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Genetic characterization of banana clones grown in Turkey based on nuclear DNA content and SRAP markers

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Summary

This study was conducted to investigate the genetic relationships among banana clones grown in Turkey based on their nuclear DNA contents and SRAP markers. Four banana clones including 'Dwarf Cavendish', 'Grand Nain', 'Azman' and local 'Erdemli' were used as plant material. Nuclear DNA content of the banana cultivars was estimated by flow cytometer and varied between 1.766 pg ('Erdemli') and 2.028 pg ('Grand Nain'). 'Azman' contained 1.973 pg and 'Dwarf Cavendish' 1.936 pg nuclear DNA. Genetic similarities of 4 banana clones were between 0.63-0.91 based on SRAP molecular markers. The local 'Erdemli' banana clone was the most distinct from the others. In conclusion, there is a high level of genetic variation among Erdemli and other banana clones grown in Turkey and the local clone 'Erdemli' is the most distinct one. This study showed that nuclear DNA content analysis together with molecular markers could be useful to assess the relationships among banana clones.

Introduction

Fruits have long been valued as part of a nutritious and tasty diet. The flavours provided by different fruit species are among the most preferred in the world, and it is increasingly evident that fruits not only taste good, but also good for human health (Bacvonkralj et al., 2014; Rop et al., 2014).

Bananas and plantains belong to the genus *Musa* and they are the most significant food and cash crops worldwide, especially in tropical and sub-tropical regions. World total annual banana production is around 102 million tons (FAO, 2013). The genus *Musa* is divided into two sections: *Musa* and *Callimusa*; the *Musa* section includes 33 species (Hakkinen, 2013). The section contains triploid varieties evolved from two wild diploids ($2n = 2x = 22$) as of seminiferous species, *M. acuminata* Colla (genomes AA) and *M. balbisiana* (genomes BB). In general, bananas consist of two major categories as of those consumed as dessert (constituting about 43% of world production) and cooking bananas (constituting the remaining 57% of world production) (Jones, 2000).

Some banana clones can be cultivated in subtropical regions between 20° and 30° north and south of the equator. Several subtropical regions protected from cold weather, such as the Mediterranean coastline, are also suitable for growing bananas. Banana has been grown in some subtropical parts of Turkey since 1934 (Gubbuk et al., 2004) and annual banana production is increasing each year and has reached 210.000 tons in Turkey (FAO, 2013).

Several direct or indirect methods have been used to identify the genomes in *Musa*. These include molecular markers, isozymes, retrotransposons, *in situ* hybridization and flow cytometry.

A number of techniques have been used to evaluate the level of genetic variability in *Musa* via molecular markers, including Random Amplified Polymorphic DNA (RAPD) (Das et al., 2009), Restriction Fragment Length Polymorphism (RFLP) (Hippolyte et al., 2010), Amplified Fragment Length Polymorphism (AFLP) (Opara et al., 2010), Inter Simple Sequence Repeats (ISSR) (Lu et al., 2011), Sequence Related Amplified Polymorphism (SRAP) (Phothipan et al., 2005) and Simple Sequence Repeats (SSR) markers (Miller et al., 2010). Sequence Related Amplified Polymorphism (SRAP) was first used in Brassicaceae for marker development and mapping (Li and Quiros, 2001). Subsequently this system has been used in many fruit crops such as citrus (Uzun et al., 2009), apricot (Uzun et al., 2010) etc. for genetic diversity and fingerprinting studies because it is a simple and also efficient marker system that can be adapted for a variety of purposes in different crops, including map construction, gene tagging, genomic and cDNA fingerprinting and map based cloning (Li and Quiros, 2001).

Flow cytometry is among the most common methods used to assess numerous cellular characteristics and to analyze cells individually (Loureiro et al., 2006). The method was considered as the fastest and the most reliable technique (Suda and Travnicek, 2006). The method was initially developed for human cells but then was adapted for plant cells as a reliable tool for estimation of nuclear DNA content and ploidy level constitutions in plants (Dolezel et al., 1994; Tuna et al., 2006; Ozkan et al., 2010; Tiryaki and Tuna, 2012). Dolezel et al. (1994) modified the technique for analysis of nuclear genomes of bananas and plantains. The method has been employed in finding out the DNA nuclear contents of various banana varieties since the early 1980s (Dolezel, 1991).

