

# Analysis of Genetic Variation, Agronomic Traits, and Nitrate-Nitrogen in Landraces and Commercial Sudangrass (*Sorghum sudanense* (Piper) Stapf) under Varying Levels of Cattle Compost Application

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## Abstract

Sudangrass possesses advantageous traits, including slender stalks and vigorous summer growth. However, its high nutrient absorption efficiency predisposes it to nitrate accumulation in the stems and poses a risk of nitrate poisoning in cattle. This study evaluated 42 lines of 33 sudangrass varieties, comprising germplasm from the National Institute of Agricultural Sciences (NIAS) gene bank and commercial varieties. Sudangrass lines exhibited diverse phenotypic characteristics in the field trials. Genotyping-by-sequencing (GBS) was used to analyze genomic DNA, categorizing sudangrass into three groups based on 3975 single-nucleotide polymorphisms (SNPs). Cluster I, with 22 lines, such as Haysudan, displayed shorter and thicker culms resembling sorghum with fewer tillers. Cluster II, comprising eight lines, including Balesudan, exhibited enhanced tillering and biomass production. Cluster III, represented by 12 lines, such as Piper, showed moderate traits but greater susceptibility to northern leaf blight. Cultivation of sudangrass under varying cattle compost application rates revealed differences in nitrate-nitrogen concentrations, confirming a trend of increased accumulation with higher fertilization levels. Nitrate-nitrogen levels were higher in the first harvest but declined in the second. To meet the regulatory limit of 0.2% total nitrate-nitrogen (dry weight), compost applications below  $5 \text{ t} \cdot 10 \text{ a}^{-1}$  are recommended. Among the tested lines, some exhibited nitrate-nitrogen concentrations near the regulatory threshold even under a high compost application rate of  $15 \text{ t} \cdot 10 \text{ a}^{-1}$ , while maintaining high dry matter yields. Lines such as GB13, which combine high biomass production with low nitrate accumulation, showed great potential as valuable breeding materials.

**Discipline:** Animal Science

**Additional key words:** biomass production, genotyping-by-sequencing, northern leaf blight, single-nucleotide polymorphisms

## Introduction

Sudangrass, a member of the sorghum subgroup, features tall, slender stalks with multiple tillers. A  $C_4$  grass species, it thrives under the intense heat of summer and performs exceptionally well in temperate regions, producing abundant leaves compared with forage sorghum. Originating from Southern Egypt and the Sudan, sudangrass was first introduced to the United

States in the early 1900s and later spread across Texas (Kasuga 2010). The  $F_1$  sudangrass hybrid is extensively used for producing silage and as a green fertilizer. Introduced to Japan from the USA in the 1960s, sudangrass is now primarily used for producing roll bale silage, which is valued for its thin stalks and vigorous regrowth. In Japan in 2024, sudangrass produced higher dry matter yields ( $6\text{--}8 \text{ t} \cdot 10 \text{ a}^{-1}$ ) than other pasture grasses ( $3.4 \text{ t} \cdot 10 \text{ a}^{-1}$ ) ([https://www.e-stat.go.jp/stat-](https://www.e-stat.go.jp/stat)

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search/file-download?statInfId=000040379817&fileKind=2). Furthermore, its rapid growth makes it a viable option for use as a green fertilizer and cleaning crop to remove excess soil nutrients, especially in greenhouse environments.

Forage crop production in Japan, including sudangrass, is closely linked to animal husbandry, with organic fertilizers derived from livestock waste commonly used for cultivation. Sudangrass adapts well to various soil types, from heavy clay to sandy soils, and is tolerant of low-fertility conditions. However, in Japan, the overuse of livestock waste in confined areas has often led to excessive nitrate accumulation in crops (Harada 1992, Ito et al. 1982). Studies on nitrogen uptake and nitrate concentrations in sorghum suggest that sudangrass tends to accumulate high nitrate levels (Harada et al. 2000). Soil nitrogen levels primarily influence this accumulation and depend on plant growth and genetic background (Harada et al. 2000, Sunaga et al. 2008). A positive correlation has been observed between nitrate-nitrogen and total nitrogen concentrations. However, nitrogen uptake in sudangrass is more closely associated with dry matter yield than with overall nitrogen concentration (Sunaga et al. 2005).

Although relatively nontoxic to animals, excessive nitrate accumulation can lead to nitrate poisoning in cattle (Thompson 2021). During digestion, nitrates in forage are converted to nitrite, a highly toxic compound. Nitrite transforms hemoglobin in cattle blood into methemoglobin, which cannot bind oxygen, resulting in oxygen deprivation and causing considerable harm (Block 2020).

In Japan, nitrate-nitrogen concentrations must be carefully monitored during silage production. According to the Japan Grassland Agriculture and Forage Seed Association (2001), nitrate-nitrogen levels in forage should remain below 0.2%, with an ideal concentration under 0.1% of dry weight. Effective nutrient management and cultivation techniques, such as adjusting fertilizer based on soil nutrient diagnostics, can help regulate nitrate-nitrogen levels. Younger plants tend to have higher nitrate concentrations that decrease as they mature (Harada et al. 1998). However, mature plants may still accumulate excessive nitrate under favorable environmental or soil conditions. Nitrate is predominantly concentrated in the lower third of the stem (Block 2020), indicating that using upper plant parts is effective in reducing nitrate-nitrogen levels in forage. Cultivars with high dry matter yield, efficient nitrogen uptake, and low nitrate-nitrogen concentrations are particularly effective for reducing nitrate-nitrogen levels (Sunaga et al. 2005).

Sudangrass, a member of the sorghum subgroup,

shares a genome size of 750 Mbp with sorghum. The sorghum genome has been fully sequenced, enabling advanced genetic analyses (Paterson et al. 2009). Single-nucleotide polymorphisms (SNPs) among sudangrass varieties can be identified using next-generation sequencing techniques. Genotyping-by-Sequencing (GBS), a high-throughput approach, efficiently provides genome-wide SNP data. Large-scale SNP genotyping aids in classifying varieties based on genetic differences and mapping quantitative trait loci.

This study identified 3975 SNPs through GBS analysis, which were used to group sudangrass lines into three distinct clusters that exhibit different agronomic traits and nitrate-nitrogen accumulation characteristics, depending on the applied levels of cattle compost fertilizer, as observed during field tests in 2017. Although the recommended compost application rates range from 3 to 5 t · 10 a<sup>-1</sup> according to standard fertilization guidelines (Kiyosawa 2023), higher application levels of 10 and 15 t · 10 a<sup>-1</sup> were also tested to assess the varietal responses under excessive fertilization, which is associated with high nitrate-nitrogen levels. This study aims to analyze the genetic variation, agronomic traits, and nitrate-nitrogen content of landraces and commercial sudangrass under varying levels of cattle compost application using the GBS technique.

