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Research Paper

Identification and characterization of lactic acid bacteria associated with tropical grass silage produced in Okinawa

Identificación y caracterización de bacterias ácido lácticas asociadas con ensilaje de gramíneas tropicales en Okinawa, Japón

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Abstract

In Okinawa, rate of increase in gross agricultural production during 2011–2016 was the highest in Japan and sales of calves ranked fourth throughout Japan. Raising cattle by feeding high quality silage is beneficial both nutritionally and economically. However, little is known about lactic acid bacteria (LAB) present in silages made from tropical grass in Okinawa. To improve understanding of fermentation processes in silages, the LAB present in a range of silages (*Digitaria eriantha*, *Megathyrsus maximus*, *Chloris gayana*, *Urochloa mutica*, *Sorghum* sp. and *Zea mays*) were identified. All isolates were Gram-positive and mainly catalase-negative bacteria. According to morphological and biochemical characters, 37 isolates were divided into 16 groups and on the basis of 16S rDNA sequence analysis, 7 were identified as *Lactobacillus plantarum*, 3 as *L. paraplantarum*, 1 as *L. brevis*, 1 as *L. acidipiscis*, 3 as *L. casei*, 1 as *L. fermentum*, 9 as *Weissella paramesenteroides*, 1 as *W. kimchii*, 5 as *Lactococcus lactis* subsp. *lactis*, 2 as *Lactococcus garvieae* and 4 as *Pediococcus pentosaceus*. Some of this wide variety of LAB in Okinawan silage could be beneficial for improving quality of silages and further studies are planned to determine benefits of inoculating forage with particular strains at ensiling.

Keywords: Ensilage; fermenting bacteria, tropical forage.

Resumen

En Okinawa, el crecimiento de la producción agropecuaria durante 2011–16 fue la más alta en Japón y las ventas de terneros ocuparon el cuarto lugar en todo el país. La producción de ganado bovino basado en la alimentación con ensilaje de alta calidad es beneficiosa tanto nutricional como económicamente. Sin embargo, en la región se sabe poco acerca de las bacterias ácido lácticas (BAL) presentes en los ensilajes de pastos tropicales. Para mejorar el conocimiento de los procesos de fermentación en los ensilajes, se identificaron las BAL presentes en los ensilajes de una variedad de gramíneas (*Digitaria eriantha*, *Megathyrsus maximus*, *Chloris gayana*, *Urochloa mutica*, *Sorghum* sp. y *Zea mays*). Todos los aislamientos fueron Gram-positivos y mayormente catalasa-negativos. De acuerdo con los caracteres morfológicos y bioquímicos, los 37 aislamientos se dividieron en 16 grupos y, con base en el análisis de la secuencia del gen 16S rDNA, 7 fueron identificados como *Lactobacillus plantarum*, 3 como *L. paraplantarum*, 1 como *L. brevis*, 1 como *L. acidipiscis*, 3 como *L. casei*, 1 como *L. fermentum*, 9 como *Weissella paramesenteroides*, 1 como *W. kimchii*, 5 como *Lactococcus lactis* subsp. *lactis*, 2 como *Lactococcus garvieae* y 4 como *Pediococcus pentosaceus*. Parte de esta amplia diversidad de BAL en el ensilaje en Okinawa podría ser útil para mejorar la calidad de los ensilajes y planear estudios para determinar si el uso de eventuales inoculantes basados en cepas particulares resulta beneficioso en el ensilado de forrajes.

Palabras clave: Bacterias fermentadoras, ensilado, forrajes tropicales.

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Introduction

In Okinawa, the southernmost part of Japan, rate of increase in gross agricultural production between 2011 and 2016 was the highest in Japan. In particular, production from the beef industry increased by 6.4 billion yen over the past 5 years and sales of calves during the past 10 years ranked fourth throughout Japan. To ensure breeding cows are healthy with high reproductive rates and milk yields, feeding of high quality silage or grass plays an important role. Farmers produce and store silage to feed cows during times when pasture growth is slow, such as the winter season in Okinawa. Lactic acid bacteria (LAB) are the key to producing high quality silage (Cai 2001) because good preservation depends on the production of sufficient organic acids to inhibit the growth of undesirable microorganisms, such as spoilage bacteria, food-borne pathogens, yeasts and molds, under anaerobic conditions (Li and Nishino 2011; Dunière et al. 2013). In general, silages made from tropical grasses have higher pH than silages from cool temperate grasses due to lower lactic acid or higher acetic acid and butyric acid concentrations (Panditharatne et al. 1986). This could result from low water-soluble carbohydrate (WSC) concentrations and limited populations of LAB in the silage (Cai 2001).

In order to improve the fermentation quality of silage, it is necessary to understand the characteristics of silages stored in Okinawa and the fermentation processes which occur. Then steps can be taken to modify the fermentation process. It is obvious that LAB play a critical role in producing good quality silages with low pH values (Cai 2001). Moreover, LAB influence not only the quality of silage but also nutrition and metabolism in cattle because some LAB can play an important role as probiotics with beneficial outcomes for farm animals (Perdigon et al. 1995). In fact, it is well established that some LAB improve the intestinal microflora and promote the growth and health of animals (Mitsuoka 1990; Perdigon et al. 1995).

The environment in the Prefecture of Okinawa, consisting of many islands in the subtropical region, is unique and microflora in the area could also be unique. Consequently, creating good silages in Okinawa could also be a unique process, different from other regions. As such, determining the types of microorganisms native to Okinawa and their culturing for silage making are really important. However, at present, little is known about LAB related to silages made of tropical grass grown in Okinawa (Hanagasaki and Cai 2009). To shed light on this issue, we analyzed the fermentation characteristics of silage and identified LAB involved in the fermentation process in silages made of tropical grasses and other crops grown in the Main Island and Ishigaki Island, Okinawa.

Materials and Methods

Our study was based on 2 types of silage: (i) silage prepared in the field (round bale silage); and (ii) silage prepared in the laboratory.

Round bale silage preparation

Round bale silages from: (i) Transvala grass; (ii) a mixture of Rhodes grass and Para grass; (iii) sorghum; and (iv) corn were obtained in the field at several sites in the Okinawa Prefecture (Figure 1).

