Multilocus sequence typing reveals a novel subspeciation of *Lactobacillus delbrueckii*

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Currently, the species *Lactobacillus delbrueckii* is divided into four subspecies, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *indicus* and *L. delbrueckii* subsp. *lactis*. These classifications were based mainly on phenotypic identification methods and few studies have used genotypic identification methods. As a result, these subspecies have not yet been reliably delineated. In this study, the four subspecies of *L. delbrueckii* were discriminated by phenotype and by genotypic identification [amplified-fragment length polymorphism (AFLP) and multilocus sequence typing (MLST)] methods. The MLST method developed here was based on the analysis of seven housekeeping genes (*fusA*, *gyrB*, *hsp60*, *ileS*, *pyrG*, *recA* and *recG*). The MLST method had good discriminatory ability: the 41 strains of *L. delbrueckii* examined were divided into 34 sequence types, with 29 sequence types represented by only a single strain. The sequence types were divided into eight groups. These groups could be discriminated as representing different subspecies. The results of the AFLP and MLST analyses were consistent. The type strain of *L. delbrueckii* subsp. *delbrueckii*, YIT 0080^T, was clearly discriminated from the other strains currently classified as members of this subspecies, which were located close to strains of *L. delbrueckii* subsp. *lactis*. The MLST scheme developed in this study should be a useful tool for the identification of strains of *L. delbrueckii* to the subspecies level.

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INTRODUCTION

Lactobacillus delbrueckii is a species of lactic acid bacteria that is important to the production of many fermented foods of both plant and animal origin. In 1983, on the basis of differences in phenotypic features, this species was classified into three subspecies: *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* (Weiss *et al.*, 1983). *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* are usually found in dairy products and *L. delbrueckii* subsp. *delbrueckii* is usually found in fermented vegetables. In 2005, *L. delbrueckii* subsp. *indicus* was described as a novel subspecies of *L. delbrueckii* (Dellaglio *et al.*, 2005). *Lactobacillus delbrueckii* subsp. *indicus* was isolated from Indian dairy products.

Abbreviations: AFLP, amplified-fragment length polymorphism; GlcNAc, *N*-acetylglucosamine; MLST, multilocus sequence typing, RAPD, randomly amplified polymorphic DNA; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences reported in this paper are AB568647–AB568687 (*fusA*), AB568688–AB568728 (*gyrB*), AB568811–AB568851 (*hsp60*), AB568606–AB568646 (*ileS*), AB568729–AB568769 (*pyrG*), AB568770–AB568810 (*recA*) and AB568852–AB568892 (*recG*).

A supplementary figure is available with the online version of this paper.

The identification of *L. delbrueckii* strains is essential both for basic research and for the food industry. These subspecies have been classified by using phenotypic identification methods, especially the sugar fermentation test. However, phenotypic tests often give ambiguous results (Tanigawa *et al.*, 2010). Many researchers have been trying to discriminate between these subspecies by using molecular methods. These methods have included the use of randomly amplified polymorphic DNA PCR (RAPD-PCR) and *pepIQ* gene sequences to discriminate *L. delbrueckii* subsp. *bulgaricus* from the other subspecies of *L. delbrueckii* (Torriani *et al.*, 1999); SDS-PAGE profiles of cell-wall proteins to separate *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *bulgaricus* (Gatti *et al.*, 2001); restriction fragment length polymorphism (RFLP) of protein-coding genes to characterize and discriminate the strains of *L. delbrueckii* subsp. *lactis* from *L. delbrueckii* subsp. *bulgaricus* (Giraffa *et al.*, 2003); analyses of 16S rRNA sequence mutations and the expression of β -galactosidase and cell-wall-anchored protease; and characterization of the lactose operon locus and the sequence of the *lacR* gene, galactose metabolism, and the distribution of insertion sequences (IS-elements) in *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* (Germond *et al.*, 2003). Unfortunately, none of these methods has proved to be useful for

discriminating *L. delbrueckii* subsp. *delbrueckii* from the other three subspecies.

16S rRNA gene sequencing is the technique most commonly used for identification at the species level. At the subspecies level, however, 16S rRNA gene sequence similarity is often very high, and these sequences therefore cannot be used for identification purposes (Stackebrandt & Ebers, 2006).

The amplified-fragment length polymorphism (AFLP) method was used to identify *L. delbrueckii* subsp. *indicus* in 2005 (Dellaglio *et al.*, 2005). The AFLP method is known to be a highly sensitive and reproducible technique for bacterial genotyping (Savelkoul *et al.*, 1999). In this study, we selected AFLP to classify the subspecies of *L. delbrueckii* due to the success of this technique in identifying *L. delbrueckii* subsp. *indicus* and also because AFLP is regarded as a reliable nucleic acid fingerprinting method (Tindall *et al.*, 2010).