## Materials and methods

### 1. Plant materials and field conditions

In total, 17 sudangrass varieties were obtained from the NIAS gene bank, whereas 25 lines of 16 commercial varieties were sourced from seed companies for analysis (Table 1). Among the commercial varieties, slight differences in growth characteristics were observed among accessions with the same product name, suggesting potential underlying genetic variation. These lines were categorized based on seed coat color, specifically white or black, and analyzed separately. Agronomic characteristics were evaluated in 2017 at the Nagano Animal Industry Experiment Station, Shiojiri, Nagano (36° 7' 14" N, 137° 58' 53" E, altitude 760 m). The field soil was classified as Anosol, a typical volcanic soil type found in the region. Sudangrass seeds were sown on 28 May 2017, with each line occupying a 1.5 m<sup>2</sup> plot and spaced 0.75 m apart. Within each line, 25 plants were grown at 8 cm intervals, achieving a density of 1667 · 100 m<sup>-2</sup>. Cattle compost was applied as the basal fertilizer at the rates of 0, 5, 10, and 15 t · 10 a<sup>-1</sup>.

The early growth phase was assessed on 5 July 2017, with the first and second harvests conducted on 7 August and 10 October 2017, respectively. The cumulative daily

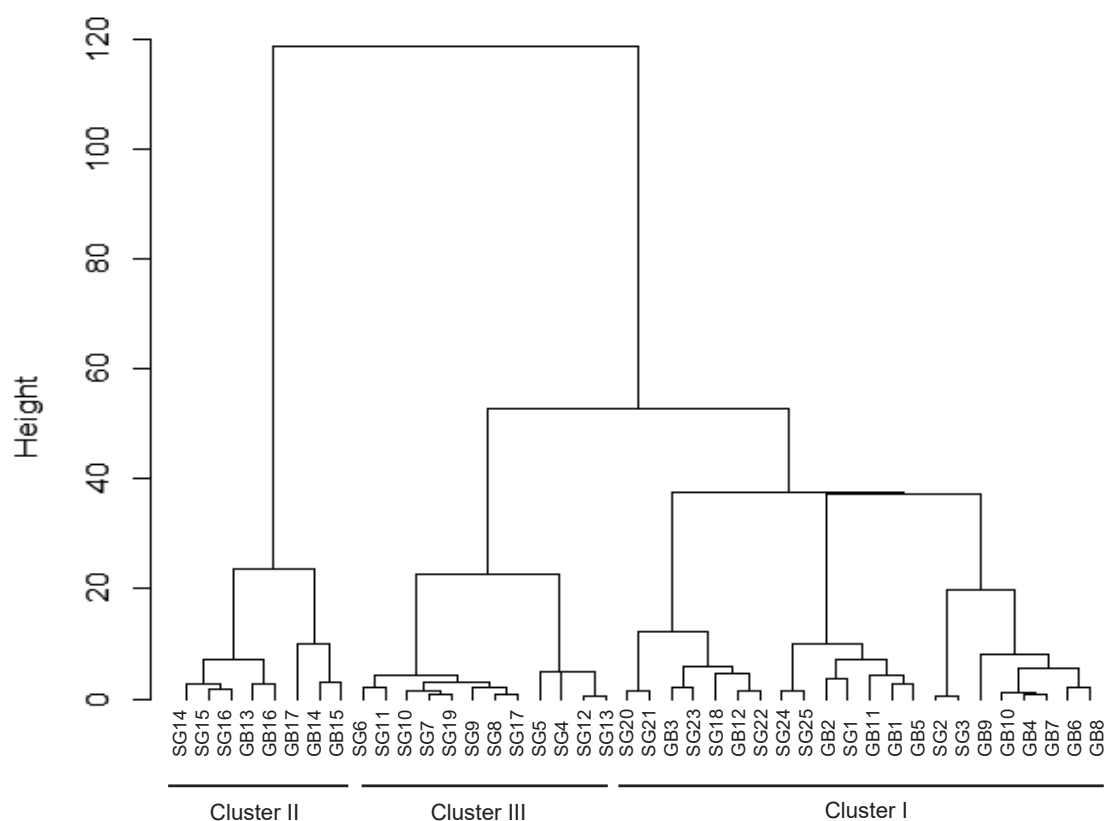
**Table 1. Sudangrass and sorghum used in this study and the classification by GBS analysis**