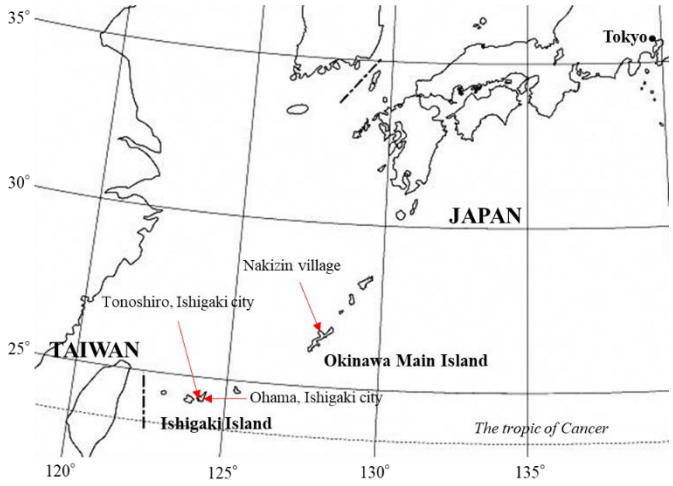

Figure 1. Map of Okinawa indicating where the field silage used in this study was produced.

Transvala. Forage of *Digitaria eriantha* cv. Transvala was obtained from an experimental field at Okinawa Livestock Research Center (Nakizin, Okinawa, Japan) on 9 May 2007 (Figure 1). The material was drawn from the third harvest in 2007 and the grass had regrown for 40 days following the second harvest.

The process of producing round bale silage was as follows: Transvala forage was mowed, then aerated using a tedder rake and allowed to dry for 1 day. The wilted material was then converted into approximately 580 kg round bales using a roll baler (Rollant 46, CLAAS, Harsewinkel, Germany) according to manufacturer's instructions. Bales were then wrapped with white polyethylene film and stored for 37 days (Silage A) or 61 days (Silage B) upright on their flat base. On each of 15 June and 9 July 2007, a single bale was opened and 500 g samples for analysis were collected from the top, middle and bottom of the bale. Bales were about 100 cm tall and samples were drawn at about 25 cm from the top, 50 cm from the top and 25 cm from the bottom in about

the center of the bale, by removing outer layers of silage and extracting material close to the center.

Grass mix. A mixed pasture of Rhodes grass (*Chloris gayana*) and Para grass (*Urochloa mutica*, syn. *Brachiaria mutica*) (1:1) on a farm in Ishigaki Island (Tonoshiro, Ishigaki) (Figure 1), which had regrown for 60 days from the previous harvest, was mowed, aerated using a tedder rake and allowed to dry for 1 day. The height of grass was about 80 cm and vegetative growth was virtually complete but pasture was not wilting. Round bale silage was produced using a roll baler (RF130, VICONJAPAN, Saitama, Japan) according to manufacturer's instructions, including wrapping in white polyethylene film. Bales were stored upright for 105 days, until 8 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

Sorghum (*Sorghum vulgare*, BMR Sweet) on a farm in Ishigaki Island (Ohama, Ishigaki) (Figure 1) was mowed at 79 days after sowing, aerated using a tedder rake and allowed to dry for 2 days. Round bale silage was produced using a roll baler (Rollant 46, CLAAS) according to manufacturer's instructions, including wrapping in white polyethylene film. Bales were stored upright for 43 days until 9 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

Corn. Corn (*Zea mays*) on a farm in Ishigaki Island (Ohama, Ishigaki) (Figure 1) was mowed at 84 days after sowing, aerated using a tedder rake and allowed to dry for 2 days. Roll bale silage was produced using a roll baler (Rollant 46, CLAAS) according to manufacturer's instructions including wrapping in white polyethylene film. Bales were stored upright for 38 days until 9 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

Laboratory silage preparation

Laboratory silages were prepared using a small-scale system of fermentation (Tanaka and Ohmomo 1994) with material from a third grass harvest in 2007 (40 days following the second harvest). Approximately 100 g portions of material of 2 grasses, Transvala and *Panicum maximum* (now *Megathyrsus maximus*) cv. Natsuyutaka, were chopped into about 20 mm length and packed into 3 nylon and polyethylene bags (Hiryu KN type, 180 × 260 cm; AsahiKASEI, Tokyo, Japan). Air was withdrawn and bags were sealed with a vacuum sealer (BH 950; Matsushita, Tokyo, Japan). After 4, 9 and 30 days of storage at 25 °C, a bag was opened and 3 samples per storage day treatment were taken for chemical and microbiological analysis.

Table 1 presents a summary of the different silages used in the study.

Chemical analysis

The dry matter concentration (DM) of fresh material and silages was determined by the removal of moisture using toluene distillation with ethanol correction (Dewar and McDonald 1961). The organic acid concentrations were measured by high-performance liquid chromatography (JASCO Corp., Tokyo, Japan) using Shodex Rspak KC-811 column (8 × 300 mm; Showa Denko K.K., Tokyo, Japan). Concentration of ammonia-nitrogen (ammonia-N) was determined by the Kjeltec system (Kjeltec Auto Sampler System 1035 Analyzer, Tecator, Hoganas, Sweden).

Microbiological analysis

Numbers of microorganisms were measured by the plate count method (Yamazato et al. 1986). Samples (10 g) of silage were blended with 90 mL of sterilized distilled water and 10^{-1} to 10^{-8} serial dilutions were made in sterilized distilled water. Analyses on samples were performed in triplicate. From each dilution, 0.1 mL of suspension was spread on agar plates. LAB were counted on MRS agar plates (DIFCO Laboratories, Detroit, USA) after incubating in an anaerobic box (TE-HER Hard Anaerobox, ANX-1; Hirasawa Ltd, Tokyo, Japan) at 30 °C for 2 days. Aerobic bacteria and *Escherichia* spp. were counted on nutrient agar and blue light agar plates (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), respectively, after incubating at 30 °C for 2 days. Yeast and mold were counted on potato dextrose agar plates (Nissui Pharmaceutical Co. Ltd) with pH 3.5 by adding 10% (w/v) tartaric acid, after incubating at 30 °C for 2 days. Molds were distinguished from yeast by colony appearance and cell morphological observation. For bacilli, the suspension was heated at 75 °C for 15 min before spreading on nutrient agar plates, which were incubated at 30 °C for 2 days, when bacilli were counted. For clostridia, the suspension was heated at 75 °C for 15 min before spreading on clostridia count agar plates (Nissui Pharmaceutical Co. Ltd) and incubated in an anaerobic box at 30 °C for 2 days, when clostridia were counted. Colonies were counted as viable numbers of microorganisms [log colony-forming units (log cfu)/g fresh matter (FM)]. For the next test for LAB, the cultivated LAB, following the counting described above, were purified on another MRS agar plate.

