CHROMOSOME NUMBER OF SACCHARUM SPONTANEUM L. 
COLLECTED FROM JAPAN

By
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KEYWORDS: Germplasm, High Ploidy, Hotplate Treatment.

Abstract

THE AUTHORS proposed a method of improving glass slide preparation used in chromosome counts that calls for a hotplate treatment. Using this newly developed preparation, we surveyed the chromosome number of Saccharum spontaneum L. collected from different locations of the Southwest Islands, Japan. Fresh root tips were collected, pretreated in cold water for 24 hours, then fixed and stored. After enzyme digestion, chromosomes were spread over a slide in 2:1 v/v ethanol: acetic acid fixative, and then quickly dried on a hotplate at 45°C. After drying, slides were dipped in ethanol and fixative solutions. Cold water treatment effectively accumulated metaphase and pro-metaphase stages. Most countable chromosome spreads were round or oval shaped, with an average of 36 μm diameter. Ethanol and fixative treatment prevent chromosome loss during downstream preparation. Compared with the conventional air-dried treatment, high-quality chromosome spreads were obtained irrespective of environmental conditions. Mild heating by hotplate enhanced spreading due to a constant evaporation of the fixative. Treatment with ethanol and fixative solution enhanced chromosome attachment to the glass slide and removed cytoplasmic debris. Chromosome numbers of S. spontaneum were 2n = 96–112, with aneuploidy, and varied within some of the islands. Geographic distribution, chromosome number and evolution of S. spontaneum are discussed.

Introduction

Saccharum spontaneum L., mainly used as the paternal parent in development of sugarcane (Saccharum) interspecific hybrid clones, has several desirable agronomic traits.

There is strong association between geographic distribution and chromosome number in Asia, as chromosome number increased from India to eastern sectors (Panje and Babu, 1960).

The Southwest Islands are located between Taiwan and the Kyushu region in Japan, between 24–30°N and 122–132°E, and are composed of over 200 islands.

So far, few reports were published on chromosome number in Japanese S. spontaneum which has cytotypes with chromosome number ranging from 2n = 104 in Amami-Ohshima to 2n = 112 in Okinawa and Kamakura (Moriya, 1959).

Chromosome observation and counting are cumbersome, especially in the case of small chromosomes and high ploidy level. Environmental conditions, such as humidity and temperature, affect the quality of chromosome spread.

Recently, Henegariu et al. (2001) reported a precise and controlled preparation method for spreading human chromosomes by means of a steam-metal plate treatment and chemical aging.

For further convenience, we propose a method to simplify and improve chromosome preparation. With this improved method, we investigated chromosome number and its relationship with geographical distribution of S. spontaneum clones collected from the Southwest Islands.

Materials and methods

Plant materials

Thirty accessions of S. spontaneum were collected from seven islands of the Southwest Islands (Iriomote, Ishigaki, Miyako, Okinawa, Tokunoshima, Amami-Ohshima and Kikaijima island) and were
examed (Table 1). Accessions from the Amami-Ohshima island were kindly provided by the Japan International Research Center for Agricultural Sciences (JIRCAS), located at the Okinawa Subtropical Station.

**Slide glass preparation**

Chromosomes were prepared as described by Fukui (1996) and Henegariu et al. (2001) with some modifications. Fresh root tips from germinated buds were collected, pretreated in 4°C distilled water for 24 h, fixed in 3:1 ethanol: acetic acid fixative, and stored in 70% ethanol until use.

Stored root tips were softened in enzyme solution (2% w/v Cellulase Onozuka RS; 1% w/v Macerozyme R200; 0.3% w/v Pectolyase Y-23; 10 mM citrate buffer, pH 4.8) at 37°C for 50-70 min, transferred onto a pre-cleaned glass slide over a drop of cold fixative (2:1 v/v ethanol: acetic acid, instead of 3:1 v/v), and tapped by fine forceps.

When the fixative started to dry, slides were quickly heated on a hotplate at 45°C for a few seconds (hotplate treatment). For comparison, slides were air-dried overnight. Dried slides were dipped into 96% ethanol, and 3:1 ethanol: acetic acid fixative for 10 min, respectively, and air-dried. Cells were stained with Giemsa or aceto-orcein solution.

Twenty-five countable cells were observed for chromosome counting, and measured for the long and the short axes for both hotplate and air-dried treatment. Images were processed with Adobe Photoshop® or NIH ImageJ software.

