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RESEARCH ARTICLE

Genetic and nuclear DNA content variation of stevia (*Stevia rebaudiana* Bertoni) accessions grown in Turkey

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ABSTRACT

The objectives of this study were to make morphological and molecular characterization of Turkish stevia accessions. The field data showed significant variations ($P < 0.05$) for all morphological parameters (plant height, number of main stems per plant, number of secondary stems, fresh and herb yields) exception with number of secondary stems. The primers of *MtP5CS, AtNHX1, MtProDH, Mt-Actin MtSOS1* and *MtSOS2* genes were used as loci specific DNA markers. All markers were amplified in *Stevia* genome and provided a mean of 74.16% polymorphism rate. Flow cytometer analysis showed no statistically significant differences ($P < 0.16$) for 2C DNA content with a mean of 1.62 pg. The UPGMA analysis revealed that Samsun accession was distinctly separated from the others and had the lowest 2C DNA content (1.52 pg). This study first time showed that the stevia accessions grown in Turkey have limited genetic and no nuclear DNA content variations. The results also revealed that gene targeted functional markers associated with salt and drought tolerance have a great potential to be used as DNA markers to determine the genetic variation in plants.

Keywords: Nuclear DNA content; Gene-targeted functional markers; Genetic relataionship; Sweet herb

INTRODUCTION

Stevia rebaudiana Bertoni belongs to the family of Asteraceae and is grown as a perennial medicinal plant (Soejarto et al. 1982). The plant is commonly known as sweet herb, candy leaf, honey yerba and sweet leaf (Carakostas et al. 2008). It was first discovered by Dr. Moises Santiago Bertoni in 1888 in Paraguay and was scientifically named as *S. rebaudiana* by Dr. Rebaudi in 1905 (Gupta et al. 2013; Singh and Rao, 2005). As a short-day plant, stevia plant grows optimally in tropical and subtropical climates, with temperatures ranging from -6 to 43°C (average of 23°C) and sandy soils with a pH range of 6.5 - 7.5 (Kaur et al. 2015). The leaves of *Stevia rebaudiana* accumulates important secondary metabolites such as steviolbioside, stevioside, rebaudioside A-F, rebaudioside M, dulcoside A and rubusoside (Mohd-Radzman et al. 2013; Adari et al. 2016; Lopez et al. 2016; Ameer et al. 2017; Latha et al. 2017). Those secondary metabolites have functional properties and beneficial effects on human health with some medical applications such as antibacterial, antifungal, anti-inflammatory, antiviral, anti-yeast, cardiotonic, diuretic anti-HIV, anti-tumor, hepatoprotective properties (Lopez et al. 2016; Mohd-Radzman et al. 2016; Latha et al. 2017). Therefore, stevia plant has been introduced to many countries all around the world, including Turkey due to its potential use for alternative medicinal purposes. The first adaptation study of stevia plant in Turkey dated back to 2009 in Antalya and then has been successfully adapted to other regions of Turkey including Black Sea and Aegean regions (Turgut et al. 2015).

Open pollination behavior of the stevia species creates the phenotypic variability within the population and the phenotypic differences present in the stevia populations are not suffiecient to separate them into a valid taxonomic variety (Tateo et al. 1998; Yadav et al. 2011). Plant characterization based on morphological properties are also not sufficient to determine genetic diversity within and among plant species since many of those properties are affected by growth stage and several environmental factors (Hunter, 2018). On the other hand, DNA-based markers which are not influenced by plant growth

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stage or environment provide invaluable help to assess genetic relationships (Hunter, 2018; Kumar, 1999) and diversity analysis while flow cytometry helps to determine nuclear DNA content and ploidy level (Vlacilova et al. 2002; Dolezel et al. 2004). Not only transferability of molecular markers among the species has been studied (Raveendar et al. 2015) but new alternative molecular marker techniques have also been developed in diverse plant species (Silva et al. 2014; Poczai et al. 2013; Wang et al. 2015). The recent advancements in sequencing technologies opened new era to determine functional genes in various plant species (Chai et al. 2017; Abbasi et al. 2015; Chung et al. 2013; Liu et al. 2014). These efforts made available new and alternative molecular markers generated based on untranslated regions of expressed sequence tags (ESTs) called as gene-targeted markers (GTMs) (Poczai et al. 2013) or the gene markers involved in variation of phenotypic traits due to their functional gene sequences called as gene-targeted functional markers (GTFMs) (Arnholdt-Schmitt, 2005).