More recently, multilocus sequence typing (MLST) has also been used for discriminating bacterial strains. MLST is generally regarded as a bacterial identification method that, depending on the genomic properties or experimental design (such as the kinds of genes analysed), provides higher resolution than RAPD or AFLP methods. MLST uses automated DNA sequencing to characterize the alleles present at different housekeeping gene loci. Since it is based on nucleotide sequences, it is highly discriminatory and provides unambiguous results that are directly comparable among laboratories. This method was first described in 1998 (Maiden *et al.*, 1998) and there are now a number of publicly accessible MLST databases, such as PubMLST (<http://pubmlst.org/>). The MLST method has been used in a number of important studies on the identification of lactobacilli: *Lactobacillus casei* (Cai *et al.*, 2007; Diancourt *et al.*, 2007), *Lactobacillus plantarum* (de las Rivas *et al.*, 2006) and *Lactobacillus sanfranciscensis* (Picozzi *et al.*, 2010). In these studies, strains could be classified with high resolution by using MLST based on the sequences of the housekeeping genes *ftsZ*, *gyrB*, *g6pd*, *ddl*, *dnaE*, *gdh*, *metRS*, *mutL*, *mutS*, *nrdD*, *pgm*, *polA*, *purK*, *rpoB*, *recP* and *tkf4*.

The aims of this study were to: (i) further re-examine the subspecies composition of *L. delbrueckii*; (ii) develop an MLST method for *L. delbrueckii*; and (iii) compare the discriminatory powers of AFLP and MLST.

METHODS

Bacterial strains and growth conditions. A total of 41 bacterial strains that had been putatively assigned as *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *indicus*, *L. delbrueckii* subsp. *lactis* or *L. delbrueckii* sp., or derived from *sunki* (a traditional Japanese non-salted pickle) in Japan or from 'stinky tofu' brines in Taiwan, were obtained from the Culture Collection of the Yakult Central Institute (YIT; Tokyo, Japan) (Table 1). All bacterial strains were grown in MRS broth (BD Difco) at 37 °C for 24 h.

Sugar fermentation tests. The ability of the strains to ferment *N*-acetylglucosamine (GlcNAc), glucose, lactose, maltose, sucrose and

trehalose was confirmed by acid formation. First, a 4 ml sample of each bacterial culture (24 h culture at 37 °C) was pelleted by centrifugation at 2000 *g* for 20 min. The cell pellet was then suspended in 2 ml saline and 130 µl of this bacterial suspension was inoculated into 4 ml test medium (modified MRS broth, with the glucose replaced by 2% of the test substrate), supplemented with 0.005% chlorophenol red as the pH indicator, and then incubated at 37 °C for 24 or 96 h. Acid production in the test medium was determined by a change in colour of the medium from red (pH 6.8) to yellow (pH 5.4).

Genomic DNA extraction. The genomic DNAs of the isolates were extracted by a previously described method (Watanabe *et al.*, 2008). Briefly, 1 ml stationary-phase bacterial culture was harvested by centrifugation at 20 000 *g* for 3 min. The cell pellet was suspended in 250 µl extraction buffer (100 mM Tris/HCl, 40 mM EDTA, pH 9.0) and 500 µl benzyl chloride; 0.7 g of glass beads (0.1 mm in diameter) was added to the suspension and the mixture was shaken vigorously for 30 s with a FastPrep FP120 homogenizer (Bio 101) at a speed setting of 6.5 m s⁻¹. Subsequently, 50 µl 10% SDS was added to the suspension, which was then vortexed vigorously at 50 °C for 20 min in a MicroIncubator M-36 (Taitech). The mixture was cooled on ice for 15 min after the addition of 150 µl 3 M sodium acetate. After centrifugation of the mixture at 20 000 *g* for 15 min, the supernatant was collected and DNA was obtained by 2-propanol precipitation. Finally, the DNA was diluted to 10 µg ml⁻¹ in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

AFLP analysis. AFLP analysis for the discrimination of subspecies was performed by using an AFLP *EcoRI* Ligation/Amplification Module (Applied Biosystems) and an AFLP *MseI* Ligation/Amplification Module (Applied Biosystems). Total DNA was digested with *EcoRI* and *MseI* restriction enzymes, and the DNA fragments were ligated to the following double-stranded restriction-site-specific adaptors, *EcoRI* adaptors and *MseI* adaptors. For preselective and selective PCR amplification, the primers *EcoRI*-A and *MseI*-CA were used. PCR products were analysed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) in standard fragment analysis mode and with a GENESCAN-500 LIZ size standard (Applied Biosystems). After electrophoresis, the AFLP patterns were analysed and extracted with GeneMapper software v.4.0 (Applied Biosystems). A threshold fluorescence value of 100 arbitrary units was used to eliminate background fluorescence and DNA fragments of between 51 and 500 bp were analysed. Bands positioned at the same length (DNA size) in different strains were assumed to be similar and were classified as the same alleles. Bands of different sizes were treated as independent loci with two alleles (present or absent). Data were exported in binary format, with '1' for the presence of a band/peak and '0' for its absence; the data were analysed phylogenetically with the MEGA4 program (Tamura *et al.*, 2007) by using an unweighted pair-group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) and Pearson's correlation coefficient, with a bootstrap analysis of 100 repetitions.

DNA amplification and sequencing for MLST. MLST analyses were performed with seven housekeeping genes: *fusA*, *gyrB*, *hsp60*, *ileS*, *pyrG*, *recA* and *recG*. For the strains examined, parts of these genes were amplified by using the primers listed in Table 2. These primers were designed by using the consensus sequences of the seven housekeeping genes of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T (GenBank accession no. CR954253) and *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (NC008529). The PCR amplification program consisted of an initial denaturation step at 94 °C for 2 min; 30 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s; and a final extension step at 72 °C for 3 min. The PCR-amplified housekeeping genes were purified with a Wizard SV Gel and PCR Clean-Up System (Promega) and then sequenced with an ABI PRISM BigDye Terminator v.3.1 cycle sequence kit (Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Table 1. Bacterial strains used in this study

–, Unknown.