**Results and discussion**

**Improved chromosome preparation**

An efficient spreading of chromosomes was obtained with the hotplate treatment (Table 1 and Figure 1). Most chromosomes were round or oval shaped. Chromosome number had a little influence on spreading (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>Long axes</th>
<th></th>
<th>Short axes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Av†</td>
<td>SD</td>
<td>CV</td>
<td>Av†</td>
</tr>
<tr>
<td>Air-dried</td>
<td>32.8</td>
<td>4.5</td>
<td>0.14</td>
<td>27.9</td>
</tr>
<tr>
<td>Hotplate</td>
<td>36.7</td>
<td>3.9</td>
<td>0.11</td>
<td>30.1</td>
</tr>
<tr>
<td>Signif.</td>
<td>*</td>
<td>*</td>
<td>—</td>
<td>*</td>
</tr>
</tbody>
</table>

Twenty-five cells from six slides were measured for each accession. In total, seven accessions were examined. Long axes, Short axes: mean diameter of twenty-five cells in each treatment; † Av: average diameter; SD: standard deviation; CV: coefficient of variance; *: Significant at P <0.05; ns: not significant; —: not examined.

The hotplate treatment effectively lengthened chromosome spreading, on an average 10% and 7% for long and short axes, respectively. The standard deviation of long axes with this treatment was statistically different as compared with the air-dried treatment.

The 2:1 fixative evaporated slower than the 3:1 fixative, and resulted in absorption of more atmospheric moisture and water-induced swelling of cells.

A constant evaporation rate of the fixative solution resulted in regular chromosome spreading on the hotplate, and high-quality chromosome spreads were obtained as a result, irrespective of environmental conditions.

In addition, cytoplasmic debris was removed effectively. In comparison, uneven spreading chromosomes were frequent in the air-dried treatment (Table 1).

Some chromosomes were overspread. A major problem with the air-dried treatment is the uncontrollable chromosome spread. Chromosome spreading was affected by environmental factors, especially temperature and humidity.
Fig. 1—Chromosome spread with the proposed method of hotplate treatment (left) and conventional air-dried method (right). Bars indicate 10 μm.

In spite of chemical reagents, a cold-water treatment is an efficient method for accumulating small chromosomes at the metaphase and prometaphase stages, since the latter phase has more information than the former, particularly for karyotype analysis (Hara et al., 1999).

After chromosome spreading, slides were usually aged overnight at room temperature. Omitting this step brought about a loss of chromosomes during downstream preparation. Dipping slides into ethanol and fixative was simple and did not require any special equipment.

The procedure was completed in a similar time to that taken for ‘chemical aging’, as described in Henegariu et al. (2001), and resulted in the improvement of chromosome attachment to the glass slide when compared with the air-dried treatment.

**Chromosome number and geographical distribution**

Chromosome numbers in *S. spontaneum*, originating from Southwest Islands, ranged from 96 to 112 (Table 2). Frequent cytotypes were $2n = 96$, 104, and 112, and aneuploids were found in the accessions from Amami-Oshima island. Chromosome number was variable within each island. There seemed to be no clear correlation between chromosome number and geographical distribution.

<table>
<thead>
<tr>
<th>Island</th>
<th>$2n$</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iriomote</td>
<td>96(4), 112(2)</td>
<td>24° 23'</td>
<td>123° 44'</td>
</tr>
<tr>
<td>Ishigaki</td>
<td>112(3)</td>
<td>24° 20'</td>
<td>124° 09'</td>
</tr>
<tr>
<td>Miyako</td>
<td>96(2), 112(2)</td>
<td>24° 47'</td>
<td>125° 16'</td>
</tr>
<tr>
<td>Okinawa</td>
<td>112(3)</td>
<td>26° 12'</td>
<td>127° 41'</td>
</tr>
<tr>
<td>Tokunoshima</td>
<td>104(2), 112(2)</td>
<td>27° 40'</td>
<td>128° 58'</td>
</tr>
<tr>
<td>Amami-Oshima</td>
<td>104(4), 108(2), 112(2)</td>
<td>28° 19'</td>
<td>128° 55'</td>
</tr>
<tr>
<td>Kikaijima</td>
<td>104(2)</td>
<td>28° 22'</td>
<td>129° 29'</td>
</tr>
</tbody>
</table>

Twenty-five cells were examined for each accession.

Although the number of samples was small, the results showed that chromosome number and distribution in the Southwest Islands were more variable than expected (Moriya, 1959).

Some factors that contribute to geographical distribution are known to be:

1. an increase in chromosome number as a result of polyploidisation, natural hybridisation, and 2n gamete formation;
2. migration or emigration;
(3) paleogeographical events; or
(4) a combination of these factors.

For instance, aneuploids (2n = 108) are expected to originate from 2n = 112 in descending chromosome number, or progeny of hybridisation between two different cytotypes. Panje and Babu (1960) reported 2n = 112 spontaneum from other regions of Asia, such as China, Indonesia, and Taiwan, and considered that distribution was attributed to migration.

In contrast, Moriya (1959) concluded, with respect to chromosome number, morphology and flowering time, Japanese S. spontaneum clones were different from those in adjacent areas.

