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Research Article

The Difference in Subpopulation Structures in Sex-Sorted and Non-Sorted Semen by Discriminant Analysis of Bull Sperm Motility Data Collected by a Computer-Assisted Sperm Analysis System

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We examined the motility characteristics of sperm from sex-sorted bull semen as a model for low-quality (expected low conception rate) sperm compared to that of sperm from non-sorted semen (expected average conception rate) using cluster analysis followed by discriminant analysis. The results indicated that sex-sorted semen contained sperm with hyperactivation-like motility as the main subpopulation immediately after thawing, and this subpopulation decreased after 2 h of incubation. The main subpopulation in the non-sorted semen had progressive motility that was maintained during incubation. A conventional comparison of the mean values of kinematic parameters could not distinguish the samples. In conclusion, discriminant analysis using data from a cluster analysis of motile sperm may accurately describe differences in the structures of sperm motility subpopulations between low and normal fertility semen.

1. Introduction

Motility is a commonly used characteristic of sperm to predict male fertility [1]. Computer-assisted sperm analysis (CASA) is widespread and used to evaluate human and animal sperm motility [2]. In humans, sperm kinematic parameters estimated by using CASA are possible predictors of pregnancy by intrauterine insemination and support the decision about whether to proceed with *in vitro* fertilization (IVF) or intracytoplasmic sperm injection [3, 4]. Moreover, the results of CASA show a good correlation with the fertilization rate in IVF [5]. However, CASA has not become the gold standard for the prediction of male fertility in the World Health Organization (WHO) manual because there is insufficient evidence to allow the use of CASA in wide clinical practice [6]. In addition, there is no clear standard sperm motility correlation with male fertility in animal breeding. Although sperm motility is one of the essential factors in male fertility and a general parameter in the evaluation of semen quality, other sperm functions and

molecules are also key factors for fertilizing capacity (e.g., membrane integrity, acrosome integrity, DNA integrity, RNAs, and proteins) [7–9].

Recently, sex-sorted bull semen, including highly gender-separated (over 90%) X- or Y-bearing sperm by flow cytometry detecting the difference in DNA content [10, 11], have been used worldwide in the dairy industry. However, it has about a 10% lower pregnancy rate in artificial insemination (AI) [12, 13] and shorter longevity than non-sorted semen [14]. The low pregnancy rate is due to damage to sperm by mechanical and chemical stress during the sex sorting process, such as a decrease of motility, acrosome integrity, and DNA integrity [15–17] induced by reducing the activity of the antioxidative enzyme [15–18]. In addition, we showed the shorter duration of artificial insemination timing for achieving pregnancy in sex-sorted semen than in non-sorted semen [19]. Therefore, we attempted to use sex-sorted semen as a low-quality model, expected a low conception rate, and analyze differences in motility characteristics between sex-sorted and non-sorted semen.

CASA analyzes individual sperm trajectory accurately and objectively [20], which enables the analysis of sperm motility in detail [21]. Cluster analysis has been used to evaluate sperm subpopulations based on motility using CASA data in humans [22], boars [23], bulls [24], deer [25], donkeys [26], and stallions [27]. A cluster analysis is useful to understand sperm heterogeneity based on their flagellar beat, which is used to assess motility and evaluate bull semen quality [24, 28–31]. Moreover, analysis of sperm subpopulation can be a powerful tool to resolve some problems in human andrology and animal breeding by combination with molecular analyses because heterogeneity in sperm is affected by several molecules related with spermatogenesis, seminal plasma, capacitation, and fertilization [32]. However, the typical structure of clusters by sperm motility have not been defined. It is desirable to characterize clusters with a defined biological meaning for the development of automated supervised classification of sperm using machine learning systems [33], which will predict male fertility in clinical and veterinary fields using the analysis of sperm motility.

In the present study, we examined the motility characteristics of sperm in sex-sorted and non-sorted bull semen by cluster analysis using CASA data to clarify the difference in their sperm subpopulations and attempted to find the subpopulation feature of sex-sorted semen.