Subspecies/strains	Source	Reference
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>		
YIT 0080 ^T (=ATCC 9649 ^T)	Sour grain mash	Weiss <i>et al.</i> (1983)
YIT 11851 (=NCIMB 701744)	Wort	Weiss <i>et al.</i> (1983)
YIT 12133 (=LMG 22235)	Human urine	
YIT 12134 (=LMG 22236)	Human	
<i>L. delbrueckii</i> subsp. <i>lactis</i>		
YIT 0086 ^T (=ATCC 12315 ^T)	Swiss cheese	Weiss <i>et al.</i> (1983)
YIT 0020 (=ATCC 10697)	–	
YIT 0058 (=NCIMB 702457)	Calf abomasum	
YIT 0059 (=NIRD L-29)	–	
YIT 0087 (=ATCC 4797)	–	Weiss <i>et al.</i> (1983)
YIT 0167 (=ATCC 7830)	–	Weiss <i>et al.</i> (1983)
YIT 11852 (=ATCC 8000)	–	
YIT 11853 (=ATCC 11983)	–	
YIT 11854 (=ATCC 10697)	–	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>		
YIT 0181 ^T (=ATCC 11842 ^T)	–	Weiss <i>et al.</i> (1983)
YIT 0044 (=NCIMB 702478)	–	
YIT 0067 (=NCIMB 702474)	Calf abomasum	
YIT 0459 (=NCIMB 701006)	Bulgarian yoghurt	
YIT 0460 (=NCIMB 701373)	–	
YIT 0463 (=NCIMB 702074)	–	
YIT 0478 (NCIMB 702772)	–	
<i>L. delbrueckii</i> subsp. <i>indicus</i>		
YIT 11751 ^T (=DSM 15996 ^T)	Traditional fermented dairy products (India)	Dellaglio <i>et al.</i> (2005)
YIT 12135 (=LMG 22084)	Traditional fermented dairy products (India)	Dellaglio <i>et al.</i> (2005)
YIT 12136 (=LMG 22085)	Traditional fermented dairy products (India)	Dellaglio <i>et al.</i> (2005)
YIT 12137 (=LMG 22086)	Traditional fermented dairy products (India)	Dellaglio <i>et al.</i> (2005)
<i>L. delbrueckii</i> sp.		
YIT 0057	–	
YIT 0373	Human faeces	
YIT 11220	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11221	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11435	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11451	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11466	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11468	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11501	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11502	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11639	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11673	Traditional pickle product, <i>sunki</i> (Japan)	Watanabe <i>et al.</i> (2009)
YIT 11291	Fermented stinky tofu brine (Taiwan)	Chao <i>et al.</i> (2008)
YIT 11293	Fermented stinky tofu brine (Taiwan)	Chao <i>et al.</i> (2008)
YIT 11294	Fermented stinky tofu brine (Taiwan)	Chao <i>et al.</i> (2008)
YIT 11300	Fermented stinky tofu brine (Taiwan)	Chao <i>et al.</i> (2008)
YIT 11403	Fermented stinky tofu brine (Taiwan)	Chao <i>et al.</i> (2008)

*The original strain numbers are noted here, if known. ATCC, American Type Culture Collection; LMG, Laboratorium voor Microbiologie, Ghent University; NCIMB, National Collection of Industrial Food and Marine Bacteria; NIRD, National Institute for Research in Dairying; DSM, German Collection of Microorganisms and Cell Cultures.

Data analysis for MLST. For each MLST locus, an allele number was given to each distinct combination of alleles of the seven genes by using BioNumerics v.6.0 (Applied-Maths). Each allele number was assigned a sequence type (ST). The same ST was used for several

strains when they shared the same allelic profiles. The mean G+C content of the DNA, the number of polymorphic sites and the nucleotide diversity were calculated by using DnaSP v.5.1 (Librado & Rozas, 2009). This software was also used to calculate the ratio of the

Table 2. Genes and primers used for MLST

Gene	Enzyme function	Position	Primer	Sequence (5'–3')	Analysis size (bp)
<i>fusA</i>	Protein elongation factor EF-2	293–312	<i>fusA</i> -F	TGCGTGTCTTACGGTGCC	474
		892–873	<i>fusA</i> -R	TGCCTTCCTTGTCTGGGCG	
<i>gyrB</i>	DNA gyrase, subunit B	588–607	<i>gyrB</i> -F	AAGCTGGGCAACTCCGACGC	380
		1617–1598	<i>gyrB</i> -R	CATCGGCCGCATGTAGCGGT	
<i>hsp60</i>	Heat-shock protein 60	4–25	<i>hsp</i> -F	ACTGTTTTGACCCAGGCCATCG	515
		552–533	<i>hsp</i> -R	AACAGCAACMACGTTGAAGG	
<i>ileS</i>	Isoleucyl-tRNA synthase	965–984	<i>ileS</i> -F	GCCTGGTCCACACCGCTTCC	496
		1619–1600	<i>ileS</i> -R	ACCCCGCTCCAGGATGACCC	
<i>pyrG</i>	CTP synthase	542–561	<i>pyrG</i> -F	TGCGGGCAGCCAAGGAACG	392
		1073–1054	<i>pyrG</i> -R	CCGAAACCGCTGGCAGCAT	
<i>recA</i>	Recombinase A	129–148	<i>recA</i> -F	CTCACTGGCACTGGACGCGG	412
		1088–1069	<i>recA</i> -R	CTTCAGGGCCCCACCACCTG	
<i>recG</i>	ATP-dependent DNA helicase	545–564	<i>recG</i> -F	GGAAATTCGCCGCCAGCCT	490
		1219–1200	<i>recG</i> -R	TGACCAGCAGATGCAGCGCC	

*Numbers denote the positions of the first and last bases of the gene on the *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T (=YIT 0181^T) chromosome.

number of synonymous changes per synonymous site (d_s) to the number of non-synonymous changes per non-synonymous site (d_N ; d_s/d_N ratio) and to perform Tajima's *D* test.