Table 1. Summary of silages produced for analysis in this study.

Silage	Round bale silage									Laboratory silage					
Forage	Transvala						Grass mix[1]	Sorghum	Corn	Transvala			Natsuyutaka		
Mowing place	Nakizin						Tonoshiro	Ohama		Nakizin					
Storage days	37[2]			61[3]			105	43	38	4	9	30	4	9	30
Sampling part[4]	T	M	B	T	M	B		M		All in the pack					

[1]Mixture of Rhodes grass and Para grass (1:1); [2]Silage A in the text; [3]Silage B in the text; [4]T = Top, M = Middle, B = Bottom.

Morphological, physiological and biochemical tests

Morphology (strain shape of rod or cocci) and Gram stain were examined after 24 h of incubation on MRS agar plates. Catalase activity and gas production from glucose (hetero- or homo-fermentative) were determined according to Kozaki et al. (1992). The isomers of lactic acid that bacteria formed from glucose were determined by enzymatic analysis using a UV method (F-kit, D-lactic/L-lactic acid; Boehringer Manheim GmbH, Mannheim, Germany). Carbohydrate assimilation and fermentation of 49 different compounds (plus a Control) were conducted using API 50 CH strips (bioMerieux Japan Ltd, Tokyo, Japan).

16S ribosomal DNA (rDNA) sequencing

Cells grown for 8 h in MRS broth at 30 °C were used for DNA extraction and purification (Saitou and Miura 1963). Genome DNA was extracted from cells after enzymatic and detergent digestion. The 16S rDNA region was amplified by polymerase chain reaction (PCR) performed in a PCR Thermal Cycler (GeneAmp PCR System 9700; Applied Biosystems, Waltham, USA) using the prokaryotic 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) with a Takara Taq PCR Kit (Takara Shuzo Ltd, Shiga, Japan) by the PCR method according to Suzuki et al. (1969). Sequencing was performed twice on both strands by the dideoxy method (Sanger et al. 1977), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in combination with an Applied Biosystems model 310A automated sequencing system.

Sequence alignments and phylogenetic inference

Sequence similarity searches were performed in the GenBank data library using the BLAST program. Nucleotide substitution rates (*K*nuc values) were calculated (Kimura and Ohta 1972) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL W software program (Hitachi Software Engineering Co., Ibaraki, Japan) for assembly and alignment. The topologies of trees were evaluated by bootstrap analysis of the sequence data with CLUSTAL W software based on 100 random resamplings (Thompson et al. 1994).

Results

Fermentation quality and microbiological analysis

The dry matter (DM) concentration average for Silage A was about 35% but values for Silage B differed for different sampling points (Table 2), decreasing from top (42.9%) to bottom (33.6%) of the bale, i.e. moisture drained to the bottom of the bale. The pH values for all samples of Silage A were 4.77, while values for Silage B were 5.08–5.28. Lactic acid concentrations of Silage A and Silage B were 0.27–0.39% and 0.03–0.22% of fresh matter (FM), respectively. Ammonia-N concentrations of Silage A and Silage B were 0.44–0.53% and 0.48–1.06% of FM, respectively, while butyric acid concentrations of Silage A and Silage B were 0.05–0.08% and 0.16–0.19%. Silage A had 6.7–6.9 log cfu LAB/g FM, more than Silage B with 4.8–6.8 log cfu LAB/g. Clostridia were detected in Silage B but not in Silage A. There were nd–4.8 log cfu yeast/g, 3.9–4.5 log cfu aerobic bacteria/g, 2.3–3.9 log cfu bacilli/g, nd–2.5 cfu *Escherichia*/g and no mold in both silages.

Regarding the 3 round bale silages in Ishigaki, grass silage had the highest ammonia-N and the lowest lactic acid concentrations, while corn silage had the lowest ammonia-N and the highest lactic acid concentration (Table 3). LAB concentrations were highest in corn silage and lowest in grass silage. There were nd–5.5 log cfu yeast/g, 3.1–4.6 log cfu aerobic bacteria/g, 2.5–3.6 log cfu bacilli/g and nd–1.5 log cfu *Escherichia*/g but no mold and no clostridia in the 3 silages. Laboratory silages had high ammonia-N and low lactic acid concentrations (Table 4). Lactic acid concentration was 0.09% FM by 9 days of storage but could not be detected at 30 days, while ammonia-N continued to increase during storage. This means that the quality of both laboratory silages deteriorated during storage for 30 days. However, LAB populations increased from about 3 log cfu/g to about 7 log cfu/g in only 4 days and then decreased slightly to about 6 log cfu/g at 30 days of storage. There were nd–3.2 log cfu yeast/g, 4.9–7.5 log cfu aerobic bacteria/g, 4.0–5.8 log cfu bacilli/g, 4.5–6.9 log cfu *Escherichia*/g, 1.3–3.0 log cfu mold/g and nd–1.0 log cfu clostridia/g in both laboratory silages.

Table 2. Fermentation quality and microbiological analysis of Transvala round bale silages in Nakizin.

	Fresh grass	Silage A stored for 37 days			Silage B stored for 61 days		
		Top	Center	Bottom	Top	Center	Bottom
Fermentation quality							
DM (% FM[1])	25.8	35.1	35.0	35.3	42.9	40.9	33.6
pH	5.56	4.77	4.77	4.77	5.21	5.08	5.28
Lactic acid (% FM)	nd[2]	0.39	0.27	0.36	0.21	0.22	0.03
Acetic acid (% FM)	nd	0.15	0.14	0.11	0.22	0.04	0.18
Propionic acid (% FM)	nd	nd	nd	nd	0.05	nd	0.05
Butyric acid (% FM)	nd	0.05	0.08	0.05	0.16	0.19	0.18
Ammonia-N (% FM)	0.04	0.53	0.49	0.44	1.06	0.48	0.67
Microorganism composition [log cfu (colony-forming unit) per gram of FM]							
Lactic acid bacteria	3.00	6.90	6.86	6.76	4.83	6.79	5.38
Clostridia	nd	nd	nd	nd	1.78	1.48	3.20
Escherichia	6.68	nd	2.00	1.00	nd	2.45	nd
Mold	3.90	nd	nd	nd	nd	nd	nd
Yeast	3.78	4.83	4.00	4.59	nd	4.67	3.50
Aerobic bacteria	7.04	4.48	4.30	4.46	3.90	4.15	4.51
Bacilli	4.83	2.90	2.78	2.85	3.43	2.34	3.89

[1]FM: fresh matter (green forage or silage); [2]nd: not detected.