Although popularity of stevia increases, its large-scale commercialization is still limited due to unavailability or lack of planting materials because of no viable or very low seed germination along with its poor seedling establishment (Tamura et al. 1984; Galo, 2019). Therefore, vegetative propagation such as stem cutting is a cheap and better alternative method to grow as annual or perennial transplanted crops (Singh and Rao, 2005). Although there are several accessions of stevia grown in different parts of Turkey, their genetic diversity and ploidy levels are not known yet. Therefore, the present study first time aimed to reveal agronomic parameters of *Stevia rebaudiana* accessions under Çanakkale conditions, and to determine genetic diversity as well as nuclear DNA content variation by using loci specific DNA markers and flow cytometer analysis.

MATERIALS AND METHODS

A field experiment was carried out in 2016 in Dardanos Research and Application Station of Faculty of Agriculture at Çanakkale Onsekiz Mart University, Çanakkale (40° 4'25.82"K, 26°21'49.63"D).

Plant materials

The seedlings of stevia (*Stevia rebaudiana*) accessions with 3-4 leaves were obtained from Atatürk Central Horticultural Research Institute (ACHRI), Yalova, Turkey. Since the seedlings of each accession were previously cloned by ACHRI, the possible genetic variation within each accession was eliminated. All stevia accessions distributed in Turkey and presented for reproduction in ACHRI by naming them according to their locations of origin namely Adana, Antalya, Bilecik and Samsun were used in this study (Fig. 1).

Field experiments and plant cultivation

The field experiment was conducted during the growing season of 2016 at Dardanos Research and Application Station in Çanakkale, Turkey (40° 4'25.82"K, 26°21'49.63"D) (Fig. 1). The soil of the experimental field was clay loam texture, medium in lime, low in salt and alkaline. The 0-20 cm layer of soil had low concentrations of organic material, sufficient amount of potassium and phosphorus. Average monthly temperature and precipitation in 2016 were given in Fig. 2 (Anonymous, 2016). The seedlings with 3-4 original leaves were planted on May 13, 2016 in a completely randomized block design with three replications. Each plot consisted of four rows with a total of 112 plants, 28 plants for each accession. The row and row spacing were 0.65 x 0.45 m and there was a 1 m gap between plots, respectively (Turgut et al. 2015). The experimental field was fertilized at planting with 10 kg/da by ammonium nitrate. After planting, the

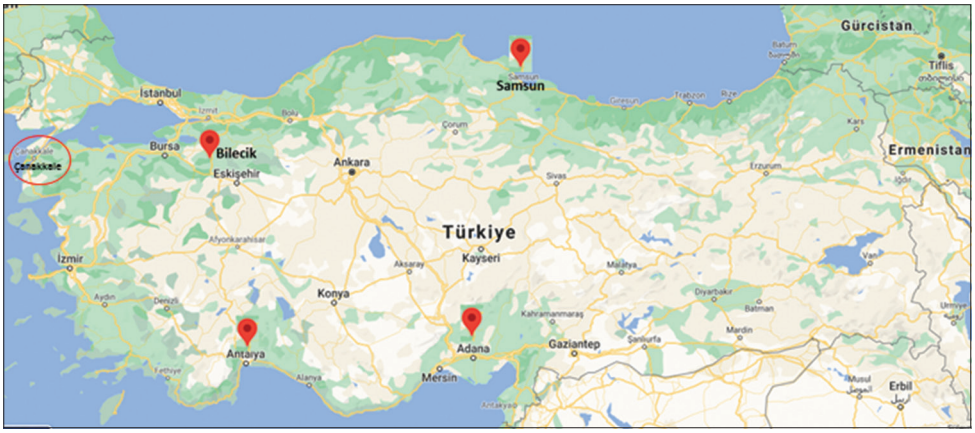

Fig 1. Distribution and locations of *Stevia rebaudiana* accessions used in the study. The field experiment was carried out in the city of Canakkale which was circled on the map.