2. Materials and Methods

All frozen semen samples were donated by an AI center (Genetics Hokkaido, Kitahiroshima, Japan). Sex-sorted and non-sorted frozen semen derived from three Holstein bulls (5-6 years old) were used in the present study (3 straws for both semen in a bull, 18 straws in total). Before semen freezing, sperm motility was evaluated by several technicians under light microscopic observation, and all semen indicated >65% progressively motile sperm. Egg-yolk-based extenders and ejaculates were used for the production of non-sorted and sex-sorted frozen semen. The samples were contained in a 0.5 ml straw, and their fertility was proven commercially by acceptable conception rates (≥35%, which is considered normal following the criteria by Sun et al. [34]) in the field.

2.1. Preparation of Sperm and CASA Analysis for Sperm Motility. Preparation of sperm was performed following our previous study [35]. Straws containing frozen semen were immersed in water at 37°C for 1 min. Frozen-thawed semen was expelled onto a 45/90% Percoll (GE Healthcare, Buckinghamshire, UK) layer diluted by modified Brackett and Oliphant media (BO; 112.00 mM NaCl, 4.02 mM KCl, 0.83 mM NaH_2PO_4, 2.25 mM $CaCl_2$, 0.52 mM $MgCl_2$, 37.00 mM $NaHCO_3$, 13.90 mM glucose, 1.25 mM sodium pyruvate, and 50 μg/ml gentamicin sulfate) [36]. Samples were then centrifuged at $700 \times g$ for 20 min to select motile sperm. The supernatants were removed, and the resulting sperm pellets were resuspended in BO and centrifuged again at $500 \times g$ for 5 min for washing. After a second centrifugation, the supernatants were removed, and sperm concentrations were calculated using a hemocytometer. Samples were diluted to 10×10^6 cells/ml by BO with 3.0 mg/ml BSA. BO was used after equilibration under 5% CO_2, 5% O_2, and 90% N_2 at 39°C for at least 2 h. Recovered motile sperm were incubated in 50 μl droplets of BO under 5% CO_2, 5% O_2, and 90% N_2 at 39°C. After 0-, 2-, and 4 h incubations, sperm motility was analyzed by the CASA system (SMAS, DITECT, Tokyo, Japan) based on the digitalized images obtained by a charge-coupled device camera (HAL-L2M 5 M DMF 031, DITECT) equipped with a ×10 negative-phase contrast microscope (E200, Nikon, Tokyo, Japan). Sperm motility and kinematic variables were evaluated as described in a previous study [29]. In brief, a 3 μl of each sample was introduced into a 20 μm-deep chamber (SC20-01-04-B, Leja, GN Nieuw-Vennep, Netherlands) and warmed at 37°C on a hot plate (Kitazato Corporation, Shizuoka, Japan).

The percentage of motile sperm was recorded, and the following kinematic parameters were analyzed: straight line velocity (VSL: the straight-line distance from the beginning to end of a sperm track for 1 sec, μm/sec), curvilinear velocity (VCL: the actual path velocity of sperm for 1 sec, μm/sec), average path velocity (VAP: the average path velocity of sperm for 1 sec, μm/sec), amplitude lateral head (ALH, μm), beat cross frequency (BCF, Hz), linearity (LIN = VSL/VCL, %), and straightness (STR = VSL/VAP, %). The CASA system recorded at 150 frames per second, and sperm with more than 120 frames were used in the analysis. The number of sperm analyzed per sample was at least 100 (sex-sorted) or 200 (non-sorted), including immotile sperm, in three fields for evaluation of sperm motility by CASA because the number of sperm contained in a straw was different because of commercial reasons (average numbers of sperm: 2 and 30×10^6 cells in sex-sorted and non-sorted semen, respectively).

2.2. Preparation of Datasets for Discriminant Analysis. Datasets for discriminant analysis were obtained from 44,570 motile sperm derived from the semen of three bulls with an acceptable conception rate in AI that were analyzed in our previous study [35]. For cluster analysis, the sperm kinematic parameters obtained by the CASA system (VSL, VCL,

Table 1: Kinematic parameters of sperm in each subpopulation for supervised classification.