Table 3. Fermentation quality and microbiological analysis of round bale silages made from grass, sorghum and corn in Ishigaki.

	Grass mix[1]	Sorghum	Corn
Fermentation quality			
DM (% FM[2])	46.5	34.9	36.5
pH	5.25	4.68	4.43
Lactic acid (% FM)	0.14	0.42	0.60
Acetic acid (% FM)	0.06	0.09	0.06
Propionic acid (% FM)	nd[3]	nd	nd
Butyric acid (% FM)	nd	nd	nd
Ammonia-N (% FM)	0.44	0.11	0.04
Microorganism composition [log cfu (colony-forming unit) per gram of FM]			
Lactic acid bacteria	4.59	5.23	6.28
Clostridia	nd	nd	nd
Escherichia	1.48	nd	nd
Mold	nd	nd	nd
Yeast	nd	4.38	5.48
Aerobic bacteria	3.08	4.60	4.36
Bacilli	2.48	3.26	3.59

[1]Mixture of Rhodes grass and Para grass (1:1); [2]FM: silage fresh matter; [3]nd: not detected.

Table 4. Fermentation quality and microbiological analysis of fresh grass and laboratory silage.

	Transvala from Nakizin				Natsuyutaka from Nakizin			
	Fresh grass	Laboratory silage (storage days)			Fresh grass	Laboratory silage (storage days)		
		4	9	30		4	9	30
Fermentation quality								
DM (% FM[1])	25.8	25.5	23.2	24.5	24.9	25.1	25.5	23.6
pH	5.56	5.68	5.49	5.67	6.60	5.97	5.95	5.41
Lactic acid (% FM)	nd[2]	0.08	0.09	nd	nd	0.05	nd	nd
Acetic acid (% FM)	nd	0.10	0.17	0.22	nd	0.20	0.33	0.67
Propionic acid (% FM)	nd	nd	0.01	0.03	nd	nd	0.01	0.09
Butyric acid (% FM)	nd	nd	0.07	0.39	nd	nd	0.07	0.37
Ammonia-N (% FM)	0.04	0.25	0.33	0.77	0.20	0.60	0.79	1.07
Microorganism composition [log cfu (colony-forming unit) per gram of FM]								
Lactic acid bacteria	3.00	7.30	7.30	6.20	3.53	7.04	7.30	6.08
Clostridia	nd	1.00	nd	1.00	nd	nd	nd	nd
Escherichia	6.68	6.85	6.08	5.38	5.30	7.23	7.34	4.45
Mold	3.90	2.41	2.70	2.48	3.48	3.04	2.28	1.30
Yeast	3.78	1.70	nd	3.23	3.61	nd	nd	nd
Aerobic bacteria	7.04	7.11	6.26	6.04	6.90	7.38	7.46	4.93
Bacilli	4.83	5.77	4.00	4.85	4.00	4.40	4.81	4.18

[1]FM: fresh matter (fresh grass or silage); [2]nd: not detected.

Physiological properties of isolated LAB

The physiological properties of 37 of the presumptive LAB strains isolated in this study are shown in Tables 5, 6 and 7. Okn1 to Okn8 were isolated from Silage A and Okn9 to Okn14 from Silage B, while Okn15 was isolated from fresh Transvala and Okn23 to Okn26 from fresh Natsuyutaka. Okn16 to Okn22 were isolated during storage of the Transvala laboratory silage and Okn27 to Okn29 during storage of the Natsuyutaka laboratory silage; Okn30 to Okn32 were isolated from the grass mix silage, Okn33 to Okn35 from sorghum silage and Okn36 and Okn37 from corn silage. All isolates were Gram-positive strains and most were catalase-negative, but Okn4, Okn6, Okn23, Okn27, Okn28 and Okn33 were catalase-positive. According to morphological and biochemical characters as well as isolation sources, the 37 strains were divided into 16 groups (A–P). Strains in Groups A, E and F were homo-fermentative rods, which did not produce gas from glucose and formed DL-lactic acid. In contrast, strains in Groups C, G and H were hetero-fermentative cocci, which produced gas from glucose and formed D(-)-lactic acid. Strains in Groups D and K were homo-fermentative cocci, which did not produce gas from glucose and formed L(+)-lactic acid, while the strain in Group B was homo-fermentative rods, which did not produce gas from glucose and formed L(+)-lactic acid. Strains in Groups I and J were hetero-fermentative cocci, which produced gas from glucose and formed D(-)-lactic acid. Strains in Groups L and N were homo-fermentative rods, which did not produce gas from glucose, while strains in Groups M and O were hetero-fermentative rods, which did produce gas from glucose, and the strain in Group P was homo-fermentative cocci, which did not produce gas from glucose. All representative strains sequenced with 16S rDNA in each group showed high-sequence homology values (100% or almost 100%) with the type LAB strain.

Carbohydrate fermentation assays using API 50 CH strips

From the result of API 50 CH strips, all strains produced acid from glucose but groups displayed a distinct carbohydrate fermentation pattern. Groups A, E and L had almost the same fermentation patterns, especially using L-arabinose and melezitose. Group E used α-methyl-D-glucoside, lactose and melibiose (Tables 8, 9 and 10). Groups C and G had a similar pattern, especially using α-methyl-D-glucoside and saccharose. Groups D, K and P had the same pattern, especially using L-arabinose, β-gentiobiose and D-tagatose. Group B used glycerol, mannitol and α-methyl-D-glucoside, Group H used amygdalin and gluconate, Group I used D-xylose and β-gentiobiose, Group J used β-gentiobiose and D-tagatose, Group M used D-xylose, β-methyl-xyloside and α-methyl-D-glucoside, Group N used arbutine and β-gentiobiose and Group O used L-sorbose and D-tagatose.

Table 5. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria isolated from Transvala round bale silages in Nakizin.