plants were equally irrigated by a drip irrigation system when needed. Weed control was done by hand and no pesticide was applied. Outer two plant rows and 50 cm from plot heads were excluded. To determine agronomic parameters, six randomly chosen plants from the center of a plot were manually harvested on September 3, 2016, and the plant height (cm), the number of main stems per plant, the number of secondary stems per plant, fresh and dry herb yields (g/plant) were determined.

DNA extraction and quality control

The DNA extraction kit (Favorgen, Pingtung, Taiwan) was used to obtain genomic DNA from a bulk of 4 young leaves of each accession as the manufacturer instructed. The DNA quality and concentrations were determined on 1 % agarose-gel and the equalized concentrations were used in PCR analysis.

Gene targeted functional markers and PCR amplification

Six gene-targeted markers (GTMs) related to drought and salt tolerance given in Table 1 were used for genetic diversity analysis. The best annealing temperature of each primer pairs was optimized by using a gradient PCR (Thermo Fisher Scientific, Inc., USA) in *Stevia* genome before they were used in PCR amplifications. The PCR reaction mixture consisted of 100 µM dNTPs; 10 mM Tris-HCl, pH 8.8; 2.0 mM $MgCl_2$; 20 mM $(NH_4)_2SO_4$; 1 unit of *Taq* DNA polymerase (Invitrogen); and 20 ng of genomic DNA (Kang et al. 2002). Two steps PCR cycles were used; 5 pre-cycles of 1 min at 95 ºC for denaturing, 45 second at 35 ºC for annealing and 30 second at 72 ºC for extension and then another 35 cycles consisted with 15 second at 95 ºC for denaturing, 1 min at the annealing temperatures (Ta) primer pairs given on Table 1, 1 min at 72 ºC for extension and 7 min at 72 ºC for final extension. PCR amplicons were resolved in a 2% (w/v) agarose gel in 1×TBE buffer by (Bio-Rad) at constant 80 V for 2-3 h and were visualized under UV light source after agarose gel was stained with ethidium bromide (2 µl/100 ml) before visualized under UV light source.

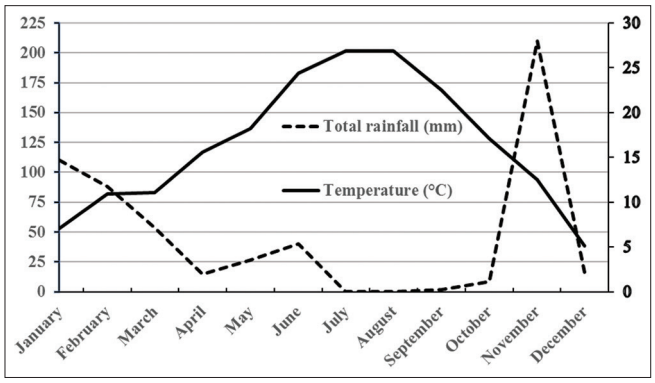

Fig 2. Average monthly temperature and precipitation of Çanakkale in 2016.