Tracing the origin of plants inhabiting islands is complicated: thus, careful consideration to geographical distribution is needed. Further studies by molecular methods and karyotype analysis will clarify the history of Japanese S. spontaneum.

Conclusion

Improved glass slide preparation provided useful results. With this method, chromosome numbers of S. spontaneum collected from the Southwest Islands were in the range of 2n = 96–112 including aneuploids. Comparisons among different populations are needed for a better understanding of the history of Japanese S. spontaneum.

Acknowledgment

The authors greatly acknowledge Mr Mitsunori Sato, JIRCAS Okinawa Subtropical Station, for kindly providing the germplasm.

REFERENCES


NOMBRE DE CROMOSOMAS CHEZ LES CLONES DE SACCHARUM SPONTANEUM L. COLECTADOS AU JAPÓN

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MOTS CLÉS: Germoplasma, Ploidie Elevée, Traitement sur Plaque Chauffante.

Résumé

Les auteurs proponent un protocol, nécessitant un traitement sur plaque chauffante, pour améliorer la préparation des lames utilisées pour le comptage de chromosomes. Cette nouvelle méthode a été utilisée pour effectuer un relevé du nombre de chromosomes chez les clones de Saccharum spontaneum L., collectés de diverses régions des îles du sud-ouest du Japon. Les pointes de racines fraîchement collectées ont d’abord été traitées à l’eau froide pendant 24 h, puis fixées et stockées. Après une digestion enzymatique, les chromosomes, dans une solution de 2:1 vol/vol d'éthanol : acide acétique comme fixateur, ont été étalés sur une lame et rapidement séchés sur une plaque chauffante à 45°C. Les lames séchées ont ensuite été plongées dans l'éthanol et les solutions fixatrices. Le traitement à l’eau froide a efficacement démontré les stades de métabase et prophase. La plupart des chromosomes étalés qui pouvaient être comptés étaient de forme ronde ou ovale et d’un diamètre de 36 µm en moyenne. L’utilisation de l’éthanol et du fixateur a permis de réduire la perte de chromosomes dans les traitements subséquents. Par rapport au traitement traditionnel qui consiste à sécher à l’air, des étaléments de chromosomes de très bonne qualité ont été obtenus, indépendamment des conditions environnementales. Le chauffage doux sur plaque chauffante a amélioré l’étalément des chromosomes grâce à une évaporation constante du fixateur, tandis que le traitement à l’éthanol et au fixateur a augmenté la fixation des chromosomes sur la lame, tout en évitant les débris cytoplasmiques. Le nombre de chromosomes chez les clones de S. spontaneum était entre 2n = 96 à 112, avec aneuploidie, et varié pour certaines îles. La distribution géographique, le nombre de chromosomes et l’évolution du S. spontaneum sont discutés.

NÚMERO DE CROMOSOMAS DE SACCHARUM SPONTANEUM L. COLECTADO EN EL JAPÓN

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PALABRAS CLAVES: Germplasma, Alta Ploidia, Tratamiento de Plato Caliente.

Resumen

Los autores proponen un método que mejora la preparación en portaobjetos de vidrio el recuento de cromosomas mediante el tratamiento en un plato caliente. Usando este nuevo sistema de preparación, se examinó el número de cromosomas de Saccharum spontaneum recogido de diversas localidades de las Islas del Sudeste, Japón. Se colectaron las puntas de las raíces, precalentaron en agua fría durante 24 horas, luego se fijaron y almacenaron. Después de efectuada una digestión enzimática, los cromosomas se extendieron en un portaobjetos de vidrio con fijador 2:1 vi/v: etanol : ácido acético, seguido por un rápido secamiento en un plato caliente a 45°C. Después de que se secó el material, los portaobjetos fueron sumergidos en soluciones de etanol y fijador. El tratamiento con agua fría acumulado de manera efectiva los estados de metafase y pro-metafase. La mayoría de los cromosomas que se extendieron eran de forma redonda u oval, con un promedio de 36 µm diámetro. El tratamiento con el etanol y el fijador previno la pérdida de los cromosomas durante el proceso de lavado. En comparación con el tratamiento convencional de secado al aire, las preparaciones de los cromosomas que se consiguieron con el procedimiento descrito eran de alta calidad independiente de las condiciones ambientales. El calentamiento suave en el plato caliente realizó la dispersión debido a la constante evaporación del fijador. Los tratamientos con la solución de etanol y el fijador realizaron la adherencia del cromosoma al portaobjeto y a su vez renovó los residuos citoplasmicos. El número de cromosomas de S. spontaneum fue de 2n = 96 - 112, con aneuploidía y varió dentro de algunas muestras de las islas. La distribución geográfica, el número de cromosomas y la evolución de S. spontaneum son discutidas.