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Cytological Distinctions Between *Timun Suri* and Cucumber Discovered by Fluorescence In Situ Hybridization (FISH) Using 45S Ribosomal DNA Gene

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ABSTRACT

The genus *Cucumis* including *timun suri*, melon and cucumber, is an important horticultural crop of flowering plants. The dispute of *timun suri* terminology is leading to false-positive results in the nomenclature of *timun suri* in Indonesia. Although molecular research on these species has been widely conducted, detailed information and precise evidence based on the molecular cytogenetic approach are poorly investigated. The FISH technique was applied to reveal the cytological distinctions among these species. Here, this research conducted physical mapping of 45S ribosomal DNA (rDNA) on six accessions of *Cucumis*. The chromosome number of cucumber is 14 chromosomes, while *timun suri* and melon are 24 chromosomes. The number of 45S rDNA loci in cucumber, melon and *timun suri* was highly conserved. All cucumber accessions in this study had nine to ten 45S rDNA loci with strong and weak signal intensities located at proximal regions of the short arms. In melon and *timun suri* the signals of two pairs of 45S rDNA were located at the terminal and interstitial regions of the short arms. These were clear shreds of evidence to confirm that timun suri was cytogenetically closed to melon instead of cucumber. It confirmed that timun suri cannot be classified as a cucumber.

INTRODUCTION

The genus *Cucumis* is one of the valuable genera in the *Cucurbitaceae* family. It includes cucumber and melon, two of the most economically valuable and widely cultivated crops in the world (Pitrat, Chauvet, & Foury, 1999). Cucumber (*Cucumis sativus* L.) is a diploid species having 14 chromosomes and well-known as *mentimun* or *timun* in Indonesia (Han et al., 2008). *Timun suri* (local name - Indonesia), based on its terminology, could be classified as a cucumber. However, that reason cannot be scientifically justified. *Timun suri* was closely related to melon (*Cucumis melo* L.) depending on its morphology, isozyme, and RAPD markers rather than to cucumber (Huda & Daryono, 2013; Rahayu & Hartana, 2002).

In addition, the fruit morphology of *timun suri* in Indonesia was closely related to *Cucumis melo* subsp agrestis var. momordica same as that has been reported by Manohar & Murthy (2012). Furthermore, the chromosome number of *C. melo* subsp agrestis var. momordica has been confirmed 24 chromosomes (Setiawan, 2018). Despite those results, the morphology, isozyme, and RAPD have some limitations, particularly morphological traits and isozyme which were affected by the environmental factor. In addition, poor reproducibility of RAPD has been informed by Penner et al. (1993) and Skroch & Nienhuis (1995).

In order to get more insights and evidence to precisely distinguish between *timun suri*, cucumber, and melon, this research has physically mapped the 45S rDNA gene using FISH and compare its chromosomal distribution between those accessions. Physical mapping of 45S rDNA provides remarkable information on the genetic relationships among the

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closely related species in several genera such as *Fabaceae, Poaceae, Solanaceae,* and *Cucumis* (Acosta, Moscone, & Cocucci, 2016; Amosova et al., 2017; Li et al., 2016; She et al., 2015; Zhang et al., 2016). 45S rDNA gene is a tandem repeat sequence and consisting of the 18S, 5.8S, and 25S ribosomal RNA (rRNA) (Ganal & Hemleben, 1986). This rRNA, together with several classes of the protein complex, established the translation factory of protein synthesis (Neves et al., 2005).

The number of rDNA loci and their location in chromosomes have great variations in *Cucumis* species, particularly between melon and cucumber which is clearly distinguishable and can be utilized to clarify and discriminate the cytological marker of these two species in addition to chromosome number (Setiawan, 2018). The number of 45S rDNA loci in melon are conserved two loci and located at Nucleolar Organizing Region (NOR) either in the terminal or interstitial region of the short arms (Li et al., 2016; Liu et al., 2010; Setiawan, 2018; Zhang et al., 2015; Zhang et al., 2016), whereas in cucumber, they were varied from eight to ten loci depending on cultivars (Chen, Staub, Adelberg, & Jiang, 1999; Koo et al., 2010; Zhao et al., 2011).