Scientific name	Name	JP No.	Origin	Line ID	Cluster
<i>Sorghum bicolor</i> ssp. bicolor	SWEET SUDAN 60	342	unknown <sup>†</sup>	GB1	I
<i>Sorghum bicolor</i> ssp. bicolor	AS 4547 JARDIRA	343	Nigeria	GB2	I
<i>Sorghum bicolor</i> ssp. bicolor	P.81607 O.P.GREENLEAF	582	U.S.A.	GB3	I
<i>Sorghum bicolor</i> ssp. bicolor	P.81481 O.P.KS 1044	584	U.S.A.	GB4	I
<i>Sorghum bicolor</i> ssp. bicolor	SORGHUM SUDANENSE PI 208578	587	U.S.A.	GB5	I
<i>Sorghum bicolor</i> ssp. bicolor	P./SUDAN	604	unknown <sup>†</sup>	GB6	I
<i>Sorghum bicolor</i> ssp. bicolor	S.S.6 SUDAN	630	unknown <sup>†</sup>	GB7	I
<i>Sorghum bicolor</i> ssp. bicolor	SUDANGRASS(LOCAL)	40848	unknown <sup>†</sup>	GB8	I
<i>Sorghum bicolor</i> ssp. bicolor	GREENLEAF	40872	U.S.A.	GB9	I
<i>Sorghum bicolor</i> ssp. bicolor	SWEET SUDAN LOT 1322	40873	U.S.A.	GB10	I
<i>Sorghum bicolor</i> ssp. bicolor	SWEET SUDAN 59-3	43722	India	GB11	I
<i>Sorghum sudanense</i>	TIFT SUDAN	88721	unknown <sup>†</sup>	GB12	I
<i>Sorghum</i> spp.	PI 269401	43191	Afghanistan	GB13	II
<i>Sorghum bicolor</i> ssp. bicolor	PER SW SUDAN SA6459-1-17-1-1-1-2-1-8-2-1	44074	U.S.A.	GB14	II
<i>Sorghum bicolor</i> ssp. bicolor	PER SW SUDAN SA6459-1-19-1-1-4-1-1-1	44075	U.S.A.	GB15	II
<i>Sorghum bicolor</i> ssp. bicolor	PER SW SUDAN SA 6459-55-5	44076	U.S.A.	GB16	II
<i>Sorghum bicolor</i> ssp. bicolor	PERENNIAL SWEET SUDAN COMMERCIAL	44077	U.S.A.	GB17	II
Scientific name	Commercial Name		Seed coat color	Line ID	Cluster
<i>Sorghum sudanense</i> (Piper) Stapf	H137		brown	SG2	I
<i>Sorghum sudanense</i> (Piper) Stapf	H137		black	SG3	I
<i>Sorghum sudanense</i> (Piper) Stapf	Haysudan		white	SG24	I
<i>Sorghum sudanense</i> (Piper) Stapf	Haysudan		black	SG25	I
<i>Sorghum sudanense</i> (Piper) Stapf	Itsudemo-sudan			SG18	I
<i>Sorghum sudanense</i> (Piper) Stapf	JN501			SG1	I
<i>Sorghum sudanense</i> (Piper) Stapf	Makimaki-sudan			SG20	I
<i>Sorghum sudanense</i> (Piper) Stapf	Richsudan			SG23	I
<i>Sorghum sudanense</i> (Piper) Stapf	Rollking			SG21	I
<i>Sorghum sudanense</i> (Piper) Stapf	Rollking II			SG22	I
<i>Sorghum sudanense</i> (Piper) Stapf	Balesudan		white	SG14	II
<i>Sorghum sudanense</i> (Piper) Stapf	Balesudan		black	SG15	II
<i>Sorghum sudanense</i> (Piper) Stapf	Balesudan		white/black	SG16	II
<i>Sorghum sudanense</i> (Piper) Stapf	Drysudan			SG19	III
<i>Sorghum sudanense</i> (Piper) Stapf	Hybridsudan			SG17	III
<i>Sorghum sudanense</i> (Piper) Stapf	Oishii-sudan		white	SG4	III
<i>Sorghum sudanense</i> (Piper) Stapf	Oishii-sudan		black	SG5	III
<i>Sorghum sudanense</i> (Piper) Stapf	Piper		white	SG12	III
<i>Sorghum sudanense</i> (Piper) Stapf	Piper		black	SG13	III
<i>Sorghum sudanense</i> (Piper) Stapf	Sudangrass dry grass		white	SG10	III
<i>Sorghum sudanense</i> (Piper) Stapf	Sudangrass dry grass		black	SG11	III
<i>Sorghum sudanense</i> (Piper) Stapf	Sugarslim		white	SG6	III
<i>Sorghum sudanense</i> (Piper) Stapf	Sugarslim		black	SG7	III
<i>Sorghum sudanense</i> (Piper) Stapf	Summerbaler thin stem		white	SG8	III
<i>Sorghum sudanense</i> (Piper) Stapf	Summerbaler thin stem		black	SG9	III

<sup>†</sup>, Countries other than Japan

mean temperature at the Nagano Animal Industry Experiment Station was 1,443.8°C during the first harvest period and 1,259.5°C during the second harvest period.

Agronomic traits, including plant height and dry weight, were recorded at harvest following sorghum guidelines provided by the Ministry of Agriculture, Forestry and



**Fig. 1. Classification of sudangrass lines based on genotyping-by-sequencing (GBS) analysis**

Hierarchical clustering of sudangrass lines was based on 3975 SNPs data. The dendrogram was constructed using Ward's method with Euclidean distance, classifying lines into three major clusters (Cluster I, II, and III). Line IDs (see Table 1) are shown at the tips of the branches, and the height scale represents the dissimilarity between clusters.

Fisheries, Japan (<http://www.hinshu2.maff.go.jp/info/sinsakijun/kijun/1610.pdf>). Damage caused by northern leaf blight was assessed using a 1-9 scale. Nitrate-nitrogen was extracted from dried samples with deionized water and quantified using ion chromatography (ICS-1500, Dionex, Sunnyvale, CA, USA), and statistical analysis was performed using STATISTICA software and Tukey's multiple-test program (<http://www.gen-info.osaka-u.ac.jp/MEPHAS/tukey.html>), provided by Osaka University.

## 2. Genotyping-By-Sequencing (GBS) analysis

GBS data for the sudangrass lines included an average of 700 Mb of 100-bp short reads per sample. GBS data analysis followed the same methods as those used for NGS data in a prior study (Kiyosawa et al. 2020). High-quality homozygous SNPs with at least two reads ( $DP \geq 2$ ;  $FQ \geq 25$ ) were retained, whereas heterozygous genotypes, non-biallelic variants, and SNPs with minor allele frequency below 0.1 were excluded. The remaining SNPs were analyzed for clustering.

## Results

### 1. Analysis of the sudangrass variations

Genetic variations among sudangrass lines were analyzed, yielding 3975 SNPs extracted from the genomic DNA of the NIAS gene bank and commercial lines using GBS. The analysis revealed that SNPs were predominantly located at the chromosome ends of the chromosomes, and a few were found in the centromeric regions. Hierarchical clustering analysis of sudangrass lines was conducted using 3975 SNP markers. The resulting dendrogram, constructed using Ward's method with Euclidean distance, delineated three distinct genetic clusters (Clusters I, II, and III), indicating clear genetic differentiation among the lines (Fig. 1). The classification of individual lines within each cluster is summarized in Table 1. Cluster I, the largest, included 22 lines from the NIAS gene bank and the commercial line Haysudan. Cluster II comprised eight lines, including the Balesudan commercial line, whereas Cluster III comprised 12 lines, including the commercial line Piper. Commercial lines