Characteristic	Group A				Group B	Group C							Group D	
	Okn1	Okn2	Okn9	Okn10	Okn3	Okn4	Okn5	Okn6	Okn7	Okn11	Okn12	Okn13	Okn8	Okn14
Source	Silage A	Silage A	Silage B	Silage B	Silage A	Silage A	Silage A	Silage A	Silage A	Silage B	Silage B	Silage B	Silage A	Silage B
Shape	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain	+[1]	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase activity	-[2]	-	-	-	-	+	-	+	-	-	-	-	-	-
Fermentation type	Homo	Homo	Homo	Homo	Homo	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo
Optical form of lactate	DL	DL	DL	DL	L (+)	D (-)	D (-)	D (-)	D (-)	D (-)	D (-)	D (-)	L (+)	L (+)
16S rDNA sequence similarity[4]	100	na[3]	100	na	100	99.6	na	na	99.9	99.8	na	na	100	100
Identified species	*Lactobacillus plantarum*				*L. acidipiscis*	*Weissella paramesenteroides*							*Pediococcus pentosaceus*	

[1]+: positive reaction; [2]-: negative reaction; [3]na: not analyzed; [4]Sequence similarity with each type strain (%).

Table 6. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria isolated from fresh grass and laboratory silages.

Characteristic	Group E	Group F			Group G		Group H	Group I					Group J		Group K
	Okn16	Okn23	Okn27	Okn28	Okn24	Okn17	Okn20	Okn15	Okn18	Okn19	Okn25	Okn21	Okn26	Okn29	Okn22
Source of grass	Tran[1]	Nats[2]	Nats	Nats	Nats	Tran	Tran	Tran	Tran	Tran	Nats	Tran	Nats	Nats	Tran
Storage day	4	0	4	9	0	4	9	0	4	4	0	9	0	9	9
Shape	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain	+[3]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase activity	-[4]	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Fermentation type	Homo	Homo	Homo	Homo	Hetero	Hetero	Hetero	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo
Optical form of lactate	DL	DL	DL	DL	D (-)	D (-)	D (-)	L (+)	L (+)	L (+)	L (+)	L (+)	L (+)	L (+)	L (+)
16S rDNA seq. similarity[6]	100	99.8	100	na[5]	99.7	100	99.9	100	na	na	99.9	na	100	na	100
Identified species	*L. plantarum*	*L. paraplantarum*			*W. paramesenteroides*		*W. kimchii*	*Lactococcus lactis* subsp. lactis					*L. garvieae*		*P. pentosaceus*

[1]Tran: Transvala; [2]Nats: Natsuyutaka; [3]+: positive reaction; [4]-: negative reaction; [5]na: not analyzed; [6]Sequence similarity with each type strain (%).

Table 7. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria isolated from the round bale silages in Ishigaki.

Characteristic	Group L		Group M	Group N			Group O	Group P
	Okn30	Okn33	Okn31	Okn34	Okn35	Okn36	Okn37	Okn32
Source	Grass mix[1]	Sorghum	Grass mix	Sorghum	Sorghum	Corn	Corn	Grass mix
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci
Gram stain	+[2]	+	+	+	+	+	+	+
Catalase activity	-[3]	+	-	-	-	-	-	-
Fermentation type	Homo	Homo	Hetero	Homo	Homo	Homo	Hetero	Homo
16S rDNA sequence similarity[4]	99.9	99.9	99.9	100	100	99.9	99.9	100
Identified species	*Lactobacillus plantarum*		*L. brevis*	*L. casei*			*L. fermentum*	*P. pentosaceus*

[1]Mixture of Rhodes grass and Para grass (1:1); [2]+: positive reaction; [3]-: negative reaction; [4]Sequence similarity with each type strain (%).

Table 8. API50 CH fermentation patterns of lactic acid bacteria isolated from Transvala round bale silages in Nakizin.

Carbohydrate	Group A				Group B	Group C							Group D	
	Okn1	Okn2	Okn9	Okn10	Okn3	Okn4	Okn5	Okn6	Okn7	Okn11	Okn12	Okn13	Okn8	Okn14
Glycerol	-[1]	-	-	-	w[3]	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	w	+	+	+	w	+	+	+	+
D-Xylose	-	-	-	-	-	+	+	+	+	+	+	+	+	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	w	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	w	+	w	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	-	-	w	-	-	-	-	-	-
Sorbitol	+	+	+	+	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-mannoside	+	+	+	+	+	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	+	+	+	+	w	+	+	+	-	-
N-Acetyl-glucosamine	+	+	+	+	+	w	w	w	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	-	-	-	-	-	-	-	+	+
Arbutine	+	+	+	+	+	-	-	-	-	-	-	-	+	+
Esculine	+	+	+	+	+	-	-	-	-	-	-	+	+	+
Salicine	+	+	+	+	+	w	-	w	-	-	-	w	+	+
Cellobiose	+	+	+	+	+	+	-	+	-	w	w	+	+	+
Maltose	+	+	+	+	w	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	-	-	-	-	-	-	+	-
Melibiose	+	+	+	+	-	+	+	+	w	+	+	+	+	-
Saccharose	+	+	+	+	-	+	+	+	w	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	w	+	+	+	-	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	+	+	+	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	w	+	+	-	-	-	-	-	-	-	-	+	-
Amidon	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	-	+	w	w	-		-	w	w	w	w	w
D-Turanose	+	+	+	-	-	+	-	+	-	+	w	+	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	w	-	-	w	-	+	-	w	w	-	-	-
2-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive.

Table 9. API50 CH fermentation patterns of lactic acid bacteria isolated from fresh grass material and the laboratory silages.

Carbohydrate	Group E	Group F			Group G		Group H	Group I					Group J		Group K
	Okn16	Okn23	Okn27	Okn28	Okn24	Okn17	Okn20	Okn15	Okn18	Okn19	Okn25	Okn21	Okn26	Okn29	Okn22
Glycerol	-[1]	w[3]	w	w	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	-	-	-	+	+	w	-	+	+	+	-	-	-	+
Ribose	+	+	+	+	w	+	-	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	+	w	+	+	+	+	+	-	-	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	w	+	-	+	+	+	+	+	w	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	-	+	+	-	-	-	-	-	+	+	+	+	+	-
Sorbitol	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-mannoside	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
N-Acetyl-glucosamine	+	+	+	+	w	+	w	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Arbutine	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Esculine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	-	-	w	+	+	+	w	-	-	-
Melibiose	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	+	w	+	-	+	+	+	+	-	-	-
Trehalose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	+	+	+	+	w	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	w	w	+	w	w	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	w	w	-	w	w	w	+	+	w	+	w	+	w
D-Turanose	+	-	-	-	w	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	w	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	w	w	w	-	w	-	w	w	w	w	-	-	-
2-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive.