FISH can be used to check the location and abundance of a DNA sequence through the hybridization of a labeled DNA probe to DNA template in chromosomes (Schwarzacher & Heslop-Harrison, 2000). It can be applied for the identification of chromosomes homologous for karyotyping and phylogenetic analysis, direct physical mapping of sequences on the chromosome, and for studying the recent evolutionary process in the genome (Jiang & Gill, 2006; Kato, Lamb, & Birchler, 2004). Therefore, FISH is a useful technique to determine the position of 45S rDNA in chromosomes and to compare its chromosomal distribution in *timun suri*, cucumber, and melon. This research provides precise evidence based on the molecular cytogenetic approach to distinguish between *timun suri* and cucumber. It is clear that *timun suri* is classified as melon with nomenclature name *Cucumis melo* subsp. agrestis var. momordica and has four 45S rDNA loci on somatic metaphase chromosomes.

Molecular research on *timun suri* has been widely conducted, but detailed information and precise evidence based on the molecular cytogenetic approach are poorly investigated. The aim of this research was to evaluate the cytogenetic relativity of *timun suri* and cucumber using the 45S ribosomal DNA gene.

MATERIALS AND METHODS

Plant Materials

One melon accession (NI19), two cucumber landraces including Okute Aodai and RAR 930024, and two accessions of *timun suri* i.e., US 205 and IPB TS1 were used for cytological studies. The seed materials were provided by Dr. Kenji Kato (Okayama University), The National Agriculture and Food Research Organization (NARO) Gene Bank of Japan, and Vegetable Crop Research Institute (Balitsa, Indonesia). (Table 1). The seeds were germinated and maintained at 25°C in the growth chamber. The research was conducted at the laboratory of genetic and plant breeding, Chiba University from March until September 2018.

Table 1. The materials used in this study and their chromosomal features

Cultivar	Accession No	Species	Origin	Status	Chro. Number (2n)	Number of 45S rDNA loci		45S rDNA location
						Strong	Weak	
Litsa Hijau	-	*Cucumis sativus* L.	IDN	Open pollinated	14	7	3	Prox (SA)
Okute Aodai	32268	*Cucumis sativus* L.	JPN	Landrace	14	6	3	Prox (SA)
RAR 930024	82368	*Cucumis sativus* L.	USSR	Landrace	14	6	4	Prox (SA)
NI19	940281	*Cucumis melo* L.	TCD	Landrace	24	2	2	Ter & Inter (SA)
US 205	PI 182952	*Cucumis melo* L. Ssp. agrestis var Momordica	IND	Landrace	24	2	2	Ter & Inter (SA)
IPB TS1	-	*Cucumis melo* L. Ssp. agrestis var Momordica	IDN	Open pollinated	24	4	0	Ter & Inter (SA)

Remarks: Chro. = chromosome; Prox = Proximal region; Ter = Terminal region; Inter = Interstitial region; SA = Short arms; IDN: Indonesia; JPN: Japan; USSR: Uzbekistan, Kazakhstan, and Kyrgyzstan area; TCD: Chad; IND; India

Chromosome Preparations

Timun suri, cucumber, and melon seeds were germinated on filter paper using distilled water, kept in petri dishes in a growth chamber at 25ºC. The main root tips were cut (0.5-1 cm). The mitotic and meiotic chromosomes were prepared in accordance with Setiawan, Teo, Kikuchi, Sassa, & Koba (2018) protocol. Modified Carnoy's solution II was used for the mitotic and meiotic chromosomes fixation method. In brief, both of flower buds and root tips were pretreated with 6:3:1 (v/v) ethanol: acetic acid: chloroform for 3-4 hours at room temperature and transferred into 3:1 (v/v) ethanol: acetic acid solution at 4°C for 5 days. The samples were washed for 10 minutes in 1 ml of enzyme buffer (40 ml of 100 mM citric acid + 60 ml of 100 mM sodium citrate, pH 4.8). The anthers were dissected from flower buds under a stereomicroscope using the forceps and the root tips were cut with a razor blade. Both of them were macerated in 15 µl of the enzyme cocktail containing 1% Pectolyase Y-23 (Kyowa Chemical, Osaka, Japan), 2% Pectinase (Sigma), and 4% Cellulose Onozuka RS (Yakult) at 37°C for 1 hour. The enzymes surrounding the roots and anthers were cleaned off using Kimwipe tissue. The squeezing and air-dry method was used for mitotic and meiotic chromosome preparations, respectively, as same as described in Setiawan, Teo, Kikuchi, Sassa, & Koba (2018).

