JARQ 59 (3), 227-231 (2025) https://www.jircas.go.jp

Development of Chromosome Observation Methods in Acerola (*Malpighia glabra* L.)

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Abstract

A chromosome preparation method using young leaves of acerola (*Malpighia glabra*) was developed. The young leaves were cut into approximately 2-mm^2 for enzymatic maceration and air-drying (EMA). For EMA, an enzyme mixture containing 2% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C for 30 min was optimal for chromosome preparation since good preparations, with all 20 chromosomes relatively extended and well-spread without cytoplasm, were observed. There were 11.2 preparations in large leaves (8 to 10 mm long and 3 to 4 mm wide) and 4.2 preparations in small leaves (6 to 8 mm long and 1 to 2 mm wide). Chromomycin A_3 (CMA)-positive (+) bands were noted in the telomeric positions of eight chromosomes. 4′-6-diamidino-2-phenylindole (DAPI)-negative bands (−) corresponded to CMA+ bands. The numbers and positions of CMA+ bands were the same in the two cultivars examined: 'Florida Sweet' and 'Sanmi-kei (Hosoba).' The methods developed in the present study are promising for further cytogenetic studies in acerola.

Discipline: Horticulture
Additional key words: Barbados cherry, CMA, DAPI, EMA, West Indian cherry

Introduction

Chromosome analysis is important for genetic and biotechnological studies, which include breeding, genome analysis, phylogeny, and taxonomy in agricultural crops. For this purpose, it is necessary to obtain clearly defined chromosome samples and identify each chromosome. Hence, advanced chromosome analysis methods such as enzymatic maceration, air-drying (EMA), and fluorescent staining have been studied.

The development of EMA (Fukui & Mukai 1988) was a major breakthrough in chromosome observation. Compared with the conventional squash method, the main advantage of the sample prepared by EMA is that chromosomes are free of cytoplasmic debris and spread well on glass slides, allowing chromosomes with fine structures to be obtained. In particular, this method is extremely useful for obtaining good preparations from plants with small chromosomes (Fukui 1996), such as many fruit trees (Yamamoto 2007, 2012). On the other hand, although root tips are common materials for preparing chromosome samples in plants (Fukui 1996), the genotype of root tips derived from seedlings is not identical to that of the original (mother) plant in fruit trees because of their heterozygosity. Thus, vegetative tissue such as a leaf is a desirable material for accurate cytological studies in fruit trees.

In karyological analysis, discrimination of morphologically similar chromosomes should be essential. For that purpose, fluorescent banding using a base-specific binding fluorochrome is effective. Guanine-cytosine (GC)-specific chromomycin A_3 (CMA) and adenine-thymine (AT)-specific 4′-6-diamidino-2-phenylindole (DAPI) banding methods are reliable and useful for identifying chromosomes of various plants (Schweizer 1976, Kondo & Hizume 1982, Hizume 1991). The CMA banding pattern of chromosomes revealed chromosome configuration and identification (Yamamoto 2007) as well as offered valuable information for ploidy breeding (Yamamoto & Tominaga 2004, Yahata et al. 2005).

Acerola (synonyms: West Indian cherry, Barbados cherry, and Puerto Rico cherry) (*Malpighia glabra* L., synonyms: *M. punicifolia* L. and *M. emarginata* DC) is a

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Received 9 August 2024; accepted 16 December 2024; J-STAGE Advanced Epub 30 April 2025.
https://doi.org/10.6090/jarq.24J12

tropical fruit tree that originated from Central America. It has well-known commercial potential as its fruit contains high levels of ascorbic acid (vitamin C) and polyphenols, known as functional components (Asenjo & Guzman 1946, Moscoco 1956, Hanamura et al. 2005, 2008). For the above-mentioned chromosome studies, EMA and CMA/DAPI staining are essential to advance knowledge on genetic resources and breeding in acerola. However, these kinds of studies have not progressed, although the number of chromosomes (2n = 2x = 20) and small size have been reported using conventional techniques such as the squash method and Feulgen staining (Mondin et al. 2010).

Therefore, it is important to establish EMA for acerola. In the present study, 1) a chromosome preparation method using young leaves of acerola was developed, and 2) CMA/DAPI staining was performed using the chromosome samples obtained by EMA.

Materials and methods

1. Plant materials

Potted acerola trees at the Faculty of Agriculture, Kagoshima University (Kagoshima, Japan) were used. Young leaves at the bud burst stage were used as materials. They were collected between 10:00 and 12:00 in July and August 2021. Fresh young leaf samples were immersed in 2 mM 8-hydroxyquinoline at 15°C for 4 h in the dark, fixed in methanol-acetic acid (3:1), and stored at –20°C.