Phylogenetic analyses. Cluster analysis based on AFLP profiles was conducted by using UPGMA (Sneath & Sokal, 1973) in the MEGA4 program (Tamura, *et al.*, 2007). Minimum spanning tree analysis was performed with BioNumerics v.6.0.

RESULTS

A total of 41 strains assigned as *L. delbrueckii* were used (Table 1). Twenty-four of the strains had been previously classified into one of the four subspecies: four strains were identified as *L. delbrueckii* subsp. *delbrueckii*, nine strains as *L. delbrueckii* subsp. *lactis*, seven strains as *L. delbrueckii* subsp. *bulgaricus* and four strains as *L. delbrueckii* subsp. *indicus*. The other 17 strains were our own original strains, which were isolated mainly from *sunki*, a traditional Japanese pickle product, in Japan in 2004 (Watanabe *et al.*, 2009) or from fermented stinky tofu brines in Taiwan in 2005 (Chao *et al.*, 2008).

Phenotypic identification

Fermentation tests were performed with GlcNAc, glucose, lactose, maltose, sucrose and trehalose. The presence of chlorophenol red caused the colour of the medium to change from red to yellow as the pH changed from 6.8 to 5.4. All strains fermented glucose (data not shown); the other results of the fermentation tests are shown in Table 3. The delineation of subspecies of *L. delbrueckii* based on the sugar fermentation profile has been reported previously (Dellaglio *et al.*, 2005). The inability to metabolize lactose distinguishes *L. delbrueckii* subsp. *delbrueckii* from the other subspecies of *L. delbrueckii*. With the exception of *L.*

delbrueckii subsp. *delbrueckii*, all strains that can ferment both maltose and trehalose are classified as *L. delbrueckii* subsp. *lactis*. In contrast, strains that can ferment GlcNAc but not sucrose are classified as *L. delbrueckii* subsp. *bulgaricus*. According to this definition, when the fermentation results from the 96 h cultures were used, only two of the four strains currently classified as *L. delbrueckii* subsp. *delbrueckii* showed the expected phenotypic characteristics of this subspecies and 12 of the unclassified isolates shared the same characteristics. Eight of the nine strains currently classified as *L. delbrueckii* subsp. *lactis* showed the phenotypic characteristics of this subspecies, two strains currently classified as *L. delbrueckii* subsp. *delbrueckii* had the phenotypic characteristics of *L. delbrueckii* subsp. *lactis* and four unclassified isolates shared the same characteristics. Five of the seven strains currently classified as *L. delbrueckii* subsp. *bulgaricus* showed the phenotypic characteristics expected for this subspecies and one strain of *L. delbrueckii* subsp. *lactis* had the same characteristics as *L. delbrueckii* subsp. *bulgaricus*. Three of the four strains currently classified as *L. delbrueckii* subsp. *indicus* had the phenotypic characteristics of this subspecies. The remaining three strains could not be identified as any known subspecies. Acid formation from lactose, maltose and GlcNAc was strain-dependent and the results varied depending on whether strains were cultured for 24 or 96 h. For example, strain YIT 0020 gave a negative result for fermentation of maltose after 24 h of culture but the result was positive after 96 h.

AFLP analysis of 41 *L. delbrueckii* strains

The AFLP method is useful for grouping bacterial strains according to their genetic relationships. The AFLP method was used to measure the extent of subspecies diversity, and a dendrogram was constructed based on the AFLP profiles

Table 3. Typing of 41 strains by MLST, AFLP and sugar fermentation test results

PT, Phenotypic tests, B, *L. delbrueckii* subsp. *bulgaricus*; D, *L. delbrueckii* subsp. *delbrueckii*; I, *L. delbrueckii* subsp. *indicus*; L, *L. delbrueckii* subsp. *lactis*; ND, not determined; G, group in minimum spanning tree of Fig. 2.