Table 10. API50 CH fermentation patterns of lactic acid bacteria isolated from the round bale silages in Ishigaki.

Carbohydrate	Group L		Group M	Group N			Group O	Group P
	Okn30	Okn33	Okn31	Okn34	Okn35	Okn36	Okn37	Okn32
Glycerol	-[1]	w[3]	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	+	+	-	-	+	-	+
Ribose	+	+	+	+	+	+	+	+
D-Xylose	-	-	+	-	-	+	-	-
L-Xylose	-	-	-	-	-	-	-	-
Adonitol	-	-	-	+	+	-	-	-
β-Methyl-xyloside	-	-	+	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	w	+	+
D-Mannose	+	+	-	+	+	-	+	+
L-Sorbose	-	-	-	+	+	-	+	-
Rhamnose	w	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	w	w	-	-	-
Mannitol	+	+	-	+	+	-	+	-
Sorbitol	+	+	-	+	+	-	+	-
α-Methyl-D-mannoside	-	+	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	+	w	w	-	w	-
N-Acetyl-glucosamine	+	+	w	+	+	+	+	+
Amygdaline	+	+	-	+	+	-	+	+
Arbutine	+	+	-	+	+	+	+	+
Esculine	+	+	-	+	+	-	+	+
Salicine	+	+	-	+	+	w	+	+
Cellobiose	+	+	-	+	+	+	+	+
Maltose	+	+	-	+	+	w	+	+
Lactose	+	+	-	-	-	-	-	-
Melibiose	+	+	w	-	-	w	-	-
Saccharose	+	+	-	w	w	w	+	-
Trehalose	+	+	-	+	+	+	+	+
Inuline	-	-	-	-	-	-	+	-
Melezitose	+	+	-	+	+	-	+	-
D-Raffinose	+	+	-	-	-	+	-	-
Amidon	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	-	+	+	w	+	w
D-Turanose	-	+	-	+	+	-	+	-
D-Lyxose	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	+	+	-	+	+
D-Fucose	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	+	-
Gluconate	-	w	-	-	-	-	w	w
2-keto-gluconate	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive.

Phylogenetic tree based on 16S rDNA sequence

In an effort to identify Okn strains at the species level, molecular phylogenetic analysis was conducted and phylogenetic trees were produced based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method (Figures 2 and 3). Following phylogenetic analysis, Okn1 and Okn9 (representative of Group A), Okn16 (Group E), Okn23 and Okn27 (Group F), Okn30 and Okn33 (Group L), Okn31 (Group M), Okn3 (Group B), Okn34, Okn35 and Okn36 (Group N) and Okn37 (Group O) were placed in a cluster, making up the genus *Lactobacillus*. Okn4, Okn7 and Okn11 (Group C), Okn17 and Okn24 (Group G) and Okn20 (Group H) made up the genus *Weissella*. Okn15 and Okn25 (Group I) and Okn26 (Group J) made up the genus *Lactococcus*, while Okn8 and Okn14 (Group D), Okn22 (Group K) and Okn32 (Group P) made up the genus *Pediococcus*. Closest related species for different Okn strains were as follows: Okn1 and Okn9 (Group A), Okn16 (Group E) and Okn30 and Okn33 (Group L) – *Lactobacillus plantarum*; Okn23 and Okn27 (Group F) – *L. paraplantarum;* Okn31 (Group M) – *L. brevis*; Okn3 (Group B) – *L. acidipiscis*; Okn34, Okn35 and Okn36 (Group N) – *L. casei*; Okn37 (Group O) – *L. fermentum*; Okn4, Okn7 and Okn11 (Group C) and Okn17 and Okn24 (Group G) – *Weissella paramensenteroides*; Okn20 (Group H) – *W. kimchii*; Okn15 and Okn25 (Group I) – *Lactococcus lactis* subsp. *lactis*; Okn26 (Group J) – *Lactococcus garvieae*; Okn8 and Okn14 (Group D), Okn22 (Group K) and Okn32 (Group P) – *Pediococcus pentosaceus*. According to a BLAST search, all representative strains in each group showed high-sequence homology values (100% or almost 100%) with the most closely related species in the phylogenetic tree. Therefore, strains in Groups A, E and L were identified as *Lactobacillus plantarum*, strains in Group F as *L. paraplantarum*, the strain in Group M as *L. brevis*, the strain in Group B as *L. acidipiscis*, strains in Group N as *L. casei*, the strain in Group O as *L. fermentum*, strains in Groups C and G as *Weissella paramesenteroides*, the strain in Group H as *W. kimchii*, strains in Group I as *Lactococcus lactis* subsp. *lactis*, strains in Group J as *Lactococcus garvieae* and strains in Groups D, K and P as *Pediococcus pentosaceus*.