**Table 2. The average phenotype of sudangrass grown in Nagano field under different compost application levels**

Compost added (10 a <sup>-1</sup> )	0 t	5 t	10 t	15 t
	n= 41	n= 42	n= 42	n= 42
Early Growth (cm, July 5)	18.4 ± 0.91 <sup>‡</sup> c	55.3 ± 2.6 <sup>b</sup>	73.9 ± 2.8 <sup>a</sup>	78.1 ± 2.7 <sup>a</sup>
Regrowth (cm, 21 days after cutting)	96.5 ± 4.1 <sup>b</sup>	125.5 ± 3.1 <sup>a</sup>	122.8 ± 2.9 <sup>b</sup>	118.3 ± 2.9 <sup>b</sup>
Northern Leaf Blight damage (1-9 severity)	3.22 ± 0.18	3.19 ± 0.21	3.26 ± 0.23	2.57 ± 0.18
Culm Width 1 (mm) <sup>†</sup>	7.45 ± 0.22 <sup>b</sup>	9.65 ± 0.35 <sup>a</sup>	9.14 ± 0.30 <sup>a</sup>	8.89 ± 0.30 <sup>a</sup>
Culm Width 2 (mm)	6.52 ± 0.15 <sup>c</sup>	7.65 ± 0.14 <sup>a</sup>	7.17 ± 0.19 <sup>b</sup>	6.81 ± 0.18 <sup>c,b</sup>
Plant Height 1 (cm)	143.5 ± 6.3 <sup>b</sup>	167.3 ± 5.9 <sup>a</sup>	186.5 ± 4.9 <sup>a</sup>	183.9 ± 5.2 <sup>a</sup>
Plant Height 2 (cm)	128.7 ± 4.3 <sup>b</sup>	207.1 ± 4.8 <sup>a</sup>	206.6 ± 4.6 <sup>a</sup>	200.0 ± 4.8 <sup>a</sup>
Tillering Number 1	1.96 ± 0.14 <sup>b</sup>	2.90 ± 0.22 <sup>b</sup>	4.34 ± 0.29 <sup>a</sup>	4.59 ± 0.36 <sup>a</sup>
Tillering Number 2	8.94 ± 1.78	6.48 ± 0.60	7.98 ± 0.48	8.92 ± 0.74
Dry Weight 1 (kg a <sup>-1</sup> )	29.5 ± 3.9 <sup>§</sup> b	42.8 ± 4.0 <sup>b</sup>	61.2 ± 4.1 <sup>a</sup>	66.5 ± 5.1 <sup>a</sup>
Dry Weight 2 (kg a <sup>-1</sup> )	49.7 ± 4.4 <sup>b</sup>	86.5 ± 6.6 <sup>a</sup>	98.4 ± 7.1 <sup>a</sup>	96.8 ± 7.9 <sup>a</sup>
Total Dry Weight (kg a <sup>-1</sup> )	79.2 ± 7.5 <sup>b</sup>	129.3 ± 9.5 <sup>a</sup>	159.6 ± 10.3 <sup>a</sup>	163.4 ± 12.1 <sup>a</sup>
Nitrate-nitrogen 1 (% dry weight)	0.13 ± 0.01 <sup>§</sup> c	0.43 ± 0.03 <sup>b</sup>	0.52 ± 0.03 <sup>a</sup>	0.56 ± 0.03 <sup>a</sup>
Nitrate-nitrogen 2 (% dry weight)	0.04 ± 0.01 <sup>c</sup>	0.13 ± 0.01 <sup>b</sup>	0.35 ± 0.02 <sup>a</sup>	0.39 ± 0.02 <sup>¶</sup> a
Average Nitrate-nitrogen (% dry weight)	0.07 ± 0.008 <sup>§</sup> c	0.23 ± 0.02 <sup>b</sup>	0.41 ± 0.02 <sup>a</sup>	0.46 ± 0.02 <sup>¶</sup> a
Absorbed Nitrate-nitrogen 1 (g a <sup>-1</sup> )	46.11 ± 9.4 <sup>§</sup> c	177.86 ± 16.50 <sup>b</sup>	312.32 ± 23.99 <sup>a</sup>	374.51 ± 32.15 <sup>a</sup>
Absorbed Nitrate-nitrogen 2 (g a <sup>-1</sup> )	19.60 ± 3.41 <sup>b</sup>	109.31 ± 12.47 <sup>b</sup>	324.25 ± 27.43 <sup>a</sup>	369.54 ± 37.76 <sup>¶</sup> a
Total Absorbed Nitrate-nitrogen 1 (g a <sup>-1</sup> )	65.71 ± 10.73 <sup>§</sup> c	287.17 ± 26.53 <sup>b</sup>	636.57 ± 47.33 <sup>a</sup>	746.37 ± 62.76 <sup>¶</sup> a

Values are presented as means ± standard errors. Different superscript letters (a, b, c) indicate statistically significant differences (Tukey HSD test;  $P < 0.05$ ).

†, The number after the abbreviation indicated the 1st harvest or the 2nd harvest.

‡, n= 42; §, n= 38; ¶, n= 41

were classified into the same cluster regardless of seed coat color. In Cluster III, some lines that belong to the same variety but have different seed coat colors, such as SG6 and SG7, exhibited greater genetic similarity to lines from other varieties (Fig. 1). Based on SNP polymorphism data, these results suggest that genetic variation within a variety can be greater than that between varieties.

## 2. Sudangrass phenotype at different compost levels

The growth and agronomic traits of sudangrass under varying levels of cattle compost are shown in Table 2. Significant differences in growth were observed between 0 t and the compost levels of 5-15 t·10 a<sup>-1</sup>, highlighting the need to determine the optimal compost application rate for improved growth. However, the tillering number did not vary significantly during the second harvest. Dry weight increased with the compost application rates of 5-15 t·10 a<sup>-1</sup>. Although no major differences were observed during the second harvest, the dry weight at 5 t·10 a<sup>-1</sup> was lower than at 10 and 15 t·10 a<sup>-1</sup> during the first harvest. Significant differences in early growth were noted between 5 and 10 t·10 a<sup>-1</sup>.

Cluster I consistently exhibited lower dry weights

than Clusters II and III across all compost levels, with reductions of 60%-82%, primarily owing to shorter plant heights and lower tillering numbers (Table 3). Cluster II achieved the highest dry weights at all compost levels, whereas Cluster III exhibited intermediate values, with dry weights between those of Clusters I and II. Cluster III exhibited greater susceptibility to northern leaf blight than other clusters at all compost application levels.