D'Hont et al. (1999) carried out a study with flow cytometry method to assess genetic differences between *Musa* genomes and reported significant differences between the sizes of *Musa* A, B, S and T genomes. He was able to indicate that S and T genomes were larger than A and B genomes and T genome was the largest one. The information provided by the researchers was then employed by the other researchers as a significant diagnostic tool to assess the differences between *Musa* genomes and it was reported in previous studies that DNA content of AA genome accessions varied between 1.22-1.27 pg (Lysak, 1999) or between 1.20-1.33 pg (Kamate, 2001). Arumuganathan and Earle (1991) also reported the similar DNA content estimations for triploid accessions with the estimations of Lysak (1999) and Kamate (2001). Lysak et al. (1999) determined DNA at a level of 1.81 pg in a triploid accession.

In this study, nuclear DNA contents of 'Dwarf Cavendsish', 'Grand Nain' and local clones 'Azman' and 'Erdemli', grown in Turkey were determined for the first time through flow cytometry and also genetic relationships among these cultivars were assessed by SRAP marker.

Materials and methods

Plant material

International 'Dwarf Cavendish' and 'Grand Nain', local 'Azman' and 'Erdemli' banana clones were used as the plant material of the

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present study. All plants were grown in protected areas of open fields and selected from a commercial orchard in Erdemli, Mersin. Some yield and leaf characteristics such as finger length (cm), finger weight (g), plant height (cm), number of hands (bunch^{-1}), number of fingers (hand^{-1}), bunch weight (kg/plant), leaf petiole length (cm) and leaf petiole width (cm) were measured by using ten plants from each clone as shown in Tab. 1. Data were analyzed statistically via JMP 7.0 (Copyright © 2007 SAS Institute Inc.).

DNA isolation

Total genomic DNA was extracted from fresh leaf tissues of four banana clones for molecular analysis by using the CTAB procedure as described by DOYLE and DOYLE (1990). DNA concentration was measured with a microplate spectrophotometer (BioTek Instruments, Inc., Vinooski, USA), and 10 ng/mL DNA templates were made using TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) buffer.

SRAP analysis

A total of 19 SRAP primer combinations were used for all banana clones (Tab. 2). PCR reaction components and PCR cycling parameters were as described by UZUN et al. (2009). PCR products were separated on 2% agarose gels in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) at 115 volts for 3 h. The fragment patterns were photographed under UV light for further analysis. A 100 bp standard DNA ladder was used to confirm the appropriate markers for SRAP analysis. Three replications were used to confirm markers. Molecular analysis was carried out as follows: each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software (ROHLF, 2000). A similarity matrix was constructed using SRAP data based on Dice's coefficient. The similarity matrix was used to construct a dendrogram using UPGMA (unweighted-pair group method with arithmetic average) to determine genetic relationships among the cultivars studied. The genetic similarity matrix and ultrametric distance matrix produced from the UPGMA-based dendrogram with COPH module nested in the same software was compared using Mantel's matrix correspondence test (MANTEL, 1967).

Nuclear DNA content

Nuclear DNA content was carried out by a flow cytometer (CyFlow® Space, Partec Münster) equipped with a 30 mW 532 nm laser at the Field Crops Department of Namık Kemal University. Propidium iodide (PI) was used as a fluorochrome to stain the DNA. Suspensions of intact nuclei were prepared using a Partec commercial kit (CyStain PI Absolute P). Briefly, the procedure consists of chopping 40 mg of banana leaf tissue using a sharp razor blade together with 20 mg of rice leaf tissue as internal standard in a petri dish containing 0.5 ml extraction buffer. The crude nuclear suspension was then transferred to a labelled test tube through 50-micron nylon mesh. 1.5 ml of staining buffer was then added to the test tube and the samples were stored at 4 °C for approximately 60 min in dark. PI fluorescence emitted from nuclei was collected through a 560 nm long-pass filter and a 630 nm band-pass filter. For each sample, fluorescence data were acquired from at least 3000 nuclei. Three independent replications were performed and histograms with coefficient of variation (CV) above 5.75% were rejected. The formula used for converting fluorescence values to DNA content was: Nuclear DNA content = (mean position of unknown peak)/(mean position of known) X DNA content of known standard (Rice, Osmancik cultivar).