2. Enzyme maceration/air-drying (EMA) and Giemsa staining

'Florida Sweet' was used as the material. In EMA, two durations of enzyme treatment (30 or 40 min) were tested. The experiment was performed in six replicates. Approximately 6 to 8 mm long and 1 to 2 mm wide leaves were cut into approximately 2-mm^2 sections for EMA. EMA was performed as described by Ohmido & Fukui (1996) with minor modifications (Yamamoto et al. 2010b). The young leaves were washed in distilled water to remove the fixative and macerated in an enzyme mixture containing 2% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), and 1 mM EDTA, pH 4.2, at 37°C. Chromosomes were stained with 2% Giemsa solution (Merck Co., Germany) in 1/30 M phosphate buffer (pH 6.8) for 15 min, rinsed with distilled water, air-dried, and then mounted with xylene. After confirmation of each chromosome's position on the slide under a microscope (Nikon Eclipse 80i, Japan), the chromosomes were destained with 70% methanol.

Next, the leaf samples were categorized into two types: approximately 6 to 8 mm long and 1 to 2 mm wide (small leaf), and 8 to 10 mm long and 3 to 4 mm wide (large leaf). The experiment was performed in nine replicates. The conditions of EMA and Giemsa staining were the same as described above. The duration of enzyme treatment was 30 min.

3. Fluorescent staining

'Florida Sweet' and 'Sanmi-kei (Hosoba)' were used as the materials. Fluorescent staining was performed according to the method of Hizume (1991) with minor modifications. The slides were preincubated for 30 min in McIlvaine buffer (6.6 mM citric acid and 88.2 mM Na_2HPO_4, pH 7.0) and treated with 0.1 g·L^{-1} distamycin A in the buffer for 10 min. The slides were incubated for 10 min in buffer containing 5 mM $MgSO_4$ and then stained for 60 min with 0.2 g·L^{-1} CMA in buffer containing 5 mM $MgSO_4$. The slides were also incubated for 10 min in buffer containing 5 mM $MgSO_4$ and then stained for 30 min with 0.2 mg·L^{-1} DAPI in the buffer. The slides were incubated for 10 min in the buffer and then mounted using Vector Shield (Vector Laboratories, USA). Chromosomes stained with CMA and DAPI were observed under a fluorescence microscope (ECLIPSE 80i) with a microscope digital camera (DP74, Olympus Co., Japan) using BV and UV filter cassettes, respectively. From the preparation stained with DAPI, five cells were selected for use in determining the chromosome length.

Results

1. Enzyme maceration/air-drying (EMA)

The duration of enzyme treatment was examined. Although good preparations, with all 20 chromosomes relatively extended and well-spread without cytoplasm, were observed with both durations (30 and 40 min), chromosomes were sometimes lost with the 40-min treatment (Fig. 1) due to excessive duration. The number

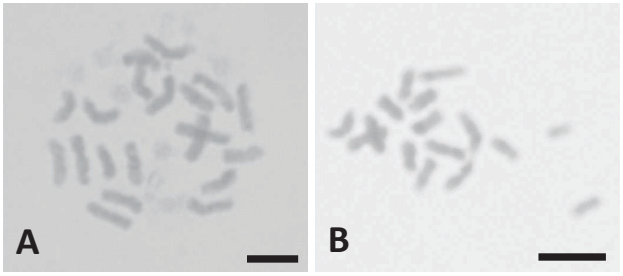

Fig. 1. Example of Giemsa-stained chromosomes prepared by enzymatic maceration and air-drying (EMA) in acerola 'Florida Sweet' (2n = 2x = 20)
A: 30 min, and B: 40 min
The bar represents 5 μm.

Table 1. Effect of duration of enzyme treatment on observed number of chromosome preparations per slide derived from young leaves[z] of acerola

Duration of enzyme treatment (min)	Number of chromosome preparations per slide
30	3.7 ± 1.1
40	1.7 ± 1.2
Significance[y]	n. s.

[z] Young leaf = 6 - 8 × 1 - 2 mm
[y] t-test

Table 2. Effect of type of sample on the observed number of chromosome preparations per slide derived from young leaves of acerola

Type of sample[z]	Number of chromosome preparations per slide
Small	4.2 ± 1.1
Large	11.2 ± 2.0
Significance[y]	**

[z] Small: young leaf = 6 - 8 × 1 - 2 mm
Large: young leaf = 8 - 10 × 3 - 4 mm
[y] t-test

of chromosome preparations in which 20 chromosomes can be clearly observed per slide was 3.7 and 1.7 in 30 and 40 min, respectively (Table 1).