Putative subspecies	Allele code							ST	MLST group (Fig. 2)	Cluster type (Fig. 3)	AFLP type (Fig. 1)	Fermentation (24 h/96 h)					Subspecies*		
	<i>fusA</i>	<i>gyrB</i>	<i>hsp60</i>	<i>ileS</i>	<i>pyrG</i>	<i>recA</i>	<i>recG</i>					Lactose	Maltose	Trehalose	Sucrose	GlcNAc	MLST	AFLP	PT
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>																			
YIT 0080 ^T	1	1	1	1	1	1	1	1	1	2	B	−/−	−/−	−/−	+/+	+/+	D	D	D
YIT 11851	2	4	1	19	6	7	22	34	2b	1c	A-2	−/−	+/+	+/+	+/+	+/+	L	L	D
YIT 12133	7	4	10	3	6	2	11	17	2a	1a	A-4	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 12134	8	4	4	3	6	2	12	18	2a	1a	A-4	+/+	+/+	+/+	+/+	+/+	L	L	L
<i>L. delbrueckii</i> subsp. <i>lactis</i>																			
YIT 0086 ^T	2	3	3	2	18	17	3	4	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 0020	3	3	3	3	3	2	3	7	2a	1a	A-1	+/+	−/+	−/−	+/+	+/+	L	L	ND
YIT 0058	2	3	3	3	3	2	3	3	2a	1a	A-1	+/+	+/+	−/−	−/−	+/+	L	L	B
YIT 0059	3	3	3	3	3	2	3	8	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 0087	2	4	4	4	4	3	4	5	2a	1c	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 0167	2	4	4	4	4	4	5	6	2a	1c	A-3	+/+	+/+	−/+	+/+	−/+	L	L	L
YIT 11852	3	3	3	3	3	2	3	7	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 11853	3	3	3	3	3	2	3	7	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 11854	3	3	3	3	3	4	3	8	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>																			
YIT 0181 ^T	4	5	5	5	5	5	6	9	3	4	C	+/+	−/−	−/−	−/−	−/+	B	B	B
YIT 0044	4	6	6	6	6	6	7	10	3	4	C	−/+	−/−	−/−	−/−	−/+	B	B	B
YIT 0067	4	8	5	8	8	6	6	12	3	4	C	+/+	−/−	−/−	−/−	−/+	B	B	B
YIT 0459	4	6	8	9	6	6	9	13	3	4	C	+/+	−/−	−/−	−/−	−/−	B	B	ND
YIT 0460	4	6	6	6	6	6	7	10	3	4	C	+/+	−/−	−/−	−/−	−/−	B	B	ND
YIT 0463	4	8	5	5	6	6	6	14	3	4	C	+/+	−/−	−/−	−/−	−/+	B	B	B
YIT 0478	4	6	6	10	6	6	7	15	3	4	C	+/+	−/−	−/−	−/−	−/+	B	B	B
<i>L. delbrueckii</i> subsp. <i>indicus</i>																			
YIT 11751 ^T	6	9	9	11	9	8	10	16	4	5	D	+/+	−/−	−/−	+/+	+/+	I	I	I
YIT 12135	6	11	12	13	11	10	10	20	4	5	D	+/+	−/−	−/−	−/−	+/+	I	I	B
YIT 12136	6	12	9	13	12	10	14	21	4	5	D	+/+	−/−	+/+	+/+	+/+	I	I	I
YIT 12137	6	13	9	13	12	10	14	22	4	5	D	+/+	−/−	−/−	+/+	−/+	I	I	I
<i>L. delbrueckii</i> sp.																			
YIT 0057	2	2	2	2	2	2	2	2	2a	1a	A-1	+/+	+/+	+/+	+/+	+/+	L	L	L
YIT 0373	5	7	7	7	7	7	8	11	7	3b	A-2	−/−	+/+	−/−	+/+	+/+	G7	L	D
<i>L. delbrueckii</i> sp. (from <i>sunki</i>)																			
YIT 11220	9	10	11	12	10	9	13	19	5	3a	A-2	−/−	+/+	+/+	+/+	+/+	G5	L	D
YIT 11221	9	10	11	17	10	9	13	28	5	3a	A-2	−/−	+/+	+/+	+/+	+/+	G5	L	D

Table 3. cont.

Putative subspecies	Allele code							ST	MLST group (Fig. 2)	Cluster type (Fig. 3)	AFLP type (Fig. 1)	Fermentation (24 h/96 h)					Subspecies*		
	<i>fusA</i>	<i>gyrB</i>	<i>hsp60</i>	<i>ileS</i>	<i>pyrG</i>	<i>recA</i>	<i>recG</i>					Lactose	Maltose	Trehalose	Sucrose	GlcNAc	MLST	AFLP	PT
YIT 11435	2	4	3	14	13	7	15	23	2b	1b	A-3	-/+	+/+	+/+	+/+	+/+	L	L	L
YIT 11451	2	4	3	14	13	7	15	23	2b	1b	A-3	-/+	+/+	+/+	+/+	-/-	L	L	L
YIT 11466	9	10	11	17	17	9	13	33	5	3a	A-2	-/-	+/+	+/+	+/+	+/+	G5	L	D
YIT 11468	2	4	3	14	13	7	15	23	2b	1b	A-3	-/+	+/+	+/+	+/+	+/+	L	L	L
YIT 11501	2	3	15	16	6	11	18	26	2a	1a	A-3	+/+	-/+	+/+	+/+	+/+	L	L	L
YIT 11502	10	4	13	15	14	7	16	24	2b	1d	A-3	-/-	+/+	+/+	+/+	+/+	L	L	D
YIT 11639	10	4	14	15	14	7	17	25	2b	1d	A-3	-/-	+/+	+/+	+/+	+/+	L	L	D
YIT 11673	9	10	11	17	10	9	13	28	5	3a	A-2	-/-	+/+	+/+	+/+	+/+	G5	L	D
<i>L. delbrueckii</i> sp. (from stinky tofu)																			
YIT 11291	2	16	3	14	6	16	21	32	2b	1b	A-3	-/-	-/-	+/+	+/+	+/+	L	L	D
YIT 11293	2	16	3	14	6	15	20	31	2b	1b	A-3	-/-	-/-	+/+	±/+	±/+	L	L	D
YIT 11294	11	14	16	14	15	12	8	27	8	1b	A-3	-/-	±/+	-/-	+/+	-/+	G8	L	D
YIT 11300	12	4	3	18	6	13	15	29	2b	1b	A-3	-/-	-/-	-/-	+/+	-/+	L	L	D
YIT 11403	12	15	17	14	16	14	19	30	6	1e	A-3	-/-	-/-	+/+	+/+	-/+	G6	L	D