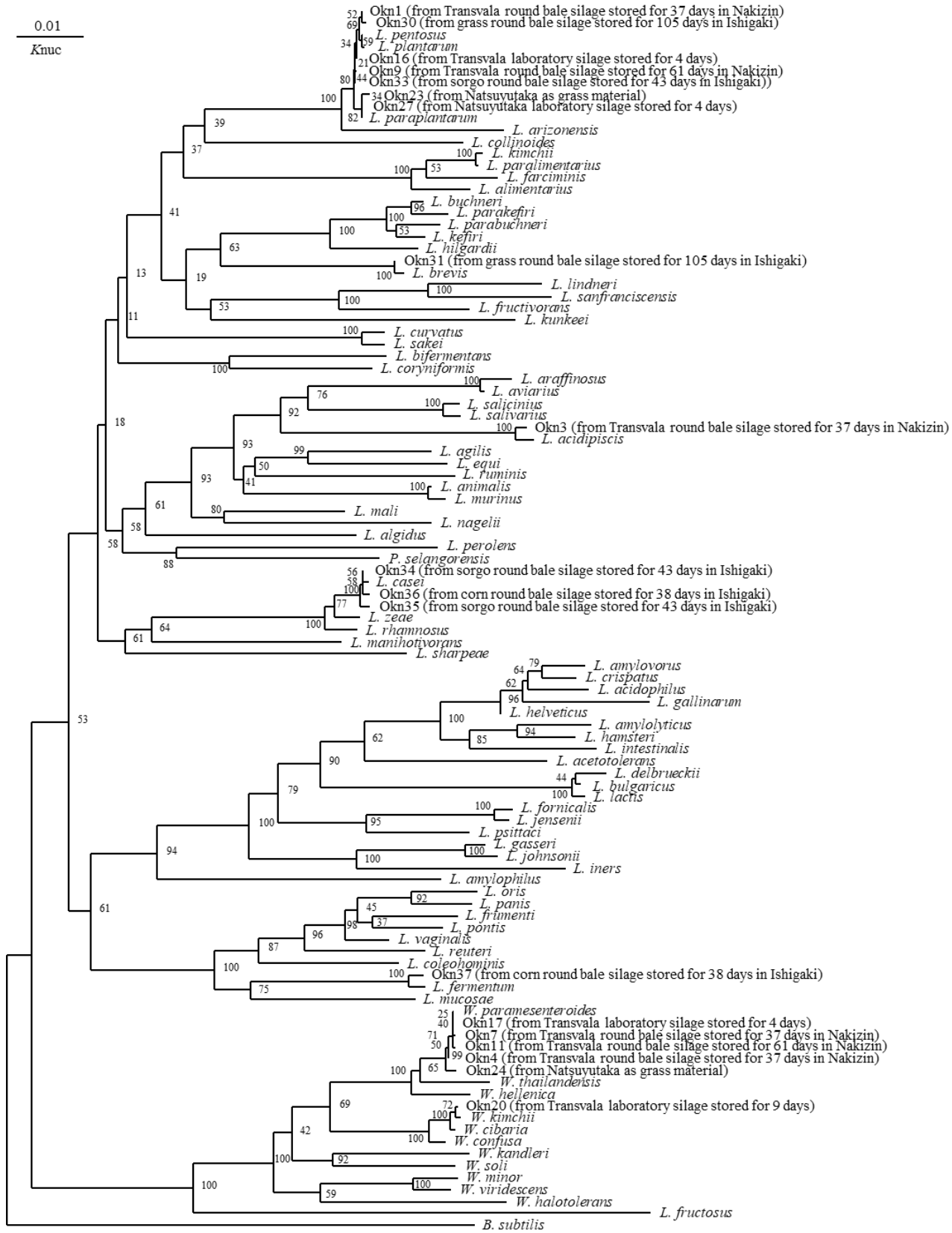

Figure 2. Phylogenetic tree showing the relative positions of isolates as inferred by the neighbor-joining method of complete 16s rDNA sequence. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *L.*, *Lactobacillus*; *W.*, *Weissella*; *K*nuc, nucleotide substitution rate.

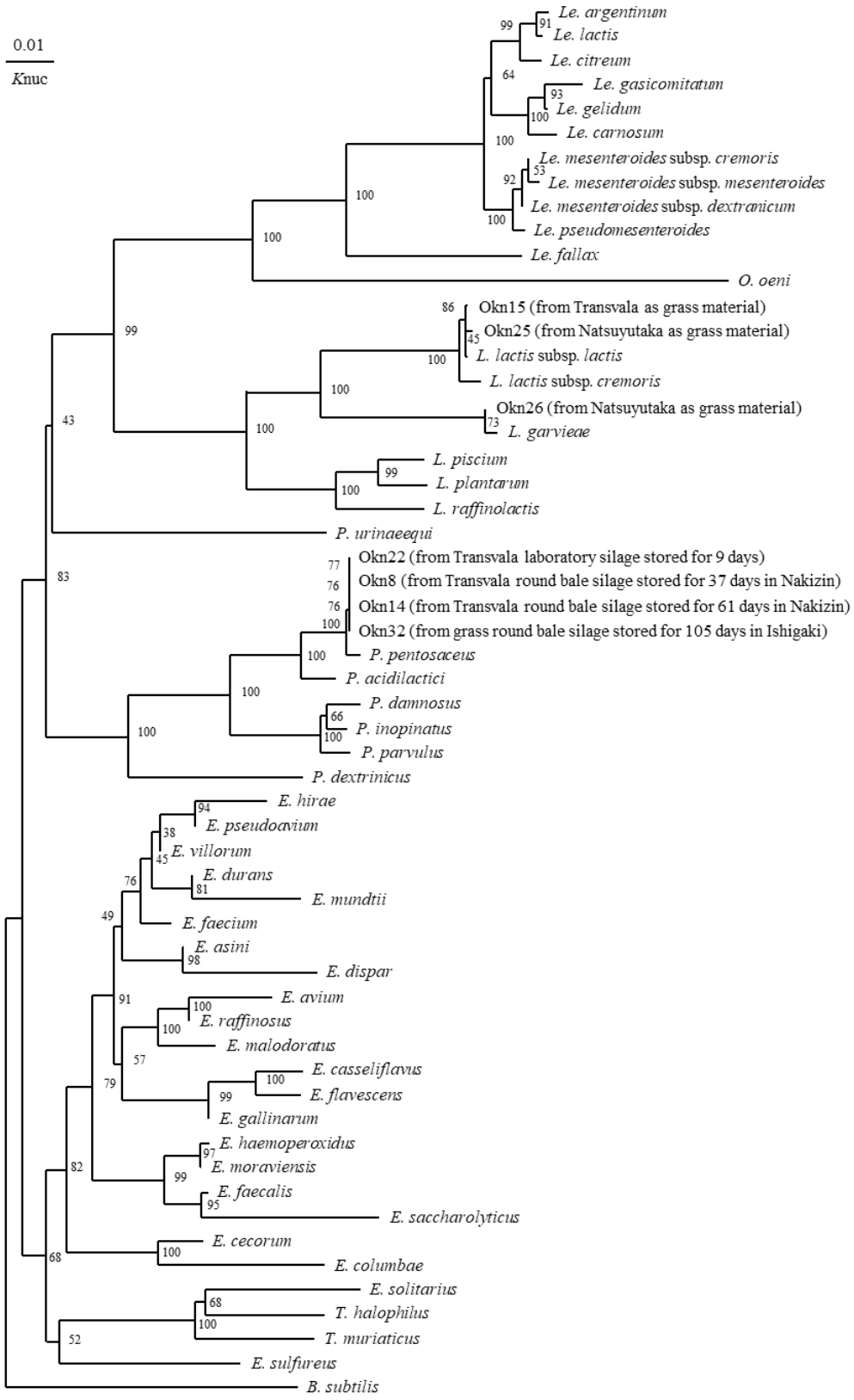

Figure 3. Phylogenetic tree showing the relative positions of isolates as inferred by the neighbor-joining method of complete 16s rDNA sequence. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *Le.*, *Leuconostoc*; *L.*, *Lactococcus*; *P.*, *Pediococcus*; and *E.*, *Enterococcus*. *K*nuc, nucleotide substitution rates.