The Probes and FISH Procedure

45S rDNA sequence (pTa71) of wheat was used as the probes (Gerlach & Bedbrook, 1979). The 45S rDNA was cloned in pGEM-T-Easy Vector (Promega), and the plasmid DNA was extracted using HiYield™ Plasmid Kit (RBC Bioscience) in accordance with the manufacturer's instructions. Therefore, the dig-nick translation mix (Roche) was used for labeling the 45S rDNA. The FISH analysis was performed as same as described in Setiawan, Teo, Kikuchi, Sassa, & Koba (2018) method, consisting of pretreatment using RNAse A (Qiagen) and pepsin (Sigma) for chromosome slides, 1% paraformaldehyde for refixation, hybridization process including denaturation at 80°C for 2 minutes, and probe detection using anti-digoxigenin rhodamine (Roche).

Image Analysis

The chromosome slides were stained with 4,6-diamidino-2-phenylindole (DAPI) mounted in a VectaShield antifade solution (Vector Laboratories). The observation was conducted using a fluorescence microscope (Olympus BX53) equipped with a cooled CCD camera (Photometrics CoolSNAP MYO). The processing and editing of FISH images were conducted using Metamorph, Metavue imaging series version 7.8, and Adobe Photoshop CS 6, respectively.

RESULTS AND DISCUSSION

The Conservation of 45S rDNA in Cucumber, Melon, and *Timun Suri*

The 45S rDNA probe was hybridized on the metaphase chromosomes of six *Cucumis* accessions. All cucumber accessions had 14 chromosomes (Fig. 1a, 1d, 1g). Three accessions of cucumber had variation in 45S rDNA loci consisting of 9 to 10 loci with different signal intensities. Seven strong and three weak signals of 45S rDNA were detected in Litsa Hijau (Fig. 1b-c). Three pairs of strong and three weak signals of 45S rDNA were discovered in Okute Aodai (Fig. 1e-f). While RAR 930024 had three pairs of strong and two pairs of weak signals (Fig. 1h-i), and some of the cucumber cultivars had been reported to have 8 rDNA loci (Chen, Staub, Adelberg, & Jiang, 1999; Lou et al., 2014; Setiawan, 2018).

The presence of eight to ten 45S rDNA loci in wild and domesticated cucumber accessions from different locations implies that the amplification of 45S rDNA loci was occurred before domestication. The different number of 45S rDNA loci in *Phaseolus vulgaris* accessions or in the same genera of *Passiflora, Sellaginella,* and *Paspalum* has been reported to have variation in 45S rDNA loci (3 to 9 loci) (De Melo & Guerra, 2003; Marcon, Leão Barros, & Guerra, 2005; Pedrosa-Harand et al., 2006; Vaio, Speranza, Valls, Guerra, & Mazzella, 2005). There are three possible mechanisms of 45S rDNA variation: (1) alteration of chromosome morphology caused by the amplification of satellite DNA without changing the order of other sequences Navrátilová, Neumann, & Macas (2003); (2) translocation, a part of chromosomal rearrangement, have been reported to shift the position of rDNA loci in *Lotus japonicas* (Hayashi et al., 2001); (3) dispersion-amplification-deletion model is proposed to be the responsible for the variation of 45S rDNA loci (Dubcovsky & Dvorák, 1995).