Cluster	No. sperm	VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)
1	6043	149.1 ± 28.1^a	318.9 ± 61.5^b	163.1 ± 21.6^a	4.0 ± 1.2^c	21.8 ± 6.2^b	47.9 ± 9.9^b	91.5 ± 12.3^a
2	9396	80.7 ± 28.2^b	168.9 ± 47.4^d	94.1 ± 27.1^c	1.6 ± 0.7^e	28.3 ± 5.8^a	49.2 ± 14.4^a	86.3 ± 17.0^b
3	2676	77.8 ± 45.9^c	391.6 ± 68.8^a	145.0 ± 29.9^b	6.9 ± 1.3^a	8.4 ± 4.7^d	20.2 ± 11.9^d	55.4 ± 31.4^d
4	5206	52.1 ± 29.5^d	238.3 ± 54.9^c	90.9 ± 27.1^d	4.3 ± 1.2^b	10.6 ± 5.8^c	23.0 ± 13.6^c	59.2 ± 29.4^c
5	7797	20.9 ± 16.0^e	123.9 ± 45.3^e	35.6 ± 17.7^e	2.6 ± 1.0^d	7.5 ± 4.5^e	16.6 ± 11.6^e	55.8 ± 29.6^d
6	13482	5.4 ± 6.0^f	42.5 ± 22.2^f	9.9 ± 7.9^f	0.7 ± 0.5^f	6.9 ± 3.6^f	12.3 ± 8.5^f	56.8 ± 28.4^d

Values are means ± SD. [a-f] Different superscripts indicate significant differences.