Discussion

This study has revealed that silages made in Okinawa contain a very wide range of species of bacteria with differing fermentation characteristics. Silage A made of Transvala smelled really good even though the data for fermentation quality were not as good as for silages made of cool temperate grasses grown in the colder regions in Japan. In fact, lactic acid concentration in Italian ryegrass silage was twice that in Silage A (0.68 vs. 0.34% FM) and acetic acid concentration was two-and-a-half times that in Silage A (0.33 vs. 0.13% FM) (Cai et al. 1999a). Nevertheless, Silage A was considered to be of better quality than conventional silages made in subtropical regions. Tamaki et al. (2007) reported that Transvala was highly nutritious and could be a replacement for oats. The pH value of Silage A was lower than that of Silage B and the laboratory silages. Moreover, lactic acid in Silage B was lower than that in Silage A and the number of LAB in Silage B was scattered with 4.8–6.8 log cfu/g FM. The laboratory silages were low in quality and had an offensive smell like that of Silage B. Possible reasons were that evacuation of bags for these silages at ensiling was incomplete and the grass was not sufficiently dried. It is known that effective wilting of forage before ensiling may be the most important aspect in making good silage and ideal dry matter concentration of forage at ensiling is a minimum of 30% (Romero et al. 2015). On the contrary, in the case of over 40% DM, it is difficult to maintain anaerobic conditions and fermentation can be lower in quality (Romero et al. 2015). This rule could apply to Silage B with 33.6–42.9% DM resulting in unacceptable quality. When moisture concentration in forage is too high at ensiling, many kinds of microorganisms can proliferate before LAB grow sufficiently to reduce pH. In the laboratory silages, lactic acid concentration was low even though high numbers of LAB (about 7 log cfu/g) were detected. Furthermore, LAB detected in the laboratory silages were mainly cocci. LAB cocci are very important in the initial stages of fermentation, because they maintain an acidic environment, which is then colonized by predominantly *Lactobacillus* (Tjandraatmadja et al. 1991; Ohmomo et al. 2002). While *Lactococcus* were present, they were unable to create an acidic environment and *Lactobacillus* was not the predominant flora during storage. Cai et al. (1999a) reported that, when *Lactobacillus* reaches a level of at least 5 log cfu/g FM, silage can be well preserved. *Lactobacillus* was obviously the predominant flora in Silage A, sorghum silage and corn silage, all of which were of acceptable quality.

Many species in the genus *Lactobacillus*, e.g. *L. plantarum* (Cai 2001; Ohmomo et al. 2002), have been found in silages. In fact, *Lactobacillus* plays a more important role in fermentation processes and promotes effective lactic acid fermentation for longer than lactic acid-producing cocci (Cai et al. 1998; 1999b). Additionally, *L. plantarum* was the predominant species and was still active 100 days after ensiling tropical grass in work reported by Tjandraatmadja et al. (1991). In fact, *L. plantarum* 'Chikusou1gou' is produced on a commercial basis in Japan. Accordingly, it is necessary that *Lactobacillus* is present in significant numbers as early as possible during storage for producing high quality silages made of subtropical grasses. Consequently, research into the fermentation effects of *L. plantarum* isolated in Silage A and sorghum silage seems warranted. In addition, *L. brevis* and *L. fermentum*, hetero-fermentative bacteria, have been reported in large numbers at the end of the ensiling process (Tjandraatmadja et al. 1991). Actually, *L. brevis* was present in grass silage and *L. fermentum* in corn silage in Ishigaki. *Pediococcus pentosaceus* was isolated from good quality silages in this study. EFSA (2014) reported that a mixture of *P. pentosaceus* and *L. plantarum* showed potential to improve preservation of nutrients in silage. In a study by Soundharrajan et al. (2019), addition of *P. pentosaceus* and *L. brevis* at ensiling produced a marked improvement in silage quality, which was attributed to their high antibacterial and probiotic properties. A combination of *P. pentosaceus* and *Lactobacillus* isolated in this study may also have the ability to improve Okinawan silage. Therefore, to increase the possibility of stimulating proliferation of these beneficial LAB as early as possible, wilting grass sufficiently to below 70% moisture seems critical. In addition, compacting fresh forage effectively and wrapping silage properly to minimize the amount of air in the silage should enhance the chances that the environment in the silage would be suitable for rapid proliferation of LAB. We plan to investigate the effects of inoculating fresh forage with these strains at ensiling in an endeavor to improve the quality of silage produced.

There have been very few reports that *L. acidipiscis* exists at ensiling, e.g. Hanagasaki and Cai (2009); Shokryazdan et al. (2018). It was originally isolated from fermented fish (Tanasupawat et al. 2000) and Greek Kopanisti cheese (Kazou et al. 2017) and when recently isolated from mulberry silage was shown to have antiproliferative and antioxidant properties (Shokryazdan et al. 2018). In addition, *L. acidipiscis* was shown to effectively absorb and expel dietary lead (Pb) from gastrointestinal tracts of chickens (Jahromi et al. 2017). Based on these findings and its isolation from Silage A, there seems merit in assessing the benefits of inoculating forage with this product at ensiling with the aim of improving silage quality in subtropical areas like Okinawa and providing nutritional benefits when fed to livestock.

This study has revealed new data on catalase activity of LAB, which were generally believed to be catalase-

negative. We have shown that the 2 strains identified as *W. paramenseteroides*, the strain identified as *L. plantarum* and all 4 strains identified as *L. paraplantarum* have catalase activity. This indicates a possibility that LAB present in Okinawan silages have unique characteristics. Further study is needed to elucidate each LAB strain's characteristics and identify the LAB strains most suitable for use in making silage in Okinawa.

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