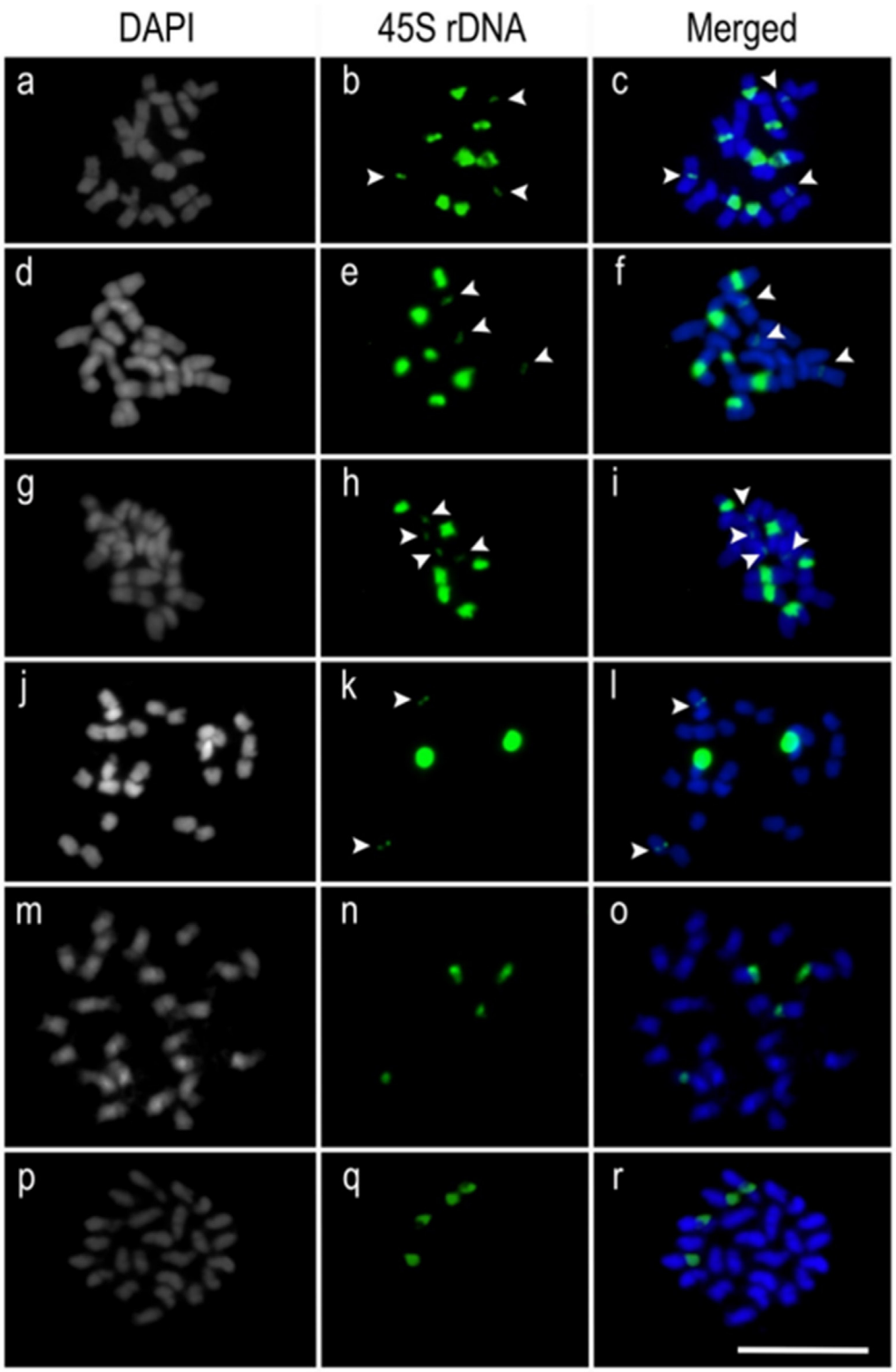

Fig. 1. Physical mapping of 45S rDNA in cucumber, melon, and *timun suri* accessions. Litsa Hijau (a-c); Okute Aodai (d-f); RAR 930024 (g-i); melon NI19 (j-l); *timun suri* US 205 (m-o); *timun suri* IPB TS1 (p-r). 45S rDNA labeled with digoxigenin (green). Weak signals of 45S rDNA (arrowheads). Scale bars = 10 μm

Although, how the dispersion occurred has not been clear yet, but this mechanism can explain the change in the number of rDNA loci and the dispersion of other repetitive DNA sequences in a genome. Several studies reported that the movement of 45S rDNA is controlled by mobile elements (Raskina, Belyayev, & Nevo, 2004a; 2004b). A recent study has revealed that the LINE element, a non-long terminal repeat (LTR) retrotransposon, has been inserted into 45S rDNA arrays in cucumber accessions, and those accessions had eight to ten of 45S rDNA loci (Setiawan, 2018). Therefore, retrotransposons are thought to cause the variation of 45S rDNA loci in cucumber.