Determination of nuclear DNA content

A commercial kit (CyStain PI absolute P) contained propidium iodide as fluorescent dye was used to isolate nucleus of accessions as the manufacture instructed in the flow cytometry (Partec CyFlow^R Space) analysis. *Solanum lycopersicum* cultivar Rio was used as internal standard. About 40 and 20 mg of young leaves of each accession and internal standard were co-chopped into small pieces by using a razor blade on a petri dish contained 500 µl of nuclei extraction buffer to have intact nuclei suspensions, respectively. The suspensions were then transferred into a glass tube through 30 µl Cell Tris filter before the samples were incubated for 1 hour at 37 °C in 2000 µl of staining buffer for running in flow cytometer. The nuclear DNA contents of samples were calculated by using G1 peak

Table 1: Salt tolerance related genes used as gene targeted functional markers in the study. The name of genes, references, forward (F) and reverse (R) primer sequences, annealing temperatures, the number of amplicons, the number of polymorphic amplicons, polymorphism rates (%) and polymorphism information content (PIC) values.

Gene	Reference	F/ R primer (5'-3')	Annealing temperature (Ta ˚C)	No. of total amplicons	No. of polymorphic amplicons	Polymorphism rate (%)	PIC
MtSOS1	(Liu et al. 2015)	GCTGACTTTCCCGTATG TGGCACCCAGTTCTTTC	48	4	3	75.0	0.37
MtSOS2	(Liu et al. 2015)	CCGTGGTATCTTCTGTT CAAGGGTTAGGTGTATT	48	5	3	60.0	0.25
AtNHX1	(Yokoi et al. 2002)	CAACACCCCAAAATCCATAC ATATCCCTTTGTTGGACCAA	52	5	3	60.0	0.56
MtProDH	(Planchet et al. 2014)	CCAACGTCCACGCTGATAAGA ACAGGTCCTATAGCCGTTGCA	57	2	1	50.0	0.25
MtP5CS	(Quan et al. 2015)	GAGAGGGAACGGCCAAGTG CAGATCCTTGTGTGTATA	52	3	3	100.0	0.12
MtActin	(Wang et al. 2017)	ACGAGCGTTTCAGATG ACCTCCGATCCAGACA	50	4	4	100.0	0.56
Mean				3.8	2.8	74.16	0.35
Total				23	17	-	-

means of sample and internal standart (Cil and Tiryaki, 2016). Three biological replications of each accession were used in the study.

Statistical analyses

Statistically significant differences of agronomic parameters and 2C DNA content data were determined by using ANOVA of SAS program (SAS, 1997). Fisher's least significant difference (LSD) test was performed for mean separation if the F test was significant at $P < 0.05$. The PCR amplicons of primer pairs were scored based on the presence (1) or the absence (0) of the alleles. The polymorphism information content (PIC) of primer pairs was calculated by using $PIC= 1-\sum(Pij)^2$ formula, where Pij is the frequency of the i^{th} band revealed by the j^{th} primer, P(ij) is summed for all the bands of each primer (Dawson et al. 1996; Powell et al. 1996). Dice coefficient was used to get genetic similarity matrix (Dice, 1945) and the dendrogram was constructed based on the unweighted pair-group with arithmetic mean (UPGMA) method by using Numerical Taxonomy Multivariate Analysis System (NTSYSpc-2.1).

RESULTS

Agronomic parameters

Significant variations ($P< 0.05$) were determined for agronomic parameters among 4 stevia accessions with one exception (Table 2). No significant difference was determined for the number of secondary stem (Table 2). The accession Bilecik had the highest values for all agronomic parameters measured. The plant height of the accessions ranged from 59.1 cm (Antalya) to 71.4 cm (Bilecik) with an average of 63.28 cm. The highest fresh herb yield was obtained from Bilecik (329.5 g/plant), while accession Adana had the lowest (168.1 g/plant). The average fresh and dry herb yields of accessions were determined as 240.68 g/plant and 68.23 g/plant, respectively.