## 3. Nitrate-nitrogen concentrations

Nitrate-nitrogen concentrations were higher in the first harvest and lower in the second, regardless of the amount of compost applied (Table 2). At 0 t·10 a<sup>-1</sup>, the nitrate-nitrogen concentration was 0.13%, which was below the recommended safety threshold of 0.2%. However, when compost was applied at 5 t·10 a<sup>-1</sup> or more, the nitrate-nitrogen concentration in the first harvest exceeded the guideline level. In contrast, the nitrate-nitrogen concentration in the second harvest remained below 0.2% at 5 t·10 a<sup>-1</sup> (0.13%), but exceeded the threshold at both 10 and 15 t·10 a<sup>-1</sup>. The total absorbed nitrate-nitrogen increased with higher levels of compost application; however, similar amounts were observed at 10 t and 15 t·10 a<sup>-1</sup> in both the first and

**Table 3. The average phenotype of each sudangrass cluster grown in Nagano field under different compost application levels**

Compost added (10 a <sup>-1</sup> )	5 t			10 t			15 t		
	I	II	III	I	II	III	I	II	III
Cluster									
Number	n= 22	n= 8	n= 12	n= 22	n= 8	n= 12	n= 22	n= 8	n= 12
Early Growth (cm, July 5)	48.3 <sup>d</sup>	51.1 <sup>b</sup>	71.0 <sup>bc</sup>	68.3 <sup>bc</sup>	60.5 <sup>bcd</sup>	93.3 <sup>a</sup>	71.5 <sup>b</sup>	71.5 <sup>bc</sup>	94.6 <sup>a</sup>
Regrowth (cm, 21 days after cutting)	122.1 <sup>abc</sup>	111.5 <sup>bc</sup>	141.0 <sup>ab</sup>	121.0 <sup>bc</sup>	110.6 <sup>bc</sup>	134.2 <sup>ab</sup>	117.2 <sup>abc</sup>	102.1 <sup>c</sup>	131.0 <sup>ab</sup>
Northern Leaf Blight damage (1-9 severity)	3.0 <sup>c</sup>	1.9 <sup>cd</sup>	4.3 <sup>ab</sup>	3.0 <sup>bc</sup>	2.0 <sup>cd</sup>	4.5 <sup>ab</sup>	2.5 <sup>bcd</sup>	1.3 <sup>cd</sup>	3.6 <sup>ab</sup>
Culm Width 1 (mm) <sup>†</sup>	9.8	10.2	9.0	9.6	9.5	8.1	9.4	9.1	7.8
Culm Width 2 (mm)	7.6	7.8	7.6	7.1	7.4	7.1	7.0	6.6	6.6
Plant Height 1 (cm)	145.5 <sup>b</sup>	184.4 <sup>a</sup>	195.8 <sup>a</sup>	172.5 <sup>ab</sup>	201.9 <sup>a</sup>	202.1 <sup>a</sup>	173.9 <sup>ab</sup>	195.0 <sup>a</sup>	195.0 <sup>a</sup>
Plant Height 2 (cm)	193.0 <sup>b</sup>	213.8 <sup>ab</sup>	228.8 <sup>a</sup>	196.4 <sup>bc</sup>	209.4 <sup>ab</sup>	224.6 <sup>a</sup>	191.8 <sup>b</sup>	198.1 <sup>ab</sup>	216.3 <sup>ab</sup>
Tillering Number 1	2.3 <sup>c</sup>	3.8 <sup>abc</sup>	3.4 <sup>bc</sup>	3.6 <sup>bc</sup>	5.5 <sup>ab</sup>	5.0 <sup>ab</sup>	3.3 <sup>bc</sup>	5.9 <sup>a</sup>	6.0 <sup>a</sup>
Tillering Number 2	5.0 <sup>c</sup>	8.9 <sup>abc</sup>	7.6 <sup>bc</sup>	7.1 <sup>bc</sup>	9.5 <sup>abc</sup>	8.5 <sup>abc</sup>	6.9 <sup>bc</sup>	13.7 <sup>a</sup>	9.5 <sup>ab</sup>
Dry Weight 1 (kg a <sup>-1</sup> )	28.0 <sup>c</sup>	58.0 <sup>abc</sup>	59.8 <sup>ab</sup>	51.1 <sup>bc</sup>	69.0 <sup>ab</sup>	74.5 <sup>ab</sup>	50.5 <sup>bc</sup>	77.9 <sup>ab</sup>	88.5 <sup>a</sup>
Dry Weight 2 (kg a <sup>-1</sup> )	70.3 <sup>c</sup>	116.0 <sup>abc</sup>	96.6 <sup>abc</sup>	88.8 <sup>abc</sup>	129.4 <sup>ab</sup>	95.2 <sup>abc</sup>	80.2 <sup>bc</sup>	138.4 <sup>ab</sup>	99.7 <sup>abc</sup>
Total Dry Weight (kg a <sup>-1</sup> )	98.3 <sup>b</sup>	173.9 <sup>ab</sup>	156.4 <sup>ab</sup>	139.9 <sup>ab</sup>	198.5 <sup>a</sup>	169.8 <sup>ab</sup>	130.6 <sup>ab</sup>	216.3 <sup>a</sup>	188.1 <sup>a</sup>
Nitrate-nitrogen 1 (% dry weight)	0.44 <sup>b</sup>	0.40 <sup>b</sup>	0.45 <sup>ab</sup>	0.51 <sup>ab</sup>	0.49 <sup>ab</sup>	0.57 <sup>ab</sup>	0.53 <sup>ab</sup>	0.51 <sup>ab</sup>	0.65 <sup>a</sup>
Nitrate-nitrogen 2 (% dry weight)	0.12 <sup>defg</sup>	0.16 <sup>cdefg</sup>	0.14 <sup>cdefg</sup>	0.32 <sup>abcde</sup>	0.26 <sup>bcd</sup>	0.45 <sup>abc</sup>	0.39 <sup>abcd</sup>	0.32 <sup>†</sup>	0.43 <sup>abcd</sup>
Average Nitrate-nitrogen (% dry weight)	0.21 <sup>cd</sup>	0.24 <sup>bcd</sup>	0.25 <sup>bcd</sup>	0.39 <sup>abc</sup>	0.34 <sup>abcd</sup>	0.50 <sup>ab</sup>	0.45 <sup>ab</sup>	0.39 <sup>†</sup>	0.53 <sup>ab</sup>
Absorbed Nitrate-nitrogen 1 (g a <sup>-1</sup> )	123.22 <sup>d</sup>	221.86 <sup>bcd</sup>	248.70 <sup>bcd</sup>	254.95 <sup>bcd</sup>	232.91 <sup>bcd</sup>	409.78 <sup>abcd</sup>	263.78 <sup>bcd</sup>	395.76 <sup>abcd</sup>	563.33 <sup>ab</sup>
Absorbed Nitrate-nitrogen 2 (g a <sup>-1</sup> )	79.89 <sup>c</sup>	146.40 <sup>bc</sup>	138.54 <sup>bc</sup>	286.13 <sup>abc</sup>	278.78 <sup>abc</sup>	420.43 <sup>ab</sup>	324.74 <sup>abc</sup>	401.45 <sup>†</sup>	433.07 <sup>ab</sup>
Total Absorbed Nitrate-nitrogen 1 (g a <sup>-1</sup> )	203.11 <sup>efg</sup>	368.25 <sup>cdefg</sup>	387.24 <sup>bdef</sup>	541.08 <sup>abcde</sup>	608.70 <sup>abcd</sup>	830.22 <sup>bcd</sup>	588.52 <sup>bcd</sup>	815.17 <sup>†</sup>	996.40 <sup>abc</sup>

Different superscript letters (a-g) indicate statistically significant differences (Tukey HSD test;  $P < 0.05$ ).