Results and discussion

Yield and leaf characteristics

Yield is the most important criterion for banana production. Some yield and leaf characteristics of 4 banana clones grown in Turkey are provided in Tab. 1. 'Grand Nain' had the highest values with regard to finger length, finger weight, hand number, and bunch weight; 'Erdemli' had the lowest value with regard to all these characteristics. 'Azman' and 'Grand Nain' clones were similar to each other, but 'Dwarf Cavendish' clones had lower values when compared with 'Azman' and 'Grand Nain' with regard to plant height and the other horticultural characteristics (Tab. 1).

SRAP analysis

The number of morphological markers for identifying the clones of banana is limited and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of traits (SMITH and SMITH, 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationship studies in plant species (REDDY et al., 2002).

In this study, genetic relationships among four banana clones grown in Turkey since 1934 were evaluated by using SRAP molecular markers. Nineteen SRAP markers gave polymorphisms ratio from 25 to 100% (Tab. 2). Genetic similarities of 4 banana clones were between 0.63-0.91 (Tab. 3). Local 'Erdemli' clone was found to be the most distant from the other clones (Fig. 1). Morphologically, this clone had short and thick fruits compared with the other clones (Tab. 1). There is no information about the clone when it was brought to Turkey. Most probably the 'Erdemli' clone had a different origin from the other bananas. 'Grand Nain' and 'Azman' were genetically closer each other than 'Dwarf Cavendish' with ~0.91 similarity ratio. 'Azman' has similar plant height (~5 m) to 'Grand Nain'. Also these two clones have the same fruit characteristics (Tab. 1). Genetic similarity

Tab. 1: Some yield and leaf characteristics of four banana clones

Clones	FL	FW	PH	NH	NF	BW	LPL	LPW
'Grand Nain'	21.4a	151.7a	380.0a	16.3a	26.0a	49.0a	252.1a	115.3a
'Azman'	21.2a	134.7ab	356.7a	15.2a	24.3a	44.7a	261.1a	107.1ab
'Dwarf Cavendish'	19.0b	116.7b	220.7b	14.7a	24.0a	38.3b	204.7b	100.1b
'Erdemli'	14.1c	80.4c	382.0a	9.7b	17.3b	17.0c	192.4b	80.9c
LSD	2.2	23.8	84	2.4	3.4	13.5	32.9	10.3

Note: Means with the same letters within columns are not significantly different at $P < 0.05$.
FL: Finger length (cm), FG: Finger weight (g), Plant height (cm), NH: Number of hands (bunch^{-1}), NF: Number of fingers (hand^{-1}), BW: Bunch weight (kg/plant), LPL: Leaf petiole length (cm), LPW: Leaf petiole width (cm)

Tab. 2: Observed polymorphism with 19 SRAP primer combinations in 4 banana clones.

Primer Combinations	Total bands	Polimorphic bands	% Polimorphism
Em14-Me3	2	1	50.0
Em2-Me5	3	2	66.7
Em1-Me1	6	3	50.0
Em2-Me1	7	7	100.0
Em3-Me2	6	3	50.0
Em6-Me3	6	3	50.0
Em3-Me3	6	4	66.7
Em4-Me3	3	3	100.0
Em6-Me2	10	6	60.0
Em6-Me11	6	3	50.0
Em7-Me10	10	7	70.0
Em8-Me4	5	5	100.0
Em10-Me7	4	4	100.0
Em11-Me6	4	1	25.0
Em12-Me9	3	2	66.7
Em14-Me4	6	3	50.0
Em16-Me3	6	3	50.0
Em3-Me4	4	3	75.0
Em8-Me8	5	4	80.0
Mean	**5.37**	**3.53**	**66.32**
Total	**102**	**67**	

Tab. 3: Similarity index of 4 banana clones obtained by UPGAMA analysis using SRAP molecular marker data

Clones	'Grand Nain'	'Azman'	'Dwarf Cavendish'	'Erdemli'
'Grand Nain'	1.0000			
'Azman'	0.9143	1.0000		
'Dwarf Cavedish'	0.8630	0.8876	1.0000	
'Erdemli'	0.6102	0.6131	0.6575	1.0000

of 'Azman' and 'Dwarf Cavendish' (0.88) was less than to 'Azman' and 'Grand Nain' (0.91) (Tab. 3). The lowest genetic similarity was observed between 'Grand Nain' and 'Erdemli'. BROWN et al. (2009) made molecular study on bana and reported that 'Grande Nain' (AAA), 'Williams' (AAA), and 'Dwarf Cavendish' (AAA) were in the same cluster. LOH et al. (2000) stated that the differences in phenotypic characters might not be due to euploidy differences but rather due to allelic differences in single or multiple genes in banana. They found a similar discrepancy among some of the banana clones. The cv. Dwarf Cavendish, Grand Nain, and Raja Udang, which were designated by SIMMOND (1966) as having the AAA genomic constitution based on morphological characterization, clustered separately.

