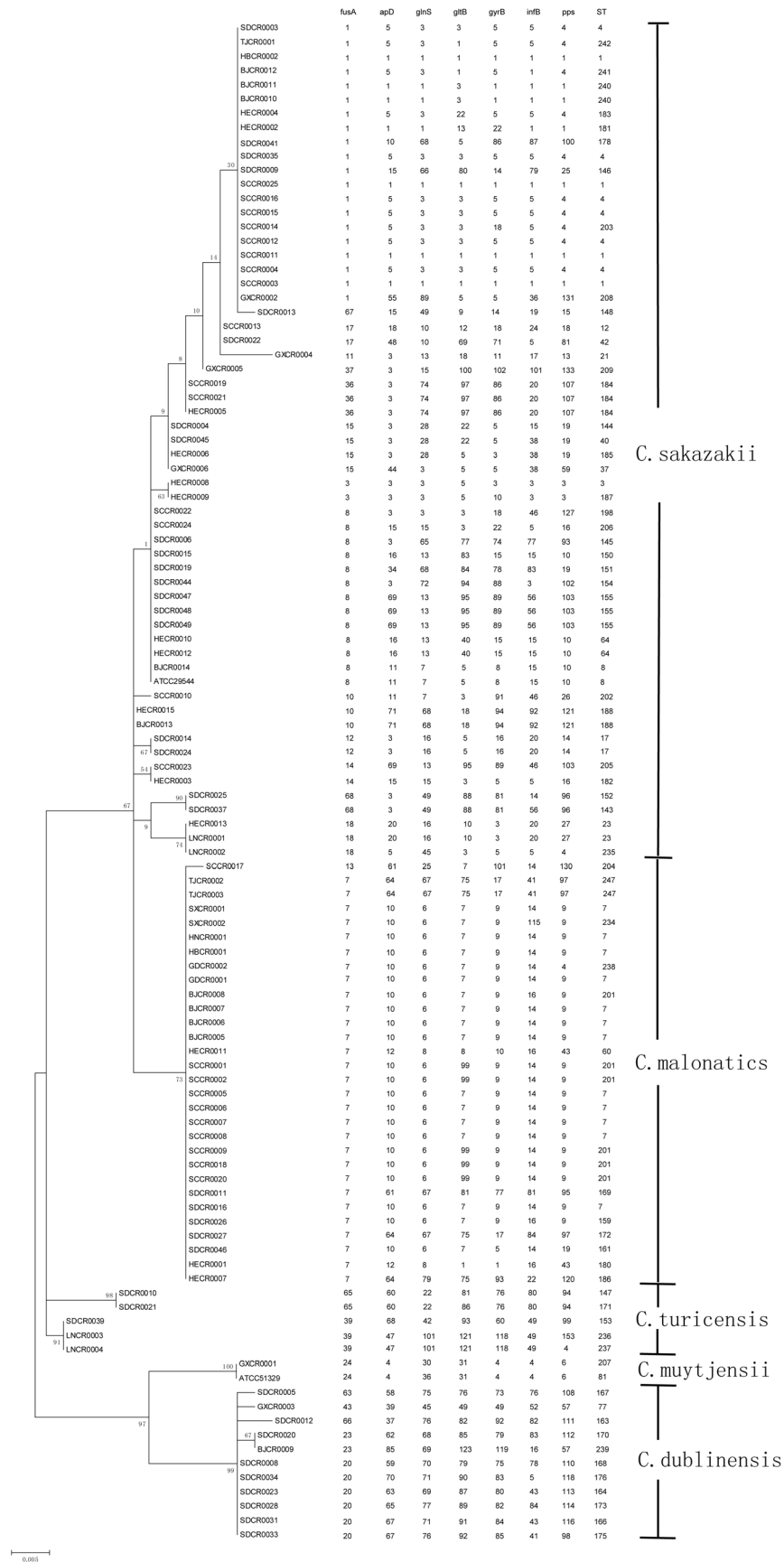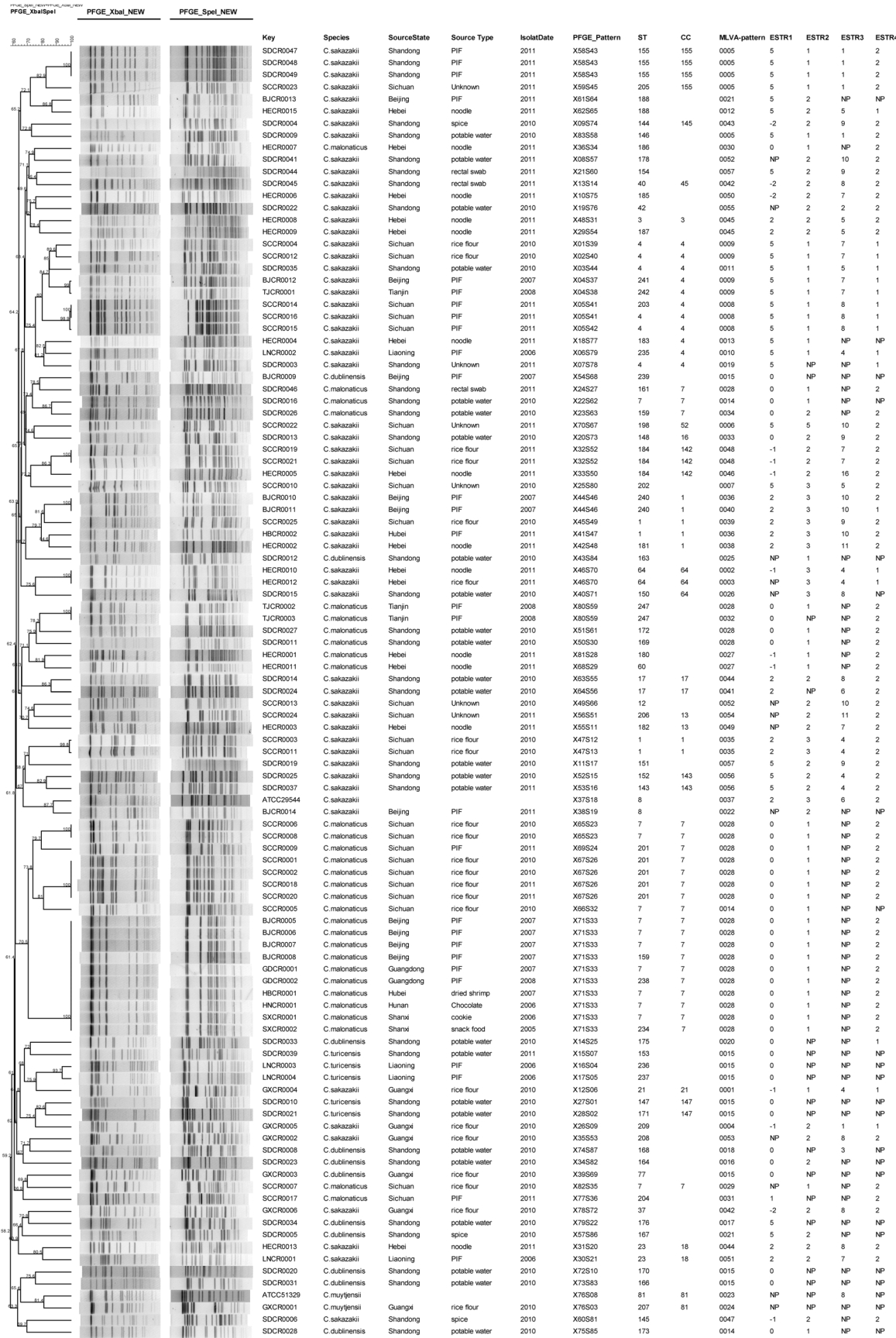
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Figure 1. Maximum likelihood tree of the *fusA* alleles and MLST profiles for the differentiation of *Cronobacter* species in this study.
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Key	Species	SourceState	Source Type	IsolatDate	PFGE_Pattern	ST	CC	MLVA-pattern	ESTR1	ESTR2	ESTR3	ESTR4
SDCR0047	C.sakazakii	Shandong	PIF	2011	X58S43	155	155	0005	5	1	1	2
SDCR0048	C.sakazakii	Shandong	PIF	2011	X58S43	155	155	0005	5	1	1	2
SDCR0049	C.sakazakii	Shandong	PIF	2011	X58S43	155	155	0005	5	1	1	2
SCCR0023	C.sakazakii	Sichuan	Unknown	2011	X59S45	205	155	0005	5	1	1	2
BJCR0013	C.sakazakii	Beijing	PIF	2011	X61S64	188		0021	5	2	NP	NP
HECR0015	C.sakazakii	Hebei	noodle	2011	X62S65	188		0012	5	2	5	1
SDCR0004	C.sakazakii	Shandong	spice	2011	X09S74	144	145	0043	-2	2	9	2
SDCR0009	C.sakazakii	Shandong	potable water	2010	X83S58	146		0005	5	1	1	2
HECR0007	C.malonaticus	Hebei	noodle	2011	X36S34	186		0030	0	1	NP	2
SDCR0041	C.sakazakii	Shandong	potable water	2011	X08S57	178		0052	NP	2	10	2
SDCR0044	C.sakazakii	Shandong	rectal swab	2011	X21S60	154		0057	5	2	9	2
SDCR0045	C.sakazakii	Shandong	rectal swab	2011	X13S14	40	45	0042	-2	2	8	2
HECR0006	C.sakazakii	Hebei	noodle	2011	X10S75	185		0050	-2	2	7	2
SDCR0022	C.sakazakii	Shandong	potable water	2010	X19S76	42		0055	NP	2	2	2
HECR0008	C.sakazakii	Hebei	noodle	2011	X48S31	3	3	0045	2	2	5	2
HECR0009	C.sakazakii	Hebei	noodle	2011	X29S54	187		0045	2	2	5	2
SCCR0004	C.sakazakii	Sichuan	rice flour	2010	X01S39	4	4	0009	5	1	7	1