NI19, a melon accession, showed 24 chromosomes with a pair of strong and weak 45S rDNA signals (Fig. 1j-l). US 205 and IPB TS1, two *timun suri* accessions derived from India and Indonesia, respectively, had the same chromosome number (2n = 24) as well as a pair of strong and weak 45S rDNA signals (Fig. 1m-r), and the interphase cells showed that US 205 had two pairs of 45S rDNA signals (Fig. 2a-c). These results are similar as has been reported by (Li et al., 2016; Liu et al., 2010; Zhang et al., 2016). In addition, all *Cucumis* accessions showed different signal intensities of 45S rDNA. The strong and weak of 45S rDNA signals are related to their copy number (Li, Fu, Hu, Huang, & Song, 2006). Gong et al. (2013) reported that weak signals in *Jatropha curcas* indicate the presence of transcriptionally inactive 45S rDNA. Therefore, cucumber has more active 45S rDNA than melon and *timun suri*. The results provide clear evidence that *timun suri* is completely had cytological distinctions in comparison with cucumber. Therefore, a word of *timun* in *timun suri* cannot be justified and classified as cucumber. In fact, *timun suri* is cytogenetically close to melon, and it was classified as *Cucumis melo* L. ssp. agrestis var. momordica.

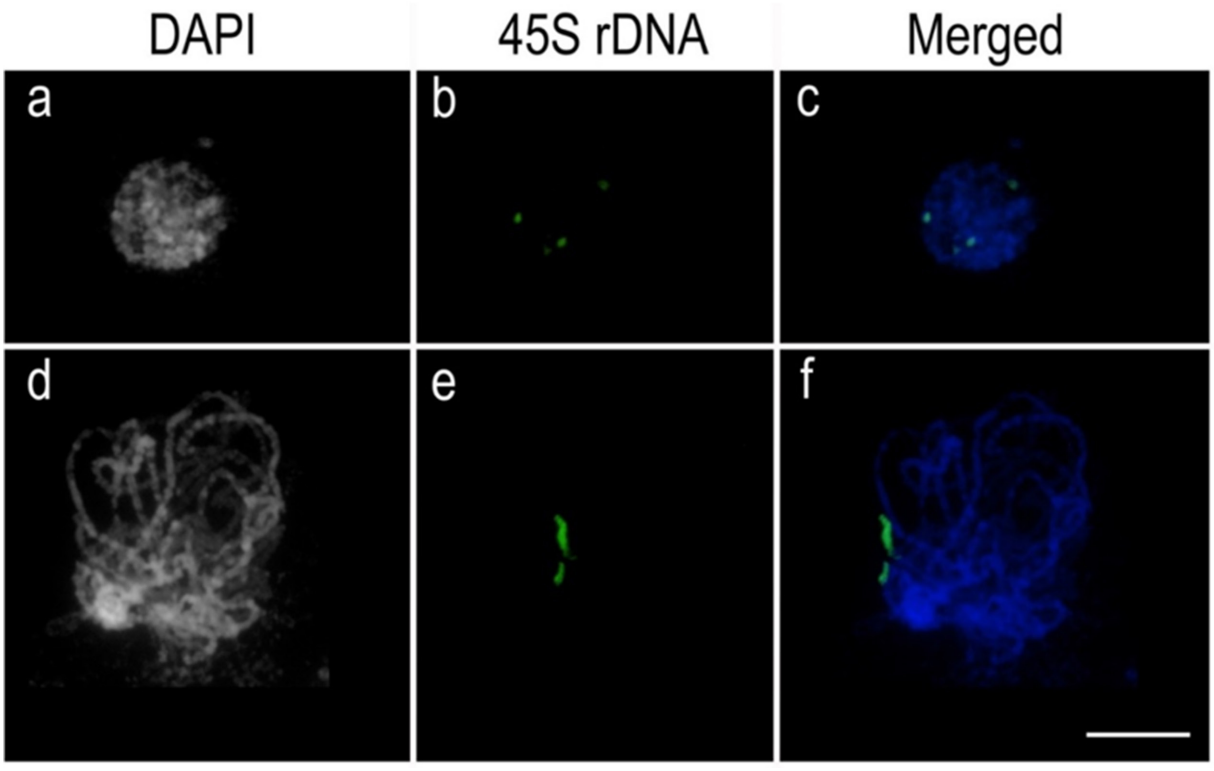

Fig. 2. Interphase and pachytene chromosomes of *timun suri* US 205. Physical mapping of 45S rDNA in meiotic Interphase (a-c) and pachytene (d-f) chromosomes. 45S rDNA labeled with digoxigenin (green). Scale bars = 10 μm

Chromosomal Distribution of 45S rDNA in *Timun Suri*, Cucumber, and Melon

FISH results on cucumber metaphase chromosomes in Litsa Hijau, Okute Aodai, and RAR 930024 showed that all 45S rDNA signals including strong and weak signals were located at proximal regions (Fig. 1c, 1f, 1i). These were coincidence with previously reported results (Han et al., 2008; Koo et al., 2010; Lou et al., 2014). Whereas in NI19, US 205, and IPB TS1, the strong and weak signals of 45S rDNA were located at terminal and Interstitial regions of the short arms (Fig. 1l, 1o, 1r), respectively. These locations of 45S rDNA are similar to that have been reported by Zhang et al. (2016).