Previously genetic similarities or differences of different banana clones have been determined by using different molecular marker techniques, but there are a few reports on SRAP molecular markers. SRAP markers were successfully used to analyze the genetic relationship among 29 accessions of banana in AA, AAB and BB groups (PHOTHIPAN et al., 2005). YOUSSEF et al. (2011) used SRAP and AFLP to analyze the genetic variation and relationships among forty *Musa* accessions (commercial cultivars and wild species of Musa). SRAP had threefold more specific and unique bands than AFLP. SRAP markers are demonstrated to be effective tools for discriminating amongst *M. acuminate*, *M. balbisiana* and *M. schizocarpa* in the *Musa* section, as well as between plantains and cooking bananas within triploid cultivars. VALDEZ-OJEDA et al. (2014) aimed to determine the genetic variability within 71 accessions of Musa (wild species and cultivars of different subgroups) using SRAP marker. The used accessions were consistently identified and separated by SRAP markers. A total of 330 polymorphic bands were detected using 12 primer combinations. The genetic similarity between accessions ranged between 0.44 and 0.97. They reported that SRAP marker system is useful to identify closely related accessions in the genus Musa and facilitated the recognition of duplicates to be eliminated and clarified uncertainties or mislabeled banana accessions introduced to the collection. MATTOS et al. (2010) characterized banana accessions from Brazil using agronomical, physical and physicochemical characteristics of fruit as well as simple sequence repeats (SSR) markers. Thirteen microsatellite primers revealed an average of 7.23 alleles, which showed high variability. LU et al. (2011) identified genetic similarity of different cultivars and monitored somaclonal variations of banana during rapid mass micro propagation using Inter Simple Sequence Repeat (ISSR) primers and found about 85.1% of total polymorphism. In another study, genetic similarities ranged from 0.3 to 1.0 with an average of 0.51 using Random Amplified Polymorphic DNA (RAPD) markers with 27 accessions in the Mauritian *Musa* germplasm (BROWN et al., 2009). RETNONINGSIH et al. (2011) used microsatellite markers to classify 116 banana accessions and placed 73 accessions in AA/AAA and AAA genomic

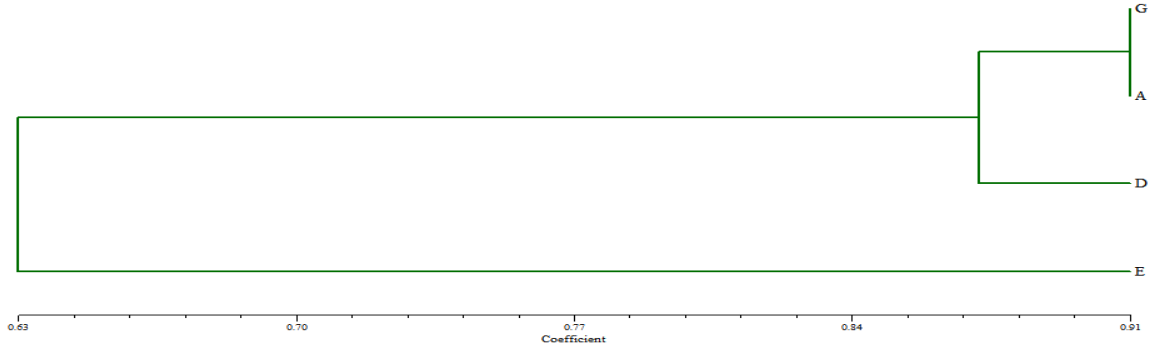

Fig. 1: Dendrogram of 4 banana clones using UPGMA method obtained from SRAP markers, E: 'Erdemli', A: 'Azman', D: 'Dwarf Cavendish', G: 'Grand Nain'.