†, The number after the abbreviation indicate the 1st harvest or the 2nd harvest.

‡, N= 7

second harvests.

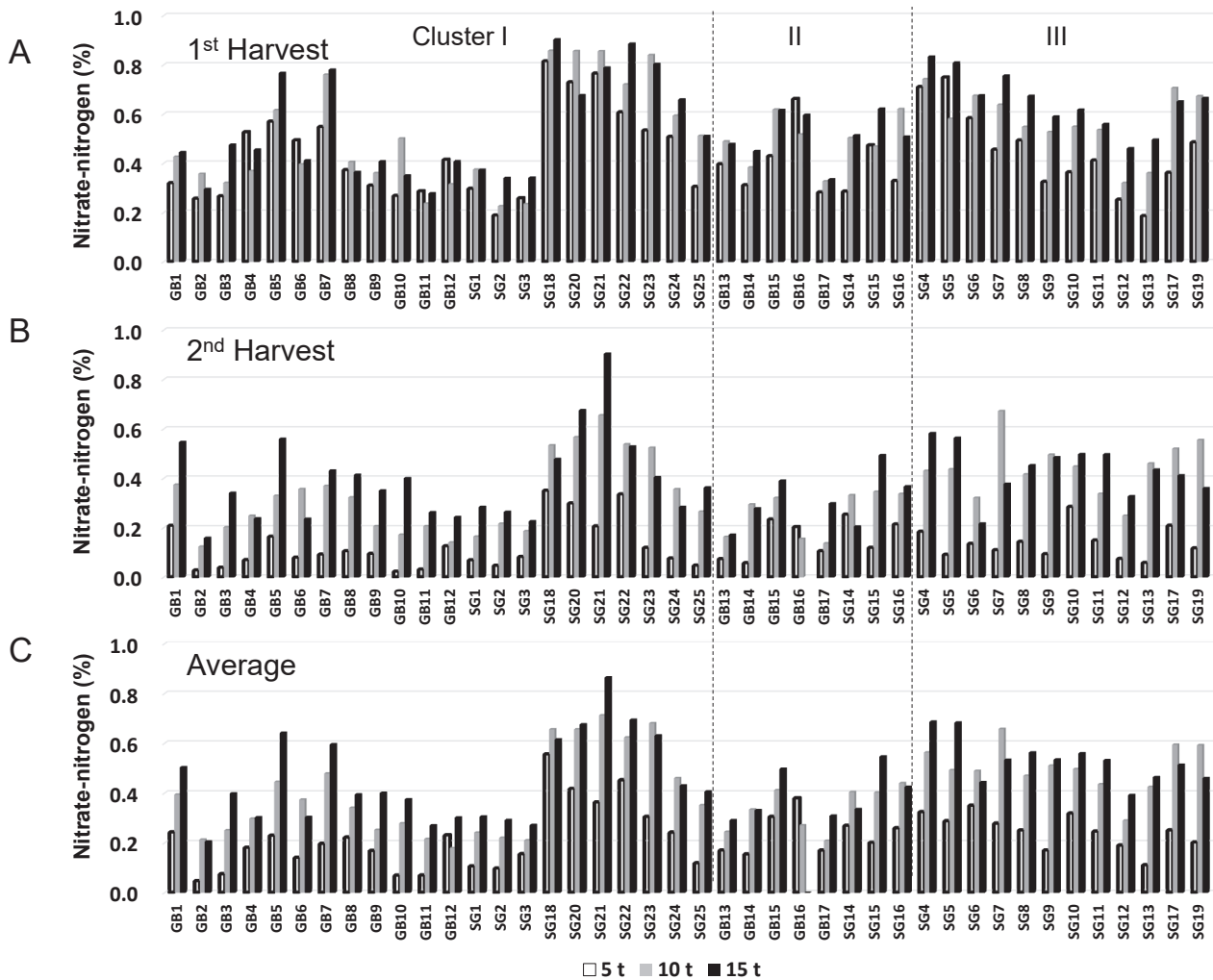
When comparing nitrate-nitrogen concentrations, all clusters exhibited an increasing trend in concentration with higher compost application rates (Table 3). In the second harvest, Cluster II showed lower nitrate-nitrogen concentrations than the other clusters at both 10 and 15 t · 10a<sup>-1</sup>, which likely reflects the vigorous growth characteristic of this cluster. In addition, Cluster II demonstrated lower absorbed nitrate-nitrogen amounts than Cluster III. In contrast, Cluster III exhibited higher levels of absorbed nitrate-nitrogen and tissue concentration at 10 and 15 t · 10a<sup>-1</sup>, suggesting that nitrate uptake remained elevated even under high compost application.

Analysis of nitrate-nitrogen concentrations by individual line revealed that over 95% of samples from the first harvest exceeded the recommended 0.2% threshold in dry weight, even with a compost application of 5 t · 10 a<sup>-1</sup> (Fig. 2A). However, during the second harvest, nitrate-nitrogen levels decreased under the same composting conditions, with many lines dropping below the 0.2% threshold. Of the 42 tested lines, 19 met the recommended nitrate-nitrogen concentration level with

5 t · 10 a<sup>-1</sup> compost application (Fig. 2B). Eight met the recommended nitrate-nitrogen concentration level with 10 t · 10 a<sup>-1</sup> composting, and two met the recommended nitrate-nitrogen concentration level with 15 t · 10 a<sup>-1</sup> compost application. Across the total harvest, nitrate-nitrogen concentrations exceeded 0.2% for 10 t · 10 a<sup>-1</sup> of compost application, except in two lines (Fig. 2C).