SCCR0012	C.sakazakii	Sichuan	rice flour	2010	X02S40	4	4	0009	5	1	7	1
SDCR0035	C.sakazakii	Shandong	potable water	2010	X03S44	4	4	0011	5	1	5	1
BJCR0012	C.sakazakii	Beijing	PIF	2007	X04S37	241	4	0009	5	1	7	1
TJCR0001	C.sakazakii	Tianjin	PIF	2008	X04S38	242	4	0009	5	1	7	1
SCCR0014	C.sakazakii	Sichuan	PIF	2011	X05S41	203	4	0008	5	1	8	1
SCCR0016	C.sakazakii	Sichuan	PIF	2011	X05S41	4	4	0008	5	1	8	1
SCCR0015	C.sakazakii	Sichuan	PIF	2011	X05S42	4	4	0008	5	1	8	1
HECR0004	C.sakazakii	Hebei	noodle	2011	X18S77	183	4	0013	5	1	NP	NP
LNCR0002	C.sakazakii	Liaoning	PIF	2006	X06S79	235	4	0010	5	1	4	1
SDCR0003	C.sakazakii	Shandong	Unknown	2011	X07S78	4	4	0019	5	NP	NP	1
BJCR0009	C.dublinensis	Beijing	PIF	2007	X54S68	239		0015	0	NP	NP	NP
SDCR0046	C.malonaticus	Shandong	rectal swab	2011	X24S27	161	7	0028	0	1	NP	2
SDCR0016	C.malonaticus	Shandong	potable water	2010	X22S62	7	7	0014	0	1	NP	NP
SDCR0026	C.malonaticus	Shandong	potable water	2010	X23S63	159	7	0034	0	2	NP	2
SCCR0022	C.sakazakii	Sichuan	Unknown	2011	X70S67	198	52	0006	5	5	10	2
SDCR0013	C.sakazakii	Shandong	potable water	2010	X20S73	148	16	0033	0	2	9	2
SCCR0019	C.sakazakii	Sichuan	rice flour	2011	X32S52	184	142	0048	-1	2	7	2
SCCR0021	C.sakazakii	Sichuan	rice flour	2011	X32S52	184	142	0048	-1	2	7	2
HECR0005	C.sakazakii	Hebei	noodle	2011	X33S50	184	142	0046	-1	2	16	2
SCCR0010	C.sakazakii	Sichuan	Unknown	2010	X25S80	202		0007	5	3	5	2
BJCR0010	C.sakazakii	Beijing	PIF	2007	X44S46	240	1	0036	2	3	10	2
BJCR0011	C.sakazakii	Beijing	PIF	2007	X44S46	240	1	0040	2	3	10	1
SCCR0025	C.sakazakii	Sichuan	rice flour	2010	X45S49	1	1	0039	2	3	9	2