groups, 2 accessions in BB genomic group, 21 accessions in AAB genomic group and 20 accessions in ABB genomic group. The researchers also, reported 99 accessions as unique genotypes and the remaining 17 accessions as synonyms. In the same study, 116 accessions were separated into two main clusters with a similarity of 0.13 on UPGMA analysis. JAIN et al. (2007) investigated the genetic relationships among four banana clones (Grand Naine, Red Banana, Nendran and Rasthali) commonly cultivated in south India through Random Amplified Polymorphic DNAs (RAPDs). Researchers indicated 43.47 % of the amplification products as monomorphic (common to all genotypes) and 30.43 % as unique; but only 26.08 % was able to reveal the genetic relationships among the investigated clones. The present findings about the genetic similarities between 'Dwarf Cavendish' and 'Grand Nain' clones comply with the results of GRAJEL-MARTIN et al. (1998), SHOSEYOV et al. (1996) and GUBBUK and PEKMEZCI (2001). Similar to current findings, GRAJEL-MARTIN et al. (1998) reported high similarity among Lacatan, Williams, Petit Nain, Grand Nain, Robusta and Valley, which belong to the Cavendish subgroup (AAA). SHOSEYOV et al. (1996) investigated genetic similarities among banana cultivars and indicated low polymorphism among Grand Nain, Williams and Natha banana clones and somaclonal variants of these cultivars. GUBBUK and PEKMEZCI (2001) used RAPD markers and reported the genetic similarity 'Dwarf Cavendish' and 'Grand Nain' as 0.929. PINAR et al. (2015) conducted a study on estimate genetic relationships and natural somatic mutations among selected banana genotypes from different region of Turkey via SRAP molecular markers. Ninety-six banana genotypes including 39 Dwarf Cavendish, 18 Grand Nain, 28 Azman and 11 other bananas were evaluated. Their study showed that there was high level of variation among the cultivars. In the present study, SRAP technique was demonstrated to be a useful tool in determining genetic diversity in 4 banana clones grown in Turkey.

Evaluation of nuclear DNA content of four banana clones using flow cytometry

Flow cytometry is the common method employed in estimation of nuclear DNA content of bananas (NOVAK, 1992). In this study, mean nuclear DNA contents of four banana clones were evaluated by flow cytometer and are presented in Fig. 2, 3, 4 and 5. Rice (Osmancik cultivar) used as an internal standard. Results showed that 'Grand Nain' had the highest 2C nuclear DNA content (2.028 pg) while 'Erdemli' had the lowest (1.766 pg). 'Dwarf Cavendish' (1.936 pg) was close to 'Azman' (1.976 pg) in nuclear DNA content (Tab. 4). 'Erdemli' clones separated from the other clones by nuclear DNA content as well as by molecular data (Fig. 1, 2, 3, 4, 5).

There is limited information in literature on nuclear DNA content and genome sizes of *Musa* cultivars (ARUMUGANATHAN and EARLE,

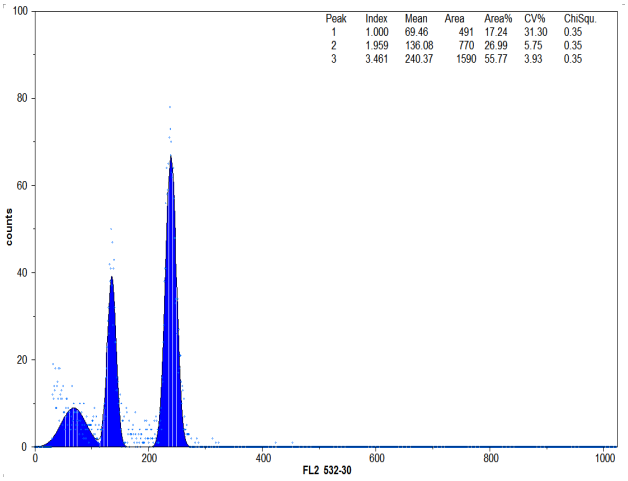

Fig. 2: Relative positions of the G1 peaks of 'Erdemli' banana clone and rice (first peak)