*Subspecies determined by AFLP, MLST and phenotypic tests in this study. Subspecies determined by AFLP were based on the locations of the type strains in the dendrogram of Fig. 1. Subspecies determined by MLST were based on the groupings of the type strains in the minimum spanning tree in Fig. 2. Subspecies determined by phenotypic test were based on the sugar fermentation profile, as reported by Dellaglio *et al.* (2005).

of the strains examined (Fig. 1). The 41 *L. delbrueckii* strains were separated into four major clusters (A, B, C and D). Cluster A consisted of three strains of *L. delbrueckii* subsp. *delbrueckii*, nine strains of *L. delbrueckii* subsp. *lactis* and 17 strains of *L. delbrueckii* sp. Cluster B comprised only a single strain (YIT 0080^T), cluster C consisted of seven strains of *L. delbrueckii* subsp. *bulgaricus* and cluster D consisted of four strains of *L. delbrueckii* subsp. *indicus*. Clusters A, B, C and D were identified in accordance with the locations of the type strains as *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *indicus*, respectively.

Cluster A was divided into four subclusters. Cluster A-1 included eight strains of *L. delbrueckii* subsp. *lactis* and one strain of *L. delbrueckii* sp.; cluster A-2 included one strain of *L. delbrueckii* subsp. *delbrueckii*, one strain of *L. delbrueckii*

sp. and four strains of *L. delbrueckii* sp. isolated from *sunki*. Cluster A-3 included 12 strains of *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* sp. and cluster A-4 included two strains of *L. delbrueckii* subsp. *delbrueckii*.

Allelic and nucleotide diversity using MLST

MLST analysis was used to examine the genetic diversity of *L. delbrueckii* and to discriminate subspecies. The sequences of the seven loci were determined for the 41 study strains. From the 41 *L. delbrueckii* strains, 34 different STs were obtained. Of these, 29 STs were assigned to single strains, three STs (ST8, ST10 and ST28) were assigned to two strains and two STs (ST7 and ST23) were assigned to three strains. Consensus sequence templates ranged in length from 380 bp (*gyrB*) to 515 bp (*hsp60*). Polymorphic

Fig. 1. Dendrogram derived from the AFLP band pattern. Bootstrap values based on 100 replications are given at nodes. Bar, number of band mismatches.

sites are shown in Supplementary Fig. S1 (available in *Microbiology Online*). Non-synonymous substitutions per non-synonymous site were relatively rare compared with synonymous changes per synonymous site (Table 4), indicating selection against amino acid changes and excluding strong positive selection on the observed allelic diversity, as is typically observed for housekeeping genes. Tajima's *D* values did not deviate significantly from zero, supporting a neutral selection of the alleles of the seven housekeeping genes. The DNA G+C content observed for all alleles of the seven genes ranged from 49.1 mol% (*fusA*) to 60.9 mol% (*recA*), which was a little higher than that of the complete genomes of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T and *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (49.0 mol%).

MLST for subspeciation of *L. delbrueckii*

The number of alleles per locus ranged from 12 (*fusA*) to 22 (*recG*). By combining the seven gene loci, 34 STs were distinguished. To explore the relationships among the 41 strains, phylogenetic analysis based on the allelic profiles was performed by using the minimum spanning tree algorithm (Fig. 2). When the distance between different STs was 6 or more allelic differences, the strains that stepped between these STs were bundled as different groups. The 41 strains of *L. delbrueckii* were thus divided into eight groups: group 1 (ST1), group 2 (ST2, ST3, ST4, ST5, ST6, ST7, ST8, ST17, ST18, ST23, ST24, ST25, ST26, ST29, ST31, ST32, ST34), Group 3 (ST9, ST10, ST12, ST13, ST14, ST15), Group 4 (ST16, ST20, ST21, ST22), Group 5 (ST19, ST28, ST33), Group 6 (ST30), Group 7 (ST11), and Group 8 (ST27).

We also performed phylogenetic analysis based on the position of mutations in the composite sequences that were constructed by concatenating the seven housekeeping genes (Fig. 3). The 41 strains were separated into five clusters: cluster 1 consisted of three strains of *L. delbrueckii* subsp. *delbrueckii*, nine strains of *L. delbrueckii* subsp. *lactis* and 12 strains of *L. delbrueckii* sp. Cluster 2 comprised only a single strain (YIT 0080^T) and cluster 3 consisted of five strains of *L. delbrueckii* sp. Cluster 4 consisted of seven

strains of *L. delbrueckii* subsp. *bulgaricus* and cluster 5 consisted of four strains of *L. delbrueckii* subsp. *indicus*. Cluster 1 was divided into five subclusters: cluster 1a included two strains of *L. delbrueckii* subsp. *delbrueckii*, seven strains of *L. delbrueckii* subsp. *lactis* and two strains of *L. delbrueckii* sp.; cluster 1b included seven strains of *L. delbrueckii* sp.; cluster 1c included one strain of *L. delbrueckii* subsp. *delbrueckii* and two strains of *L. delbrueckii* subsp. *lactis*; cluster 1d included two strains of *L. delbrueckii* sp.; and cluster 1e comprised a single strain of *L. delbrueckii* sp. Cluster 3 was also divided into two subclusters: cluster 3a included four strains of *L. delbrueckii* sp. all derived from *sunki*; and cluster 3b comprised a single strain of *L. delbrueckii* sp.