The type of sample affected the number of chromosome preparations per slide (Table 2). There were 11.2 preparations observed in large leaves and 4.2 preparations in small leaves. Thus, there was a significant difference between large and small leaves.

2. Fluorescent staining

When CMA staining was performed, eight out of 20 chromosomes exhibited CMA-positive (+) bands, chromosome regions showed bright fluorescence with CMA, and the remaining twelve chromosomes had no CMA+ or negative (–) bands in either 'Florida Sweet' or 'Sanmi-kei (Hosoba)' (Fig. 2A, C). All eight CMA+ bands were located in the telomeric regions of the eight chromosomes. These eight CMA+ bands corresponded to DAPI– bands, chromosome regions showing pale fluorescence with DAPI. No DAPI+ band was observed in either cultivars (Fig. 2B, D).

Table 3 shows the relative length of each of the 20 chromosomes of 'Florida Sweet' and 'Sanmi-kei (Hosoba)'. The relative length and chromosome type of both cultivars were similar. The relative lengths of the former and latter chromosomes ranged from 6.3% to

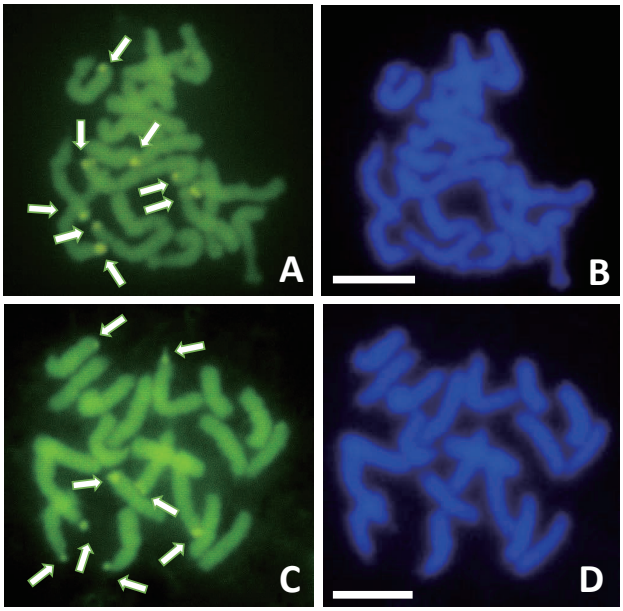

Fig. 2. CMA- and DAPI-stained chromosomes of acerola
A and B: 'Florida Sweet'; C and D: 'Sanmi-kei (Hosoba)'
A and C: stained with CMA; B and D: stained with DAPI
Arrows indicate CMA-positive bands.
The bar represents 5 μm.

Table 3. The relative length (% of the total length) of each of the 20 chromosomes derived from young leaves of acerola

Relative length (%)	
Florida Sweet	Sanmi-kei (Hosoba)
Chromosome with CMA+	
6.3 ± 0.1	6.1 ± 0.1
6.1 ± 0.2	5.8 ± 0.2
5.6 ± 0.1	5.6 ± 0.3
5.3 ± 0.2	5.0 ± 0.2
5.0 ± 0.1	4.8 ± 0.2
4.8 ± 0.1	4.6 ± 0.1
4.2 ± 0.1	4.4 ± 0.1
3.4 ± 0.4	4.1 ± 0.1
Chromosome without CMA+	
6.3 ± 0.1	6.2 ± 0.3
5.7 ± 0.0	5.8 ± 0.2
5.4 ± 0.1	5.6 ± 0.2
5.3 ± 0.1	5.3 ± 0.2
5.1 ± 0.1	5.2 ± 0.1
4.9 ± 0.1	5.0 ± 0.1
4.9 ± 0.1	4.9 ± 0.1
4.8 ± 0.1	4.7 ± 0.1
4.6 ± 0.1	4.6 ± 0.0
4.5 ± 0.1	4.4 ± 0.1
4.3 ± 0.1	4.1 ± 0.2
3.4 ± 0.4	3.7 ± 0.3

3.4% and 6.2% to 3.7%, respectively. That of the eight chromosomes with CMA+ bands of the former and latter chromosomes ranged from 6.3% to 3.4% and 6.1% to 4.1%, respectively.

Discussion

The present study established EMA and fluorescent staining in acerola chromosomes. These results provide fundamental information on acerola chromosomes and will contribute to the progress of studies on breeding, genetic resources, and cytogenetic studies.