HBCR0002	C.sakazakii	Hubei	PIF	2011	X41S47	1	1	0036	2	3	10	2
HECR0002	C.sakazakii	Hebei	noodle	2011	X42S48	181	1	0038	2	3	11	2
SDCR0012	C.dublinensis	Shandong	potable water	2010	X43S84	163		0025	NP	1	NP	NP
HECR0010	C.sakazakii	Hebei	noodle	2011	X46S70	64	64	0002	-1	3	4	1
HECR0012	C.sakazakii	Hebei	rice flour	2011	X46S70	64	64	0003	NP	3	4	1
SDCR0015	C.sakazakii	Shandong	potable water	2010	X40S71	150	64	0026	NP	3	8	NP
TJCR0002	C.malonaticus	Tianjin	PIF	2008	X80S59	247		0028	0	1	NP	2
TJCR0003	C.malonaticus	Tianjin	PIF	2008	X80S59	247		0032	0	NP	NP	2
SDCR0027	C.malonaticus	Shandong	potable water	2010	X51S61	172		0028	0	1	NP	2
SDCR0011	C.malonaticus	Shandong	potable water	2010	X50S30	169		0028	0	1	NP	2
HECR0001	C.malonaticus	Hebei	noodle	2011	X81S28	180		0027	-1	1	NP	2
HECR0011	C.malonaticus	Hebei	noodle	2011	X68S29	60		0027	-1	1	NP	2
SDCR0014	C.sakazakii	Shandong	potable water	2010	X63S55	17	17	0044	2	2	8	2
SDCR0024	C.sakazakii	Shandong	potable water	2010	X64S56	17	17	0041	2	NP	6	2
SCCR0013	C.sakazakii	Sichuan	Unknown	2010	X49S66	12		0052	NP	2	10	2
SCCR0024	C.sakazakii	Sichuan	Unknown	2011	X56S51	206	13	0054	NP	2	11	2
HECR0003	C.sakazakii	Hebei	noodle	2011	X55S11	182	13	0049	NP	2	7	2
SCCR0003	C.sakazakii	Sichuan	rice flour	2010	X47S12	1	1	0035	2	3	4	2
SCCR0011	C.sakazakii	Sichuan	rice flour	2010	X47S13	1	1	0035	2	3	4	2
SDCR0019	C.sakazakii	Shandong	potable water	2010	X11S17	151		0057	5	2	9	2
SDCR0025	C.sakazakii	Shandong	potable water	2010	X52S15	152	143	0056	5	2	4	2
SDCR0037	C.sakazakii	Shandong	potable water	2011	X53S16	143	143	0056	5	2	4	2