DISCUSSION

Various methods for discriminating subspecies of *L. delbrueckii* have been proposed by many researchers. However, several research groups have recognized that the identifications based on phenotypic and genotypic methods do not match (Giraffa *et al.*, 2004; Miteva *et al.*, 2001). Furthermore, although we had attempted to identify *L. delbrueckii* subspecies by using single housekeeping genes in the past, the resulting subspeciation was of low resolution (data not shown).

Lactobacillus delbrueckii subsp. *indicus* was first established as a novel subspecies in 2005 by using the AFLP method (Vos *et al.*, 1995). The AFLP method is based on calculating the differences in the length of restriction-enzyme-digested genomic DNA, and it is known to be a reproducible method. However, the use of housekeeping gene sequences for the identification of bacteria is often more sensitive and more accurate. AFLP and MLST techniques target different aspects of the genome. MLST targets housekeeping genes, which evolve slowly, whereas AFLP targets large genome rearrangements. In addition, the principle of clustering in these methods is also different. AFLP compares the lengths of fragments of chromosomal DNA and MLST compares the base compositions of gene sequences. Therefore, we developed

Table 4. Genetic variability at *L. delbrueckii* loci

d_s , Number of synonymous changes per synonymous site; d_N , number of non-synonymous changes per non-synonymous site.

Locus	Fragment size (bp)	Mean DNA G+C content (mol%)	No. of polymorphic sites	Nucleotide diversity (substitution/base)	d_N/d_s	Tajima's <i>D</i> value	No. of alleles
<i>fusA</i>	474	49.1	17	0.0051	0.11	-1.251	12
<i>gyrB</i>	380	60.1	22	0.0080	0.05	-1.363	16
<i>hsp60</i>	515	51.0	18	0.0066	0.13	-0.751	17
<i>ileS</i>	496	55.6	23	0.0089	0.03	-0.794	17
<i>pyrG</i>	392	53.1	22	0.0068	0.11	-1.564	18
<i>recA</i>	412	60.9	20	0.0066	0.06	-1.483	17
<i>recG</i>	490	54.3	29	0.0096	0.16	-1.200	22

Fig. 2. Minimum spanning tree of 41 strains of *L. delbrueckii* based on sequence type (ST). Each circle corresponds to the ST, and the circle size denotes the number of strains sharing the same ST. To illustrate the concordance of the MLST data with AFLP data, circles are coloured on the basis of AFLP cluster type: yellow, cluster A-1; pink, cluster A-2; blue, cluster A-3; green, cluster A-4; red, cluster B; purple, cluster C; sky blue, cluster D. Numbers between the circles indicate the number of allelic differences between the profiles. Coloured zones between some groups of circles indicate that these profiles belong to the same potential subspecies. The double line separates the strains into two groups on the basis of their ability to ferment lactose.

an MLST method based on seven housekeeping gene sequences (*fusA*, *gyrB*, *hsp60*, *ileS*, *pyrG*, *recA* and *recG*) to classify 41 strains of *L. delbrueckii*. The utility of MLST for the analysis of the genetic structure of bacterial populations is based mainly on the ability of housekeeping genes to have selectively neutral variability. Analysis of synonymous and non-synonymous changes in the allele sequences of a locus can be used to determine whether they are subject to positive selection; therefore, a d_N/d_S ratio >1 implies selection for amino acid changes. In our analysis of the divergences in the seven housekeeping genes, the d_N/d_S ratios were much lower than 1. The nucleotide diversity ranged from 0.0051 to 0.0096. By comparison, nucleotide diversity values obtained for *Lactobacillus casei* ranged from 0.0002 to 0.0076 (Diancourt *et al.*, 2007) and nucleotide diversity values for *Lactobacillus plantarum* ranged from 0.0004 to 0.0072 (de las Rivas *et al.*, 2006). The nucleotide diversity values of *L. delbrueckii* were a little higher than those of these two species.

The three methods were compared, as shown in Table 3. These subspecies were determined by the dendrogram based on AFLP (Fig. 1) and the minimum spanning tree based on MLST (Fig. 2) to be in accordance with the locations of the type strains. For the phenotypic tests, subspecies were determined by using previously reported

sugar fermentation profiles (Dellaglio *et al.*, 2005). In spite of the MLST and AFLP methods utilizing different aspects of the genome, subspecies identification by the two methods was in good concordance, with the exception of groups 5–8 in the MLST analysis. On the other hand, the phenotypic tests showed results for some strains that were discordant with the results from the other two methods. All the strains located in the four disparate groups (groups 5–8) by MLST were identified as *L. delbrueckii* subsp. *lactis* by AFLP and as *L. delbrueckii* subsp. *delbrueckii* by means of phenotypic tests. MLST analysis suggested that the strains in these four disparate groups (groups 5–8) did not belong to either *L. delbrueckii* subsp. *delbrueckii* or *L. delbrueckii* subsp. *lactis*. This finding indicated that these strains could represent a novel subspecies and that MLST has a higher resolution than AFLP for the subspeciation of *L. delbrueckii*.