#### 4. Sudangrass lines for low nitrate-nitrogen breeding

To identify sudangrass lines suitable for low nitrate-nitrogen silage production, the SG2 and SG13 lines maintained nitrate-nitrogen levels below 0.2% during the first harvest with a 5 t · 10 a<sup>-1</sup> compost application (Fig. 3A). Moreover, SG15 and GB13 exhibited high dry mass production with nitrate-nitrogen levels below 0.2% during the second harvest, with a compost application rate of 5 t · 10 a<sup>-1</sup> (Fig. 3B). Evaluating the relationship between total dry weight and nitrate-nitrogen (Fig. 3C), we note that GB13 had an annual dry weight above 300 kg and a high tiller count, with nitrate-nitrogen levels at 0.17% under 5 t · 10 a<sup>-1</sup> compost application. Furthermore, nitrate-nitrogen levels



**Fig. 2. Nitrate-nitrogen concentrations in sudangrass lines across compost application levels**

Nitrate-nitrogen concentrations as a percentage of dry weight are shown for the first harvest (A), second harvest (B), and their average (C). White, gray, and black bars indicate the compost application rates of 5, 10, and 15 t · 10a<sup>-1</sup> of compost applied in the field, respectively.

remained consistently low, at, or below 0.3%, even with increased compost application rates.

## Discussion

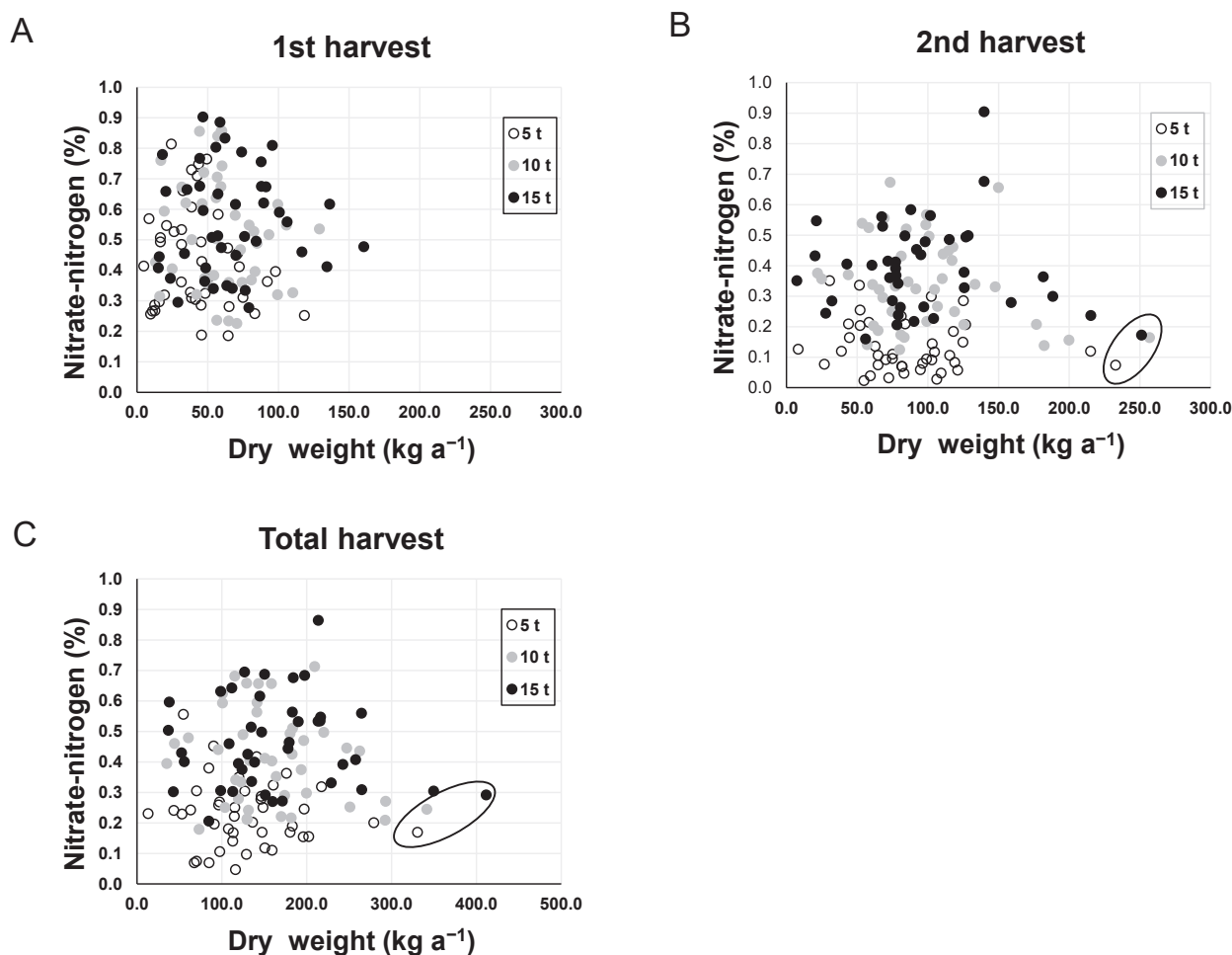
### 1. Phenotypes in the sudangrass clusters

Classifying sudangrass lines based on phenotypic characteristics is challenging because of the subjective nature of the process. To better understand morphological differences, genetic analyses among the sudangrass lines are essential. Prior studies using simple sequence repeat markers have highlighted the high genetic similarity and low polymorphism in sudangrass germplasm compared to sorghum (Zhan et al. 2008). This study used SNP data used to genetically distinguish sudangrass into three clusters. Cluster I exhibited shorter plant height and fewer tillers, resulting in lower dry weight than the other

clusters. In contrast, Cluster II had the highest biomass production and included the Balesudan commercial line, which possesses rhizomes for regrowth in addition to tillering. This perennial trait, resembling *Sorghum halepense* (L.) Pers., sets Cluster II apart. Cluster III displayed rapid early growth and regrowth, with most traits performing at moderate levels compared with the other clusters. However, Cluster III also showed higher susceptibility to northern leaf blight, indicating the potential presence of a gene linked to this susceptibility within the cluster.

### 2. Nitrate-nitrogen concentrations in sudangrass

As nitrate-nitrogen accumulation varies among sudangrass varieties owing to genetic traits (Harada et al. 2000, Sunaga et al. 2005), we evaluated the agronomic traits of sudangrass under varying levels of cattle compost



**Fig. 3. Scatter plots showing the relationship between dry weight and nitrate-nitrogen concentration in sudangrass lines**

The relationship between nitrate-nitrogen concentration and dry weight is shown for the first harvest (A), second harvest (B), and the total harvest (C), in which dry weight is the sum of the first and second harvests, and nitrate-nitrogen concentration is their average. White, gray, and black circles represent compost application rates of 5, 10, and 15 t · 10 a<sup>-1</sup> of compost applied in the field, respectively. The points in black ovals represent samples of GB13 (B, C).

application. Table 2 shows that agronomic traits had considerably lower mean values when no compost was applied. However, the complete absence of compost input is uncommon in Japan and should be considered when interpreting these results. Most agronomic traits improved as compost levels increased from 5 to 15 t · 10 a<sup>-1</sup>. However, no significant differences were observed in total dry weight or plant height, suggesting that adequate compost is essential for growth, while excessive application provides no additional benefit. Nitrate-nitrogen levels increased during the second harvest with higher compost rates, although no significant difference was found between 10 and 15 t · 10 a<sup>-1</sup>. No significant differences in nitrate-nitrogen absorption were observed between the first and second harvests with 10 t · 10 a<sup>-1</sup> and 15 t · 10 a<sup>-1</sup> of compost application. These findings indicate a saturation point for nitrogen

absorption in sudangrass, highlighting the importance of limiting compost application to 10 t · 10 a<sup>-1</sup> during cultivation. We compared agronomic traits among the clusters (Table 3) and observed variations in nitrate-nitrogen concentration, dry weight, and other traits. These findings suggest that future breeding efforts in sudangrass should strategically utilize the specific characteristics of each cluster according to targeted breeding objectives.