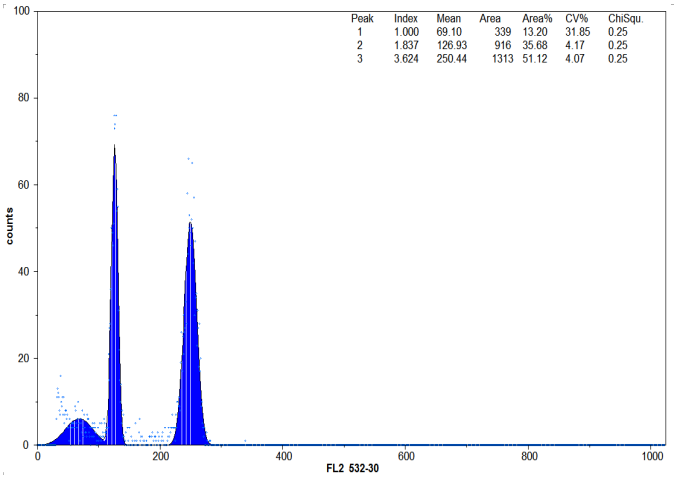

Fig. 3: Relative positions of the G1 peaks of 'Azman' banana clone and rice (first peak)

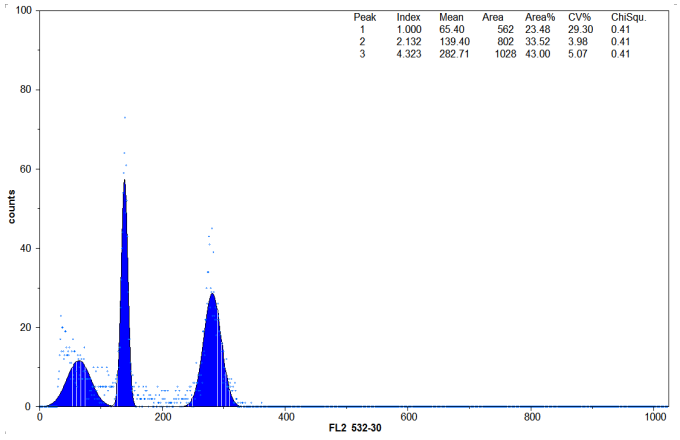

Fig. 4: Relative positions of the G1 peaks of 'Grand Nain' banana clone and rice (first peak)

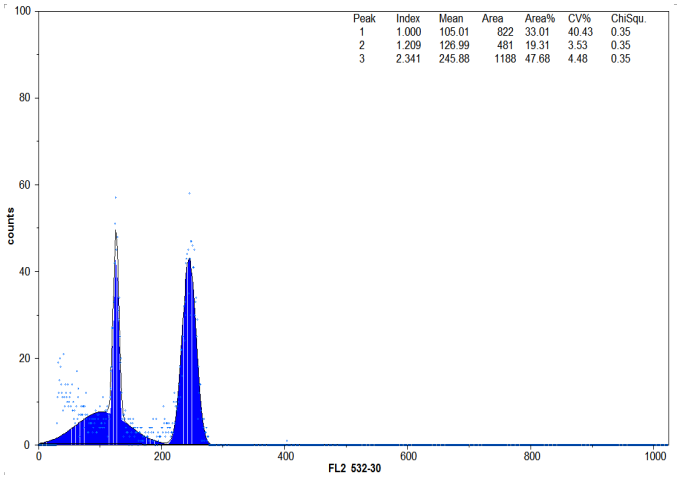

Fig. 5: Relative positions of the G1 peaks of 'Dwarf Cavendish' banana cultivar and rice (first peak)

Tab. 4: Nuclear DNA content of 4 banana cultivars based on internal standards of rice

Name of the variety	Genomic group	2C DNA Content (pg)
Grand Nain	AAA	2.028±0.008a
Azman	Unknown	1.973±0.011b
Dwarf Cavendish	AAA	1.936±0.007c
Erdemli	Unknown	1.766±0.015d
LSD		0.04