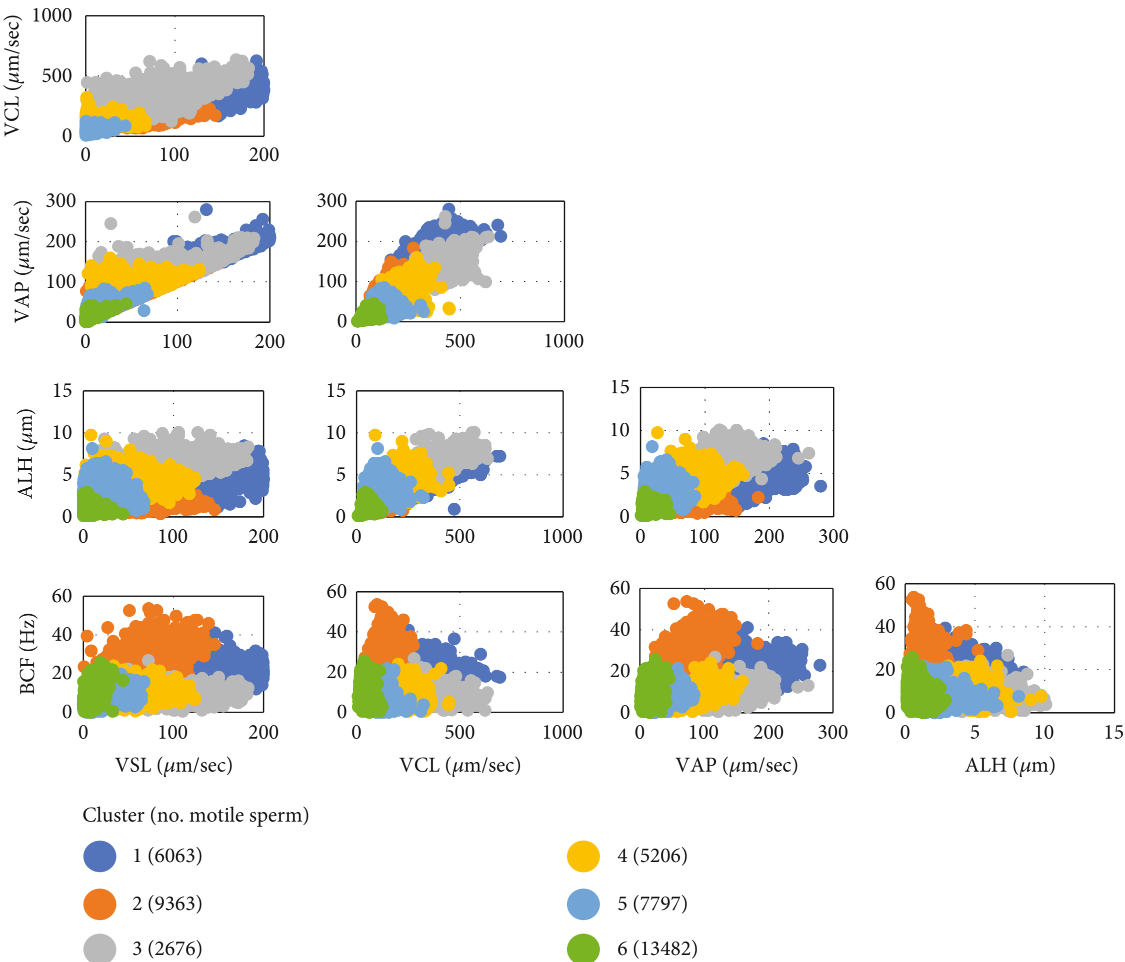

Figure 1: Assignment of individual sperm into clusters by kinematic parameters. Developed program allocates each sperm automatically to the optimal cluster by kinematic parameters (VSL, VCL, VAP, ALH, and BCF).

VAP, ALH, and BCF) were used. The kinematic parameters of sperm in each cluster [26] are shown in Table 1. The datasets included sperm kinematic parameters in BO with or without calcium ionophore A23187. Sperm were cultured in BO with A23187 to induce capacitation followed by acrosome reaction and showed hyperactivation-like motility [37]. Each cluster was numbered from the largest to the smallest, depending on the VSL value. Cluster 1 showed the highest VAP, the second highest VCL and LIN, and the third highest ALH and BCF. Cluster 2 showed the second highest STR, the third highest VCL, and the highest BCF and LIN, but the second lowest ALH and lower VAP than clusters 1, 3, and 4. Cluster 3 had the highest VCL and ALH, the second highest VAP, and the third lowest BCF and LIN. Cluster 4 had the second highest VCL and the third highest ALH, BCF, and LIN, whereas VAP was lower than in clusters 1, 2, and 3. Cluster 5 showed the second-lowest values in all parameters, except for ALH. Cluster 6 had the lowest values in all parameters.

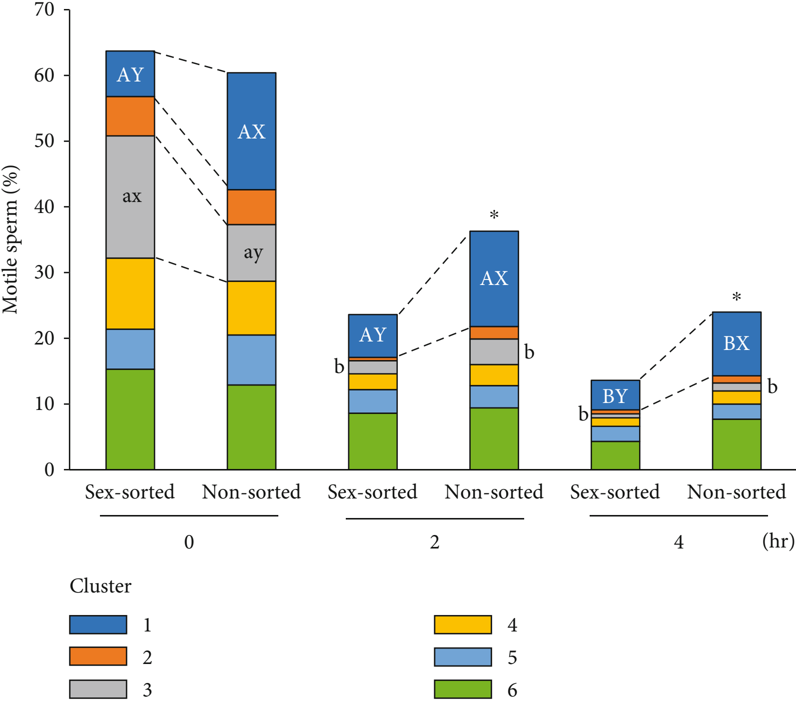

Figure 2: The structures of sperm motility subpopulations in sex-sorted and non-sorted bull semen at 0, 2, and 4 h after incubation. Three bulls are used and three replicates are performed in each bull. Number of sperm analyzed by the CASA are 1,316 in sex-sorted and 1,578 in non-sorted semen. Asterisks indicate a significant difference in the percentage of motile sperm between sex- and non-sorted sperm ($P < 0.05$). [A, B]Different letters indicate significant differences in the proportion of cluster 1 between incubation times ($P < 0.05$). [a, b]Different letters indicate significant differences in the proportion of cluster 3 between incubation times ($P < 0.05$). [X, Y]Different letters indicate significant differences in cluster 1 between sex- and non-sorted sperm ($P < 0.05$). [x, y]Different letters indicate significant differences in cluster 3 between sex- and non-sorted sperm ($P < 0.05$).