ATCC29544	C.sakazakii				X37S18	8		0037	2	3	6	2
BJCR0014	C.sakazakii	Beijing	PIF	2011	X38S19	8		0022	NP	2	NP	NP
SCCR0006	C.malonaticus	Sichuan	rice flour	2010	X65S23	7	7	0028	0	1	NP	2
SCCR0008	C.malonaticus	Sichuan	rice flour	2010	X65S23	7	7	0028	0	1	NP	2
SCCR0009	C.malonaticus	Sichuan	PIF	2011	X69S24	201	7	0028	0	1	NP	2
SCCR0001	C.malonaticus	Sichuan	rice flour	2010	X67S26	201	7	0028	0	1	NP	2
SCCR0002	C.malonaticus	Sichuan	rice flour	2010	X67S26	201	7	0028	0	1	NP	2
SCCR0018	C.malonaticus	Sichuan	rice flour	2011	X67S26	201	7	0028	0	1	NP	2
SCCR0020	C.malonaticus	Sichuan	rice flour	2011	X67S26	201	7	0028	0	1	NP	2
SCCR0005	C.malonaticus	Sichuan	rice flour	2010	X66S32	7	7	0014	0	1	NP	NP
BJCR0005	C.malonaticus	Beijing	PIF	2007	X71S33	7	7	0028	0	1	NP	2
BJCR0006	C.malonaticus	Beijing	PIF	2007	X71S33	7	7	0028	0	1	NP	2
BJCR0007	C.malonaticus	Beijing	PIF	2007	X71S33	7	7	0028	0	1	NP	2
BJCR0008	C.malonaticus	Beijing	PIF	2007	X71S33	159	7	0028	0	1	NP	2
GDCR0001	C.malonaticus	Guangdong	PIF	2007	X71S33	7	7	0028	0	1	NP	2
GDCR0002	C.malonaticus	Guangdong	PIF	2008	X71S33	238	7	0028	0	1	NP	2
HBCR0001	C.malonaticus	Hubei	dried shrimp	2007	X71S33	7	7	0028	0	1	NP	2
HNCR0001	C.malonaticus	Hunan	Chocolate	2006	X71S33	7	7	0028	0	1	NP	2
SXCR0001	C.malonaticus	Shanxi	cookie	2006	X71S33	7	7	0028	0	1	NP	2
SXCR0002	C.malonaticus	Shanxi	snack food	2005	X71S33	234	7	0028	0	1	NP	2
SDCR0033	C.dublinensis	Shandong	potable water	2010	X14S25	175		0020	0	NP	NP	1
SDCR0039	C.turicensis	Shandong	potable water	2011	X15S07	153		0015	0	NP	NP	NP
LNCR0003	C.turicensis	Liaoning	PIF	2006	X16S04	236		0015	0	NP	NP	NP