1991; KAMATE et al., 2001). Of the available studies, two (LYSAK et al., 1999; KAMATE, 2001) of them included accessions with only A and B genomes and only one involved plants with S and T genomes (D'HONT et al., 1999). ARUMUGANATHAN and EARLE (1991) presented total 2C DNA content (1.81 pg) and genome size (873 Mbp) in *Musa* for the first time. Their estimations on DNA content were similar to ones provided by KAMATE et al. (2001) for triploid accessions. LYSAK et al. (1999) in a previous study reported 2C DNA content of a triploid accession as 1.81 pg. With regard to individual genomes of *Musa*, B genome with 2C values of between 1.03-1.16 pg was reported to have the least nuclear DNA content (LYSAK et al., 1999; KAMATE et al., 2001). The average 2C value of A genome was estimated to be 1.25 pg (KAMATE et al., 2001), 1.11 pg (D'HONT et al., 1999) and 1.27 pg (KAMATE et al., 2001). Such findings reveal significant differences (about 15%) in DNA contents of A and B genomes. A triploid accession with two B genomes (ABB) was expected to have less DNA than an accession with one B genome (AAB). But the case was not as expected in LYSAK et al. (1999) and KAMATE et al. (2001). The genome size of Simili Radjah (ABB) was identical to that of a tetraploid and 11% difference was identified in DNA content of subspecies of the *M. acuminata* complex (KAMATE et al., 2001). KAMATE et al. (2001) also indicated wider range in DNA contents of cultivars with similar genomes; while the DNA contents of AAB clones were between 1.61-1.79 pg and the DNA contents of ABB clones varied between 1.70-2.23 pg. In the present study, the difference between the 'Erdemli' clone and 'Dwarf Cavendish' clone was 9%. The difference between 'Erdemli' and 'Grand Nain' was 13% and the difference between 'Erdemli' and 'Azman' was 10.5%. Such findings revealed that 'Grand Nain', 'Dwarf Cavendish' and 'Azman' were placed in the same group (AAA) because of close 2C DNA contents, 'Erdemli' probably carried 1 B genome together with 2 A genomes (AAB) or ABB because of lower 2C DNA content. Similarly in previous studies, LYSAK et al. (1999) reported nuclear DNA content of Obino l'Ewai (AAB) as 1.777 pg, Maritu (AAB) as 1.847 pg and Pelipita (ABB) as is 1.751 pg. CIZKOVA et al. (2013) reported nuclear DNA content in AAB as 1.786 pg and ABB as 1.814 pg in banana. The differences in 2C DNA contents of AAA and AAB were between 6.5-8.3% and the difference in 2C DNA within this genome group was only 1.9%. The difference in 2C DNA within AAB (5.5%) was found to be significant. The largest difference in genome variation (8.9%) was observed AA. Variation in 2C nuclear DNA content within both AA and BB genomes had significant contributions to existing differences within and between AAA and AAB genomic groups (ONYANGO et al., 2008). While the variation in 2C DNA contents of triploid AAB or ABB genomic groups was found to be significant, the variation within AAA group was not significant. DOLEZEL (2001) reported nuclear DNA content of Agbagba (AAB) as 1.737 pg, Pelipita (ABB) as 1.751 pg, Gross Michel (AAA) as 1.881 pg and Grand Enano as 1.905 pg. BARTOS et al. (2005) reported relatively small but significant differences (3.4%) between *M. acuminata* accessions. LYSAK et al. (1999) and DOLEZEL et al. (1994) reported that A and B genomes of *Musa* were different in size. LYSAK et al. (1999) indicated that B genome was generally significantly smaller (about 12%) than A genome but the reserachers were not able to detect any variations among the *M. balbisiana* cultivars. In this study, 'Erdemli' banana (ER) clone was the most distinct genotype with regard to SRAP molecular data and nuclear DNA content. This clone has different fruit and plant architecture than 'Grand Nain', 'Dwarf Cavendish' and 'Azman' as well (Tab. 1). 'Erdemli' is also more tolerant to cold weather than the other clones. As previous studies suggested, this clone may have an AAB or ABB genome because 'Erdemli' has a lower DNA content than the other banana clones grown in Turkey. 'Azman' clone has DNA content close to 'Grand Nain', and had similar fruit and plant characteristics, although 'Azman' plant height is slightly greater than 'Grand Nain' (Tab. 1). Also 'Azman' clone DNA content is close to Dwarf Cavendish clone. But there were some differences between their fruit and plant characteristics. 'Grand Nain', 'Dwarf Cavendish', and 'Azman' banana clones likely belong to the AAA genome group.

Conclusion

In conclusion, bananas were brought to Turkey as ornamentals in 1934 and subsequently put into commercial production in open fields. 'Dwarf Cavendish' was the first clone used in commercial production. After that, growers started to use 'Grand Nain' and 'Dwarf Cavendish'. 'Azman' and 'Erdemli' are local clones but their names and origins are unknown. In this study, especially, 'Azman' and 'Erdemli' banana clones were determined to be different from 'Dwarf Cavendish' and 'Grand Nain' according to SRAP molecular markers, some yield and leaf characteristics and nuclear DNA contents.

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