2.3. Evaluation of the Structures of Sperm Motility Subpopulations. To evaluate the structures of sperm motility subpopulations in sex-sorted and non-sorted semen, discriminant analysis was performed using a custom-written program (Igor Pro, Wavemetrics, Lake Oswego, OR, USA). Independent kinematic parameters (VSL, VCL, VAP, ALH, and BCF) were used for analysis. Centroids and standard deviations in each cluster in our previous study [28] were input into the program as reference data. Next, individual values of the kinematic parameters of 2,994 motile sperm (sex-sorted: $n = 1{,}316$; non-sorted: $n = 1{,}578$) derived from three bulls (three replicates in each bull) were entered into the program (Figure 1). Before entering these values, the program standardized the kinematic data of individual sperm by the following formula: (measurements–average)/standard deviation and then classified those sperm into each cluster by their motility parameters automatically. The average and standard deviation were calculated by pooling data from sex-sorted and non-sorted sperm.

2.4. Statistical Methods. All analyses were performed using JMP pro 14 (SAS, NC, USA). Sperm kinematic parameters in each cluster were compared by one-way ANOVA followed by Tukey-Kramer's HSD test. Data of sex-sorted and non-sorted sperm were analyzed by a repeated measures two-way ANOVA followed by Tukey-Kramer's HSD test or Student's t-test. Data are shown as the mean ± SD. Differences were significant at $P < 0.05$.

3. Results

The results of the discriminant analysis to evaluate the motility of sperm in sex-sorted and non-sorted semen are shown in Figure 2. The proportion of cluster 1 was lower in sex-sorted than in non-sorted semen at all incubation times ($P < 0.05$). The proportion of cluster 3 was higher in sex-sorted than in non-sorted semen immediately after thawing ($P < 0.05$). Although the percentage of motile sperm decreased with the progression of the incubation ($P < 0.05$), no reductions were observed in the proportion of cluster 1 between 0 and 2 h, regardless of sorting. The percentages of motile sperm were lower in sex-sorted than in non-sorted semen at 2 and 4 h ($P < 0.05$).

The mean averages and standard deviations of each kinematic parameter in sex-sorted and non-sorted sperm at 0, 2, and 4 h after incubation are shown in Table 2. There was no interaction between the experimental group and the incubation time. Kinematic parameters other than ALH in non-sorted semen were higher than those in sex-sorted semen ($P < 0.05$), although ALH tended to decrease with incubation ($P = 0.0567$). These results suggest that sex-sorted sperm have low kinematic activity.

Table 2: The kinematic parameters of sperm in sex-sorted and non-sorted semen during incubation.

Kinematic parameters	Experimental groups: Sex-sorted, Incubation time (h) 0	2	4	Non-sorted 0	2	4	*P* value: Group	Incubation time	Interaction
VSL (μm/sec)	72.1 ± 19.7	73.0 ± 35.6	59.0 ± 50.6	92.8 ± 23.3	108.4 ± 17.1	96.4 ± 28.0	0.0006	0.4561	0.6841
VCL (μm/sec)	237.3 ± 50.8	213.3 ± 79.9	188.2 ± 106.3	247.3 ± 43.4	263.4 ± 40.6	242.1 ± 67.7	0.0479	0.4483	0.5733
VAP (μm/sec)	93.4 ± 19.8	85.1 ± 36.4	73.0 ± 49.6	107.3 ± 23.0	120.2 ± 18.2	106.5 ± 28.8	0.0002	0.4256	0.5294
ALH (μm)	3.9 ± 0.6	3.2 ± 1.0	2.8 ± 1.3	3.5 ± 0.5	3.7 ± 0.8	3.2 ± 0.9	0.4998	0.0567	0.2941
BCF (Hz)	12.3 ± 3.1	11.5 ± 2.9	11.1 ± 4.4	14.2 ± 4.0	14.9 ± 2.8	14.8 ± 2.8	0.0022	0.9515	0.7041
LIN (%)	28.1 ± 4.5	26.1 ± 5.7	23.1 ± 11.2	32.3 ± 5.8	33.4 ± 4.0	31.4 ± 4.7	0.0005	0.3415	0.6073
STR (%)	70.7 ± 6.1	70.7 ± 8.5	68.1 ± 14.2	76.0 ± 7.8	76.5 ± 5.8	76.4 ± 5.8	0.0075	0.8808	0.8526

Values are means ± SD.