LNCR0004	C.turicensis	Liaoning	PIF	2006	X17S05	237		0015	0	NP	NP	NP
GXCR0004	C.sakazakii	Guangxi	rice flour	2010	X12S06	21	21	0001	-1	1	4	1
SDCR0010	C.turicensis	Shandong	potable water	2010	X27S01	147	147	0015	0	NP	NP	NP
SDCR0021	C.turicensis	Shandong	potable water	2010	X28S02	171	147	0015	0	NP	NP	NP
GXCR0005	C.sakazakii	Guangxi	rice flour	2010	X26S09	209		0004	-1	2	1	1
GXCR0002	C.sakazakii	Guangxi	rice flour	2010	X35S53	208		0053	NP	2	8	2
SDCR0008	C.dublinensis	Shandong	potable water	2010	X74S87	168		0018	0	NP	3	NP
SDCR0023	C.dublinensis	Shandong	potable water	2010	X34S82	164		0016	0	2	NP	NP
GXCR0003	C.dublinensis	Guangxi	rice flour	2010	X39S69	77		0015	0	NP	NP	NP
SCCR0007	C.malonaticus	Sichuan	rice flour	2010	X82S35	7	7	0029	NP	1	NP	2
SCCR0017	C.malonaticus	Sichuan	PIF	2011	X77S36	204		0031	1	1	NP	2
GXCR0006	C.sakazakii	Guangxi	rice flour	2010	X78S72	37		0042	-2	2	8	2
SDCR0034	C.sakazakii	Shandong	potable water	2010	X79S22	176		0017	5	NP	NP	NP
SDCR0005	C.dublinensis	Shandong	spice	2010	X57S86	167		0021	5	2	NP	NP
HECR0013	C.sakazakii	Hebei	noodle	2011	X31S20	23	18	0044	2	2	8	2
LNCR0001	C.sakazakii	Liaoning	PIF	2006	X30S21	23	18	0051	2	2	7	2
SDCR0020	C.dublinensis	Shandong	potable water	2010	X72S10	170		0015	0	NP	NP	NP
SDCR0031	C.dublinensis	Shandong	potable water	2010	X73S83	166		0015	0	NP	NP	NP
ATCC51329	C.muytjensii				X76S08	81	81	0023	NP	NP	8	NP
GXCR0001	C.muytjensii	Guangxi	rice flour	2010	X76S03	207	81	0024	NP	NP	NP	NP
SDCR0006	C.sakazakii	Shandong	spice	2010	X60S81	145		0047	-1	2	NP	2
SDCR0028	C.dublinensis	Shandong	potable water	2010	X75S85	173		0014	0	1	NP	NP