Genetic diversity and nuclear DNA content

All primers of loci specific DNA markers amplified in *Stevia* genome and provided a mean of 74.16% polymorphism rate (Fig. 3; Table 1). The highest polymorphism rate (100%) was obtained from *MtP5CS* (*Medicago truncatula* Delta1 pyroline-5-carboxylatesynthase) and *MtActin* (*Medicago truncatula* Actin) gene markers along with *MtSOS1* (*Medicago truncatula* salt overly sensitive gene 1) (75%) while primers of *MtSOS2* and *AtNHX1* (*Arabidopsis thaliana* vacuolar Na^+/H^+ exchanger gene 1) gave the highest numbers of alleles per marker (5 amplicons each). The average PIC values of markers ranged from 0.12 to 0.56 with an average of 0.35 (Table 1).

Four stevia accessions were divided into 2 main clades based on UPGMA analysis (Fig. 4). The cluster analysis revealed that Samsun accession was distinctly separated from the others (Fig. 4). Adana and Bilecik accessions grouped in Clade II were genetically determined as the most similar accessions (79%). The 2C DNA contents of stevia

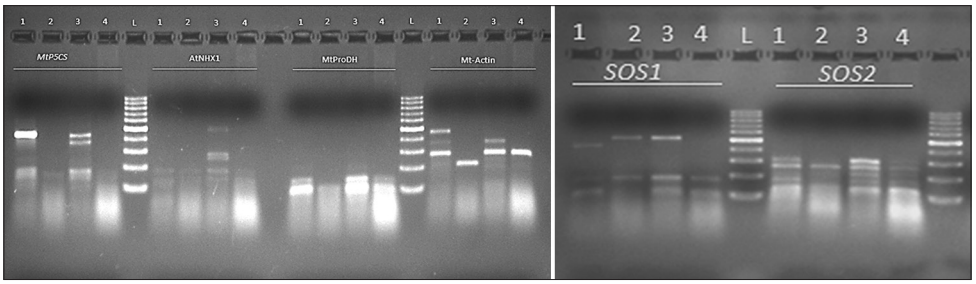

Fig 3. PCR amplification profile of 4 Stevia accessions generated with *MtP5CS, AtNHX1, MtProDH, Mt-Actin, MtSOS1* and *MtSOS2* gene markers. Lanes from 1-4: accessions (1) Adana, (2) Samsun, (3) Antalya and (4) Bilecik. L, ladder 50 bp molecular size marker (Vivantis).

Table 2: Agronomic parameters and mean 2C-DNA values of the accessions used in the study.*

Accessions	Plan height (cm)	Number of main stem (no./plant)	Number of secondory stems (no./plant)	Fresh weight (g/plant)	Dry weigth (g/plant)	Mean 2CDNA value (pg)
ADANA	61.87^{b}	1.06^{b}	18.89	168.10^{c}	51.43^{b}	1.67
ANTALYA	59.06^{b}	1.00^{b}	14.78	245.70^{b}	64.27^{b}	1.66
BİLECİK	71.44^{a}	1.61^{a}	14.00	329.50^{a}	92.94^{a}	1.64
SAMSUN	60.78^{b}	1.11^{b}	14.89	219.40^{cb}	64.3^{b}	1.52
Mean	63.28	1.19	15.63	240.69	68.23	1.62
Coeff. Var.	4.83	7.85	12.62	14.88	15.99	4.77
$LSD_{0.05}$	6.11	0.18	3.94	71.59	21.81	0.15
P value	0.0097	0.0007	0.0796	0.0082	0.0173	0.16

*Mean values with the same letter in each column are not significantly different at $P<0.05$.

accessions varied from 1.52 pg to 1.67 pg with an average of 1.62 pg (792.18 Mbp 1C genome size) (Table 2) (Dolezel et al. 2003). A sample histogram was given in Fig. 5.