Although chromosome samples of fruit trees are typically derived from the root tips (Yamamoto 2012), the genotype of root tips derived from seedlings is not identical to that of the original (mother) plant because of their heterozygosity. Although root tips derived from cutting or tissue culture are considered to show the true genotype, it takes time and effort to obtain them. Thus, vegetative tissue such as a leaf is a desirable type of material for accurate cytological studies. Therefore, a chromosome preparation method using young leaves of acerola was developed in the present study.

In EMA, elucidation of the appropriate enzyme composition and duration to obtain good chromosome samples is necessary. Based on the results of the present study, the optimum conditions of enzyme treatment for acerola were clarified: enzyme mixture containing 2% Cellulase Onozuka RS, 1.5% Macerozyme R200, and 0.3% Pectolyase Y-23 for 30 min. The number of chromosome samples obtained was influenced by leaf size. Large leaves (8 to 10 mm long and 3 to 4 mm wide) yielded many chromosome preparations. There was a large and significant difference between large and small leaves. Thus, using an adequate leaf size is necessary for conducting chromosome studies in acerola efficiently.

This is the first report on EMA chromosome sample preparation in acerola. Compared with a previous report (Mondin et al. 2010) adopting the Feulgen method, which involves conventional chromosome sample preparation, EMA chromosome samples were relatively extended. EMA was suitable for acerola, which, like other fruit trees, has small chromosomes. These chromosome samples are adequate for further studies, such as fluorescent staining and fluorescent *in situ* hybridization (FISH) (Fukui et al. 1994).

Fluorochrome staining analysis revealed that acerola has eight chromosomes with telomeric CMA+/DAPI− bands. The characteristics of the stains used are as follows: CMA and DAPI are GC- and AT-specific, respectively. The CMA+/DAPI− bands are considered to be GC-rich regions of chromosomes. These chromosomes with CMA+/DAPI− bands could be readily distinguished from other chromosomes without observing CMA+/DAPI− bands. This is the first report on CMA+/DAPI− bands in acerola. Previous studies reported that CMA+ bands generally corresponded to 18S-5.8S-25S ribosomal RNA gene (rDNA) sites (Yamamoto et al. 1999, 2012, 2019, 2020). The rDNA sites are also considered to be located in CMA+ regions of acerola.

'Florida Sweet' and 'Sanmi-kei (Hosoba)' showed the same CMA/DAPI banding pattern. However, 'Sanmi-kei (Hosoba),' which is mainly used as a rootstock in Japan, could be clearly distinguished from other accessions for fruit production by DNA analysis (Ito et al. 2014). From the results, the divergence of chromosome configuration may be very low or nonexistent in view of the fluorescent banding pattern in acerola, as in the pear, pineapple, pitaya, and *Prunus* (Yamamoto et al. 2010a, Yamamoto 2012, Yamamoto et al. 2019, 2020). The relative length of 20 chromosomes in 'Florida Sweet' and 'Sanmi-kei (Hosoba)' were similar to that of an unknown acerola cultivar (Mondin et al. 2010). This also indicates the low diversity of chromosome configuration within species in acerola. Since the chromosome length variations were continuous, identification of individual chromosomes was difficult. However, the identification of chromosomes with or without CMA+ was straightforward. This demonstrates the usefulness of fluorescent staining in chromosome studies.

Fluorescence *in situ* hybridization (FISH), which detects the location on a chromosome of any gene, and genomic *in situ* hybridization (GISH) using whole DNA as a probe, are very powerful tools for genome analysis (Le et al. 1989, Fukui et al. 1994). However, these were not carried out in the present study. In some fruit trees, chromosome preparation relatively extended and well-spread without cytoplasm by EMA is suitable for both fluorescent staining and FISH (Yamamoto et al. 1999, 2009, 2010b, 2012, 2019, 2020). Hence, the EMA method developed in the present study is considered to be applicable to FISH and GISH as well.

In conclusion, the appropriate conditions for enzymatic maceration and CMA/DAPI staining in acerola chromosomes were demonstrated. The present study provided fundamental information on the chromosomes of acerola. These results are considered to contribute to the progress of breeding and genetic resources studies of this important tropical fruit tree. Using this information and technique, extensive work on karyotyping, polyploidy breeding, and elucidation of diversity based on the chromosome configuration will progress in acerola and *Malpighia*. In general, the optimum conditions for EMA and fluorescent staining

are the same in related species or genera (Yamamoto et al. 2016). Thus, the methods developed in the present study also apply to plants belonging to *Malpighia*, as both diploid (2x = 20) and tetraploid (4x = 40) forms exist (Lombello & Forni-Martins 2003). In addition, the author has attempted to produce acerola tetraploids since tetraploid fruits tend to be larger because of their larger cell size. The methods developed in the present study will be effective for confirmation of the ploidy of candidate tetraploids.

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