Figure 2. Dendrogram combining PFGE patterns of *Xba*I- and *Spe*I- digested DNA from *Cronobacter* spp. in this study. Isolates information, STs, CC and MLVA allele string are also included.
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isolates in this study. The isolates belonging to the different species were gathered in one cluster (Fig. 3)

Discussion

Iversen *et al.* used f-AFLP fingerprints, ribopatterns, full-length 16S rRNA gene sequences, and biochemical tests for species typing in previous study [9]. While 16S rRNA gene sequences failed to distinguish *C. sakazakii* from *C. malonaticus*, and lack of continuity between *Cronobacter* biotypes and species was reported [21]. Here the isolates' species identification was determined by *fusA* allele sequence typing based on Joseph's study. Similar to other studies [22,23], *C. sakazakii* was isolated from all sources, and was the most common *Cronobacter* species. Except for four isolates from food sources, the other 12 isolates, which were classified as *C. dublinensis* or *C. turicensis*, were from environment. This indicates that different species of *Cronobacter* is prevalent in different econiche. In addition, of the three isolates from rectal swabs of healthy individuals, two were identified as *C. sakazakii* (ST40, CC45; ST154), and one was identified as *C. malonaticus* (ST161, CC7). It has been reported that the *Cronobacter* spp. that cause disease were mainly *C. sakazaikii* and *C. malonaticus*. These results support the hypothesis that *C. sakazakii* and *C. malonaticus* may be associated with humans while other species with plants and the environment [24].

PFGE is well-established and widely used for the molecular subtyping of bacteria, including *Enterobacter sakazakii* [25–28]. Brengi *et al.* modified the PulseNet standard protocol in 2012, and we performed and evaluated its applicability for analyzing the isolates from China. Most isolates with the same *Xba*I-*Spe*I PFGE pattern were isolated from the same source, region and year, except for one cluster (PFGE-pattern X71S33). The strains of this cluster were from 5 provinces, and isolated from 2005 to 2008, which maybe demonstrate this type of isolates was prevalent at that time in China. We also found that the cluster (PFGE-pattern X67S26) was existent in Sichuan Province from 2010 to 2011. Unexpectedly, the fingerprint similarity rate from the same species was not high. Although several isolates demonstrated the same PFGE pattern when they were digested with *Xba*I, they showed different PFGE patterns when using *Spe*I, and the converse was

true. Therefore, it was important to perform PFGE on *Cronbacter* using at least two restriction enzymes as a useful tool for surveillance and outbreak investigation.

The *fusA* gene was more conservative comparing with other house-keeping genes, only five new alleles were found in 105 isolates, so it was more suitable for species. While, other genes possessed more than ten types of new alleles, with the result that many novel STs, which were quite different from the alleles reported by other countries, were found in this study. In the same STs, some parts of isolates from the same region and time, such as ST155, ST17, ST201, ST 240, and ST247; however, the isolates of ST7, ST4, ST1, ST23, ST184, and ST188 were from different source, region and time. MLST method was not enough distinguishable for subtyping *Cronobacter* spp., but it was a convenient method to compare the data across laboratories worldwide. For example, *C. sakazakii* CC4 was reported to be associated with neonate meningitis by other countries [29], while it was found in food and water in China. There were 3 isolates from healthy individuals in China were identified to *C. sakazakii* ST40 (CC45), *C. sakazakii* ST154, and *C. malonaticus* ST161(CC7), which have not been reported to associated with clinical cases.

MLVA-Pattern 28 had the most number of isolates, while the isolates from this cluster were from the different regions, sources, and year. The identification rate of MLVA method was not high. Mullane *et al.* obtained similar results in their study, and they attributed this to the *C. sakazakii* strain ATCC BAA-894 VNTR-based primers, which were designed prior to the *Cronobacter* genus definition. In our study, it was found that the isolates that failed to amplify did not always belong to *C. sakazakii*. Therefore, this MLVA method was limited to the full genus as currently recognized, and should be further optimized for effective subtyping of *Cronobacter*.