4. Discussion

We compared the structure of sperm motility subpopulation in sex-sorted and non-sorted semen by discriminant analysis using the datasets of six clusters analyzed in our previous study [28]. Sperm in clusters 1 and 2 exhibited higher BCF and LIN than sperm in other clusters. In a previous study [38], sperm with high LIN maintained high activity until 6 h after incubation; thus, they were regarded as progressively motile sperm, which is related to fertilization [24, 39]. Clusters 3 and 4 included sperm with higher VCL and ALH and lower LIN and BCF than clusters 1 and 2. These values indicate a widely beating head in a circular motion, which is similar to the motility of hyperactivated sperm [40]. Also, it has been reported that circular movement may be a sign of a reduction in protein kinase-A-mediated signaling activities [41], which is related to sperm capacitation and acrosome reactions [41]. These results indicate that sperm exhibiting hyperactivation-like motility have shorter longevity than progressively motile sperm. The present discriminant analysis found that the main subpopulations of sex-sorted and non-sorted semen were clusters 3 and 1, respectively, immediately after thawing. The proportion of cluster 1 was maintained until 2 h after thawing in both semen groups and was always lower in sex-sorted than in non-sorted semen. However, the proportion of cluster 3 decreased in both semen groups immediately after thawing. Previous studies reported that sex-sorted bull sperm showed the decrease in average values of velocities, amplitude and frequency of head shaking, and linearity [42] but increase curvilinear distance [43] as evaluated by CASA. The results of the present study clearly showed that these altered of sperm kinematic parameters derived from the differences in the structures of sperm motility subpopulation in sex-sorted and non-sorted semen. Sex-sorted semen show lower fertility than non-sorted semen after AI [12, 13] and also have shorter longevity [14]. Furthermore, the shorter lifespan of sperm in sex-sorted semen was previously proposed to be associated with capacitation-like changes induced by sex sorting [44]. The results of the present study demonstrated that a higher proportion of sperm with hyperactivation-like motility was present in frozen-thawed sex-sorted semen, although a classical evaluation that compared only the mean values of kinematic parameters did not show any clear difference.

In the present study, we used the sperm treated by percoll as samples to analyze the movement pattern of motile sperm. Percoll has been used to remove immotile sperm from semen for IVF in cattle [45].

This is the first study to use discriminant analysis for the evaluation of bull sperm motility using sex-sorted semen. Our findings show the possibility of predicting semen fertility by cluster analysis. However, the data size and treated types of sperm motility were limited; therefore, further studies are required to establish the best reference data to predict semen fertility.

5. Conclusion

A discriminant analysis using the data of a cluster analysis of motile sperm appropriately described differences in the structures of sperm motility subpopulations in sex-sorted and non-sorted semen. Our findings will contribute to the development of a novel and useful examination to predict male fertility in human clinical studies to determine the strategy of assisted reproductive technology and veterinary fields to select male animals.

Data Availability

The data presented in the present study are available upon request from the corresponding author.

Disclosure

The content of the manuscript has been posted as a preprint [46].

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Conceptualization was done by C. Kanno, S. S. Kang, Y. Yanagawa, and M. Nagano. Formal analysis was worked on by C. Kanno and K. Q. Sakamoto. The funding acquisition was supervised by C. Kanno and M. Nagano. The investigation was performed by C. Kanno and S. S. Kang. The methodology was formulated by C. Kanno, S. S. Kang., K. Q. Sakamoto, Y., Yanagawa, and M. Nagano. Project administration was conducted by M. Nagano. Software was assigned to K. Q. Sakamoto. Supervision was conducted by S. Katagiri and M. Nagano. Validation was worked on by M. Nagano. The original draft was written by C. Kanno. Writing which is reviewing and editing was worked on by C. Kanno, S. S. Kang, K. Q. Sakamoto, Y. Yanagawa, S. Katagiri, and M. Nagano. All authors have read and agreed to the published version of the manuscript.

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