DISCUSSIONS

Previous reports suggested that agronomic parameters of *Stevia rebaudiana* were significantly influenced by genotype, environment and the genotype x environment interactions, and the parameters related to yield should specifically be determined for individual growth conditions (Yadav et al. 2011; Al-Taweel et al. 2021). Genetic and phenotypic variations in *Stevia rebaudiana* have been reported for plant size, flowering period, leaf yield, SVglys content and composition (Abdelsalam et al. 2016; Çıkman, 2019; Al-Taweel et al. 2021). Ninteen stevia accessions collected from nature population in Egypt showed great variation for plant height and number of branches per plant, fresh and dry leaf weight (Abdelsalam et al. 2016). The parameters of stevia accessions measured under Çanakkale conditions were comparable with other reports. For instance, a 3-year experiment conducted under Antalya conditions showed that plant height, number of main stems per plant and fresh herb yield were determined as 100-121 cm, 15-19 stem/plant and 1710-2675 kg/da, respectively (Sözmen, 2015).

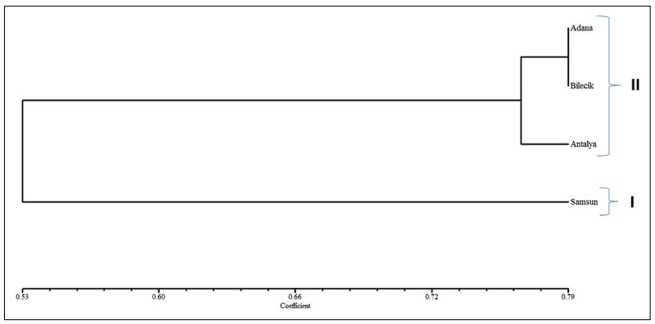

Fig 4. Similarity dendrogram (Nei, 1972) for 4 stevia accessions based on UPGMA method using 4 gene specific gene markers.

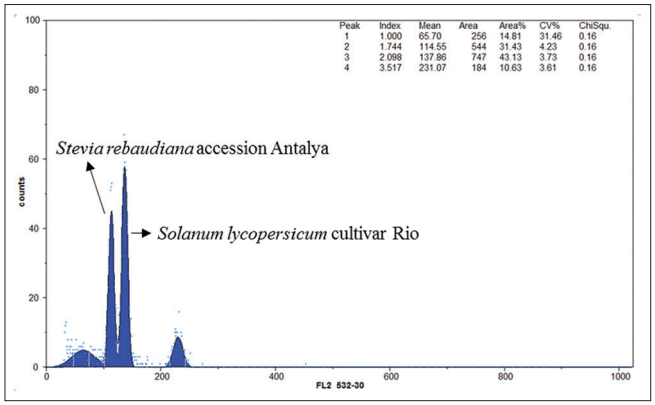

Fig 5. Flow histogram showing relative positions of G1 peaks of internal standard *Solanum lycopersicum* cultivar Rio and *Stevia rebaudiana* accession Antalya analyzed by flow cytometry.

On the other hand, the highest plant height, the number of main stem per plant and the fresh herb yield per plant were reported as 68.9 cm, 5.7 stem/plant and 256.9 g/plant under Şanlıurfa conditions, respectively (Çıkman, 2019) while the highest plant height and the highest fresh herb yield were 52.6 cm and 318.4 kg/da under Aydın ecological conditions (Selbi, 2019). Othman et al. (2018) also reported that plant height of stevia ranged from 23.4 to 36.6 cm and number of stems per plant varied from 4.5 to 13.7 in Malaysia (Othman et al. 2018). Gupta et al. (2013) indicated very high variation for plant height (65 to 180 cm) (Gupta et al. 2013).