We confirmed that the results of the MLST method corresponded well to the results of the AFLP method with respect to subspecies identification of the 41 *L. delbrueckii* strains. By the AFLP method, the 41 strains of *L. delbrueckii* were divided into four clusters. The type strain of *L. delbrueckii* subsp. *delbrueckii* (YIT 0080^T) was the single strain located in independent cluster B, whereas the other strains of *L. delbrueckii* subsp. *delbrueckii* were located in

Fig. 3. Dendrogram derived from the similarity of the seven housekeeping gene sequences. Bar, percentage sequence similarity.

cluster A, which also included the type strain of *L. delbrueckii* subsp. *lactis* (YIT 0086^T) and the other strains of *L. delbrueckii* subsp. *lactis*. By the MLST method, we also confirmed that strain YIT 0080^T (group 1) was clearly separated from the other strains in this species (group 2). To the best of our knowledge, this is the first study to demonstrate the ability of the MLST technique to classify *L. delbrueckii* species and subspecies.

MLST has a powerful ability to discriminate at strain level. This method is often used in the discrimination of strains by their origins. However, we could not find any association between a clonal complex (a cluster of linked STs; shown by groups in Fig. 3) and the source of the strains included within it. The strains in our original culture collection of *L. delbrueckii* sp. derived from *sunki* and stinky tofu brines were not bundled in the same clonal complexes. However, four strains of the *L. delbrueckii* sp.

isolated from *sunki*, strains YIT 11220, YIT 11221, YIT 11466 and YIT 11673, were bundled in the same clonal complex (group 5), which corresponded to subcluster A-2 in the AFLP dendrogram. Additionally, we found that there was a relationship between lactose utilization and the patterns of grouping in the minimum spanning tree. Group 2 was divided into two subgroups on the basis of the ability of the strains to utilize lactose: Group 2a, which comprised 13 lactose-fermenting strains and ten STs (ST2, ST3, ST4, ST5, ST6, ST7, ST8, ST17, ST18 and ST26), and Group 2b which comprised nine non-lactose-fermenting strains and seven STs (ST23, ST24, ST25, ST29, ST31, ST32 and ST34) (Table 3).

This study also confirmed that the phenotypic features based on sugar fermentation tests were not useful for the identification of subspecies because they varied with culture time and were sometimes ambiguous. In addition,

phenotypic markers can often provide discordant results, as previously reported (Tanigawa *et al.*, 2010). However, the phenotypic tests did reflect the strain's origin in some aspects. The 20 strains derived from milk products (seven strains of *L. delbrueckii* subsp. *bulgaricus*, nine strains of *L. delbrueckii* subsp. *lactis* and four strains of *L. delbrueckii* subsp. *indicus*), could all ferment lactose. Two strains of *L. delbrueckii* subsp. *delbrueckii* (YIT 12133, YIT 12134) and two *L. delbrueckii* sp. strains could also ferment lactose. In contrast, the *L. delbrueckii* sp. strains isolated from fermented vegetables, fermented brines for making stinky tofu, and human faeces were non-lactose-fermenting strains (except strains YIT 0057 and YIT 11501). These 15 non-lactose-fermenting strains were located in three clusters, cluster 1b, cluster 1d and cluster 3, in the MLST dendrogram (Fig. 3) and in the disparate groups in the minimum spanning tree based on MLST (Fig. 2), group 2b, group 5, group 6, group 7 and group 8. The two strains of *L. delbrueckii* subsp. *delbrueckii* were exceptions: strains YIT 0080^T and YIT 11851 were located in cluster 2 and cluster 1c (Fig. 3), and in group 1 and group 2b (Fig. 2), respectively.

The MLST method also had a higher resolution than the ALFP method with respect to the discrimination of the non-lactose-fermenting strains identified as *L. delbrueckii* subsp. *lactis*. By using the minimum spanning tree method, group 2 was divided into two subgroups: Group 2a consisting of the lactose-fermenting strains, which could be classified as *L. delbrueckii* subsp. *lactis*, and Group 2b consisting of the non-lactose-fermenting strains (Table 3).

From the evolutionary evidence revealed by these genotypic approaches, we confirmed that it was impossible to discriminate the strains of *L. delbrueckii* subsp. *delbrueckii* (except the type strain YIT 0080^T) from the strains of *L. delbrueckii* subsp. *lactis* because of their close relationship. In addition, eight independent groups were observed by MLST, which could be discriminated at the subspecies level. The four *L. delbrueckii* sp. strains derived from *sunki* could represent a novel subspecies of *L. delbrueckii*.

Therefore, we propose that *L. delbrueckii* subsp. *delbrueckii* YIT 0080^T should be the sole strain from among the 41 strains examined here that should be classified as *L. delbrueckii* subsp. *delbrueckii*. The other strains of *L. delbrueckii* subsp. *delbrueckii* used in this study, YIT 11851 (=NCIMB 701744), YIT 12133 (=LMG 22235) and YIT 12134 (=LMG 22236), should be reclassified as strains of *L. delbrueckii* subsp. *lactis*.

The MLST scheme proposed in this study could be a useful tool for subspeciation of strains in *L. delbrueckii*.

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