Although reducing nitrate-nitrogen concentrations is crucial, maintaining a high dry matter yield is equally important for livestock feed production. Notably, no significant correlation was observed between nitrate-nitrogen concentration and dry weight during the first or second harvest or in the total dry weight (Fig. 3). As nitrate-nitrogen concentrations were lower in the second harvest, the dry weight is critical for reducing

nitrate-nitrogen levels in sudangrass-based feed. The vigorous growth of sudangrass likely contributes to lower nitrate-nitrogen concentrations within the plants, absorbed from the soil. Among the tested lines, GB13 demonstrated substantial biomass production, particularly during the second harvest, which contributed to a dilution of nitrate-nitrogen concentrations in plant tissues. This line, classified within Cluster II, was characterized by both high dry matter yield and relatively low nitrate-nitrogen accumulation in the second harvest. These combined traits indicate that GB13 offers a favorable balance between agronomic performance and feed safety, making it a strong candidate for breeding programs aimed at minimizing nitrate-related risks in forage crops.

## Conclusions

This study categorized 42 lines of 33 sudangrass varieties into three distinct clusters based on genetic variations derived from 3975 SNPs. To our knowledge, this marks the first effort in Japan to classify commercial sudangrass cultivars and landraces from the NIAS gene bank using SNP diversity. The substantial genetic diversity observed in sudangrass enables evidence-based breeding strategies facilitated by this classification. Developing sudangrass varieties with reduced nitrate-nitrogen levels addresses the challenges associated with high nitrate-nitrogen concentrations in cattle feed. For the cultivation of sudangrass within the acceptable nitrate-nitrogen limits, compost application up to  $5 \text{ t} \cdot 10 \text{ a}^{-1}$  is feasible and recommended, depending on line and cultivation conditions. Some of the tested lines exhibited both high dry weight and nitrate-nitrogen concentrations below the regulatory threshold even under high compost application. These findings indicate considerable variation in nitrate accumulation among sudangrass varieties, and that evaluating genetic resources under such fertilization conditions enables the development of varieties with both low nitrate-nitrogen concentrations and high dry matter yields. Through our selection process, we identified promising candidates with high dry matter yields and low nitrate levels, paving the way for producing safe silage and promoting improved animal welfare in agricultural practices.

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## References

- Block, J. (2020) Nitrate poisoning of livestock. North Dakota State University Extension, V839 (Revised February 2020), <https://www.ag.ndsu.edu/publications/livestock/nitrate-poisoning-of-livestock>. Accessed on 3 February 2025.
- Harada, H. et al. (1998) Variety difference of nitrate nitrogen concentration of sorghum under a large amount of nitrogen fertilizer application. *Nihon sochi gakkaiishi (Grassl. Sci.)*, **43**, 449-451 [In Japanese with English summary].
- Harada, H. et al. (2000) Variations in nitrogen uptake and nitrate-nitrogen concentration among sorghum groups. *Soil Sci. Plant Nutr.*, **46**, 97-104.
- Harada, Y. (1992) Recycling of livestock waste. *Shisutemu nogaku (J. Jpn. Agric. Syst. Soc.)*, **8**, 44-58 [In Japanese].
- Ito, Y. et al. (1982) Effects of continuous heavy-application of farmyard manure on the fertility of a humus-rich. *Kyushu nogyoshikenjo hokoku (Bull. Kyushu Natl. Agric. Exp. Stn.)*, **22**, 259-320 [In Japanese].
- Japan Grassland Agriculture and Forage Seed Association, 3rd edition (2009) *Guidebook of Quality Evaluation for Roughage*. Nihon-Souchi-Chikusan-Syushi-Kyoukai, Tokyo, Japan, pp. 154-158 [In Japanese].
- Kasuga, S. (2010) Chapter 5, Sorghum. In Ukai, Y. & Oosawa, R. (eds.), *World history of plant breeding*, Yuushokan, Tokyo, Japan, pp. 111-136 [In Japanese].
- Kiyosawa, A. (2023) Chapter II, Section 3: Sorghum, pp. 129-134. In *Guidebook for Forage Crop Cultivation and Processing (DAIRYMAN Autumn Special Issue)*. Supervised by T. Saigusa and T. Yoshihira [In Japanese].
- Kiyosawa, A. et al. (2020) Analysis of quantitative trait loci for fertility restoration in seven  $F_2$  populations derived from sorghum  $F_1$  hybrids bred in Japan. *Breed. Sci.*, **70**, 379-386.
- Paterson, A. et al. (2009) The *Sorghum bicolor* genome and the diversification of grasses. *Nature*, **457**, 551-556.
- Sunaga, Y. et al. (2005) Varietal differences in nitrate nitrogen concentration of Sudangrass (*Sorghum sudanense* (Piper) Stapf). *Grassl. Sci.*, **51**, 169-177.
- Sunaga, Y. et al. (2008) Management of nitrogen fertilizer application rates based on soil nitrogen fertility with the goal of lowering nitrate-nitrogen concentrations in Sudangrass (*Sorghum sudanense* (Piper) Stapf). *Soil Sci. Plant Nutr.*, **54**, 543-554.
- Thompson, L. J. (2021) Nitrate and nitrite poisoning in animals. MSD Manual Veterinary Manual. <https://www.msdsmanual.com/toxicology/nitrate-and-nitrite-poisoning/nitrate-and-nitrite-poisoning-in-animals>. Accessed on 3 February 2025.
- Zhan, Q. W. et al. (2008) Diversity comparison and phylogenetic relationships of *S. bicolor* and *S. Sudanense* as revealed by SSR markers. *Plant Sci.*, **174**, 9-16.