Comparing the three molecular subtyping methods by the Simpson's diversity indexes, the D-value of PFGE method was the highest for each species (Table 1). Although certain isolates with the same PFGE-pattern were not in same STs, they were in same CCs, while the opposite results were not obtained; certain isolates with similar PFGE-Patterns were not gathered in one cluster using MLVA methods: the opposite results also were obtained.
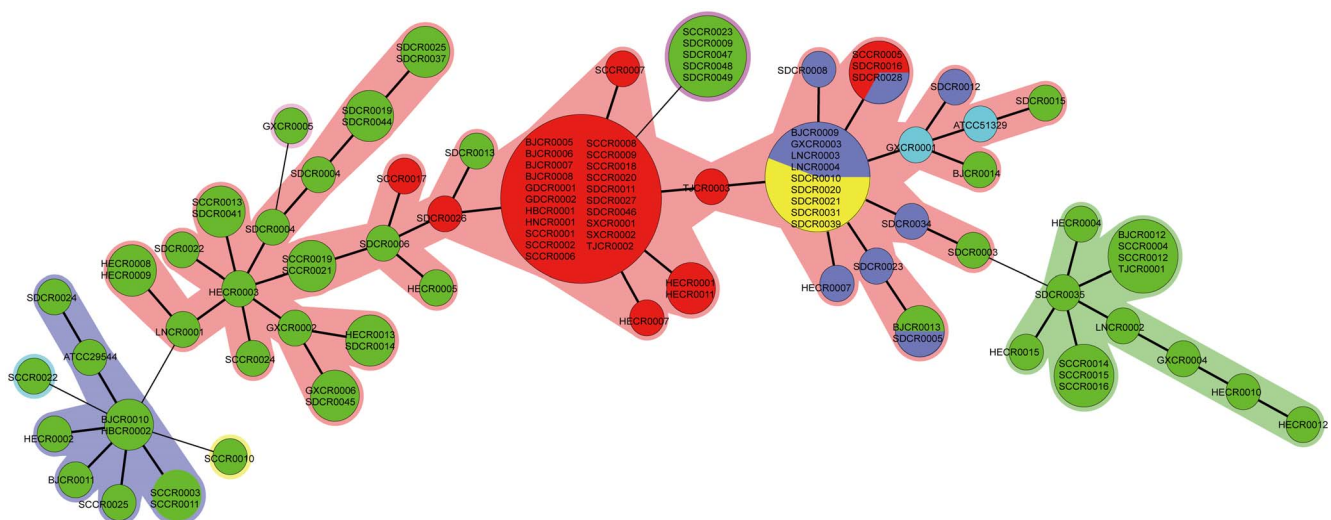
Figure 3. MST of MLVA analysis of 107 isolates of *Cronobacter* spp. in this study. Different species were labelled with different colour: *C. sakazakii* with green, *C. malonaticus* with red, *C. dublinensis* with purple, *C. turicensis* with yellow, and *C. muytjensii* with blue.
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Table 1. Numbers of patterns and D-values of PFGE, MLST and MLVA among different *Cronobacter species.*

	PFGE		MLST		MLVA	
	No. of patterns	D	No. of patterns	D	No. of patterns	D
C. sakazakii (n = 58)	52	0.9958	41	0.9806	40	0.9831
C. malonaticus (n = 30)	16	0.8782	13	0.8207	8	0.5126
C. dublinensis (n = 11)	11	1.0000	11	1.0000	8	0.8909
C. turicensis (n = 5)	5	1.0000	5	1.0000	1	0.0000
C. muytjensii (n = 1)	-	-	-	-	-	-

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Conclusions

In conclusion, there were polymorphisms between the *Cronobacter* spp. isolated in China. The PFGE method was a useful tool in identifying clusters of *Cronobacter* spp. through DNA fingerprinting, and assisted in retrospective investigations to identify food and environmental sources of *Cronobacter*. Although the D-value of the MLST method was lower than the PFGE method for each species, it was a convenient method to compare the data across laboratories worldwide. However, ESTR-1, ESTR-2, ESTR-3, and ESTR-4 were not effective loci for subtyping the *Cronobacter* spp. from China. And new loci are required to establish an MLVA-based protocol in the future.

Author Contributions

Conceived and designed the experiments: JC. Performed the experiments: XD. Analyzed the data: WL ZC. Contributed reagents/materials/analysis tools: HL GH GL BX XY. Wrote the paper: JC ZC.

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