Higher-resolution FISH of 45S rDNA was detected in the pachytene chromosome (Fig. 2d-f), and it was confirmed that *timun suri* (US 205) had one pair of 45S rDNA in pachytene cell. The homologous recombination constraint may be the cause of the location of 45S rDNA at the terminal region in melon. Therefore, the allelic and non-allelic homologous recombination may cause an important role in the homogenization of intralocus and interloci of 45S rDNA repeat (Roa & Guerra, 2012; Wendel, Schnabel, & Seelanan, 1995). Various location of 45S rDNA was not only found in plant but also in animal and this variation had no detrimental effects for the meiotic segregation and gene stability (Cabrero & Camacho, 2008; Hanson et al., 1996; Pedrosa-Harand et al., 2006).

***Timun Suri* is Cytogenetically Close to Melon**

It is clear that *timun suri* and cucumber have different cytogenetical traits including chromosome number and 45S rDNA loci (Fig. 1 and Table 1). *Timun suri* is classified as a melon group with different nomenclature i.e. *Cucumis melo* ssp. agrestis var. momordica, while cucumber is closely related to *Cucumis hystrix* L. (Sebastian, Schaefer, Telford, & Renner, 2010). Although the chromosome number between cucumber and *C. hystrix* is different, a fertile amphidiploid from interspecific hybridization, *C. hystivus* (2n = 4x = 38), between cucumber and *C. hystrix* has been successfully developed (Chen et al., 2003; Chen et al., 2004; Chen, Staub, Adelberg, Lewis, & Kunkle, 2002; Chen, Staub, Tashiro, Isshiki, & Miyazaki, 1997). Since *timun suri* is closely related to melon, it is possible to conduct the breeding for fruit quality in *timun suri*. Ripe fruit of *timun suri* generally has the major problem of fruit cracking that can cause significant loss of marketable revenue and yield (Singh et al., 2015). In addition, the sugar content or sweetness is lower than melon (Dhillon et al., 2007). Therefore, it is necessary to conduct the breeding program for *timun suri* to increase its fruit quality and marketable in the future.

CONCLUSION

Timun suri and cucumber are two different species that have significantly cytogenetic differences between them. Physical mapping of 45S rDNA in *timun suri*, cucumber, and melon successfully discovered the fundamental evidence to distinguish among these accessions. *Timun suri* breeding program for fruit quality may be improved due to the close cytogenetic traits between melon and *timun suri*.

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