Genetic diversity of *S. rebaudiana* grown in Egyptian and Indonesian was previously reported by using inter simple sequence repeats (ISSR) (Hadia et al. 2008; Kaur et al. 2015), random amplification of polymorphic DNA (RAPD) (Dyah et al. 2011; Abdelsalam et al. 2016) or both (Dyah et al. 2011; Sharma et al. 2016). A comprehensive genetic diversity analysis of 145 *Stevia* genotypes, including known cultivars and landrace populations of different origin was recently determined by using 18 EST-SSR markers (Cosson et al. 2019). We have first time used abiotic stress related gene-specific primers as DNA markers for genetic diversity analysis in stevia accessions (Table 1) (Fig. 3). Amplification of all primers of loci specific DNA markers in *Stevia* genome suggested that there was a high degree of transferability of these markers among plant species, including stevia since the primer sequences were obtained from distinct plant genomes, *Medicago truncatula* and *Arabidopsis thaliana*. The *MtP5CS* and *MtActin* genes had the highest polymorphism rate (100%) while *MtSOS2* and *AtNHX1* genes had the highest numbers of alleles per marker (5 amplicons each) (Table 1). The *SOS1* gene is known the most important loci and provides a better salt tolerance to plants compared to the other members of *SOS* genes (Shi et al. 2000). This study showed that *MtSOS2* gene has more allelic variation in *Stevia* than *MtSOS1* and may, therefore, play more critical role in control to salt tolerance in *Stevia* genome. As a most abundant member of the sodium/proton antiporter gene family, *AtNHX1* mediates salt tolerance in plants due to regulation of Na^+ homeostasis in the cell (Shi and Zhu, 2002). The primer pairs of *AtNHX1* gene used in this study provided 60.0% polymorphism rate while the primers of proline biosynthesis (*MtP5CS*) and *Medicago truncatula* proline dehydrogenase (*MtProDH*) genes had 100.0% and 50% polymorphism rates, respectively (Arabbeigi et al. 2019; Gou et al. 2016; Bouazzi et al. 2019). Stevia plants implemented several adaptation mechanisms in order to minimize the deleterious effects of salt stress at the physiological and at the biochemical levels, and allelic variation in salt tolerance related genes determined in this study may have significant contribution to this

implementation (Zeng et al. 2013; Cantabella et al. 2017). Therefore, it was suggested that saline waters or salt-affected soils might be used for stevia plant growth as well as for stevioside and rebaudioside production (Cantabella et al. 2017). On the other hand, *MtActin* gene is generally used as control in transcription analysis due to its lower level of variation in comparison to other constitutive genes under various stress conditions including salt and drought (Zhang et al. 2019; Li et al. 2011). The results of this study first time showed that primer pairs *MtActin* gene had a high level of allelic variation (100% polymorphism rate) in *Stevia* genome. These results are also suggested that high level of variation of *MtActin* gene in *Stevia* genome should be considered when it is used as a reference gene in RT-qPCR analysis.

Since Samsun accession was distinctly separated from the others in the cluster analysis based on UPGMA method (Fig. 4), this accession can be used to create genetic diversity in stevia breeding programs. The Clade II included the other three accessions which were genetically very similar to each other. Treatment of germinating seeds of *S. rebaudiana* with 0.05% colchicine for 48 h or with 0.1% colchicine for 24 h efficiently induced polyploidy and the polyploidy could be accurately identified using flow cytometry (Zhang et al. 2018). No significant 2C DNA content variation was determined among the stevia accessions with an average of 1.62 pg (792.18 Mbp 1C genome size) (Dolezel et al. 2003). Samsun accession had lower 2C DNA content as it was distinctly separated than the others in phylogenetic tree (Fig. 4; Table 2). The genome size of stevia (2C value) was previously estimated as 2.72 pg (Yadav et al. 2014) and recent genome assembly yielded a total sequence length of 411.38 Mbp (O'Neill and Pirro, 2020), suggesting that there is an intra-specific genome size variation or possible ploidy differences in stevia genotypes occurred during genome evolution although accessions grown in Turkey has very limited variation.

CONCLUSIONS

The results of this study revealed that gene specific primers can be used as DNA markers to determine genetic diversity of stevia and has a great potential to be used in DNA marker analysis for other plant species.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors Contribution

I.T. conceived the idea, performed all the numerical calculations and data analysis, interpret the data, designed the Figures and Tables. G.K. carried out the laboratory and field experiments. S.T. provided plant material, planned, and run the field experiment and drafted the manuscript.

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